nature portfolio

Peer Review File

Androgens inhibit protective CD8$^+$ T cell responses against pre-erythrocytic malaria parasites

Corresponding Author: Dr Sean Murphy

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)
Next to a reduction in number of vaccine-induced (semi)innate inflammatory mediators as well as CSP-specific CD8 T cell responses, this study shows that sex-hormonal environment at challenge is a major driver in the reduced protection obtained in male compared to female mice. Conducted in a different murine malaria model, reduced protection in male mice has been earlier reported as referenced (ref26) with a major role for testosterone. This study has further dissected the immunological basis for these observations in a different murine malaria model. This elaborate study has been carefully and systematically well- conducted and as such presented and the scientific quality is well acknowledged.
Major Comments.
1. Can the authors comment on the specific choice for this particular malaria model with focus on CD8 mediated protection as there is quite choice of Plasmodium and mouse strain combinations available. In general Balb/c mice ae known for humoral responses, while antibody responses are not reported in this study.
2. Fig 1c CD4/CD8 ratio liver and also fig3: total or per gram? Relevant since weights are different (fig 1d). Can CD4/CD 8 ratio per total organ be presented similar to parameters in the other figures
3. Fig3: don't understand the justification to calculate statistical significance of F or M versus F/M ratio representing three T-cells subsets. How to interpret this p-value? I can appreciate that the specific TRM liver response is clearly higher in F compared M while proportion of cells doesn't differ. Similar for TCM response in spleen.
4. Fig4: in my opinion unnecessary data rich with level of detail that surpasses the essential information to show that a number of inflammatory responses in F >M post P&T. Why for instance include RAS-only data while the rest of the paper only analyses P&T? Please restrict this figure to the essential elements.
5. Fig 7: 7b) I assume the specific response is absent in the P&T -/- group but p-values are lacking; 7h) An essential control is lacking: ORX56 without P&T vaccination; this allows the assessment of the stringency of the challenge; one cannot exclude that ORX may affect semi-innate immune responses and consequently the Pb liver load. This control should be included in the figure.
6. Although there are clear differences in protection between M and F after P&T vaccination, there are large standard deviations within and substantial overlaps in the indicated immune responses. How to reconcile with the impressive difference in protective efficacy between F (100%) and M (15%). In search for immune correlates of protection, study of post challenge immune responses in protected/unprotected M and F mice, might be an option for future studies?
7. Discussion 457: Capturing sex differences may not only be relevant in pre-clinical but also in clinical as such differences in immunogenicity to vaccines have been also observed in humans (e.g. doi: 10.1038/s41577-020-0338-x)

Reviewer #2

(Remarks to the Author)
In this manuscript, Duncombe et al addressed the role of androgens in the CD8+ T cell mediated protective immunity in malaria mouse model. The authors reported the striking difference in protection between male and female mice under their prime and trap vaccination strategy. By performing extensive animal experiments, immunological and transcriptome analyses the authors attempted to explain the difference observed in protection model. However, the experimental data and their interpretation are not as concrete and clear as the protection difference results between the gender. For example, the transcriptome difference at the level whole liver organ (Fig. 4) is not reflected at the level of cellular analysis that the authors focused (Fig. 2 and 3). Moreover, the whole liver organ size difference between the genders may have uniformity or difference in cell counts but not in frequency or proportion of any parental cell population – this is not addressed holistically

in the manuscript. It is also unclear why the authors tried to boost immunity in male mice in Fig 7, before fully explaining the improved protection they observe in male mice by gonad removal in Fig 5. While the study presents interesting findings based on gender, it does not comprehensively provide mechanistic details that explain the difference.

Reviewer #3

(Remarks to the Author)
In the submitted manuscript, Duncombe et al utilize a mouse model to investigate how sex hormones influence CD8+ memory responses to vaccination and subsequent challenge with the malaria causing parasite Plasmodium yoelii. Prior studies from other groups have found a female bias for mounting efficacious CD8+ immune responses against malaria, and Duncombe et al seek to build from these studies and better define how sex hormones affect CD8+ function and memory phenotype. Currently, sex differences in immune responses to infectious disease is a critically understudied topic. This is especially true for immune responses to malaria. Though the hypothesis is novel, unfortunately much of the data in the manuscript is nonsignificant and inconclusive. No significant difference was found in Trm, Tem, or Tcm cell population when comparing male and female mice and gonadectomized mice. It is noteworthy that there are observable trends toward significance, but this makes it perplexing as to why the authors did not increase the sample size to potentially gain significance. Upon concluding, the manuscript does not offer any added clarity on how sex hormones influence the CD8+ T cell response in male mice. As such, the results provided do not add to what has already been published by vom Steeg et al in 2019 (PMID 31262583). A lack of novelty coupled with some flaws in experimental design (detailed below) lead to my recommendation of rejection.

Major Issues:

Lack of significant findings:

The vast majority of the paper details figures that depict trends but are ultimately nonsignificant. The major basis of this paper was to further investigate how the cause/mechanism by which CD8+ T cell responses in vaccinated male mice failed to protect while the CD8+ cells in females did protect. While there is some promising transcript data in figure 4. The authors fail to show differences in Trm, Tcm, and Tem populations in vaccinated males. The key positive data in the paper was demonstrating that vaccinated female mice have more TNF+, IFNy+, 107a+ cells and that gonadectomies resulted in male protection upon challenge. Both of these take aways have already been demonstrated in the vom Steeg et al paper referenced earlier (PMID 31262583).

Examples of lack of significance:

Figure 2: The title of this figure is misleading. Only subfigure “e” in the entirety of this figure demonstrates a significance between vaccinated male and female mice. There is a difference in subfigures C and D, however without naïve controls for C, I am unsure if this difference is due to the vaccine or not.

Line 172: The difference is modest, but not significant when compared males or even unvaccinated for that matter.

Figure 3D: I don’t understand how this figure is demonstrating anything different from 3C. Both are the % of Tetramer+, CD44hi, CD8+ cells. 3D just breaks down the proportions of each memory phenotype, but the total of the % positive should add up to what is in 3C. Somehow it does not and is now significant in 3D where it was not in 3C.

Figure 3: Nowhere in the figure is there a significant sex difference despite the figure title. Not only that but the authors fail to achieve a significant difference between vaccinated and unvaccinated controls in 3C. At the very least there is an observable trend, but I am perplexed as to why the authors did not increase the number of mice in their study to achieve statistical significance.

Figure 5E: Once again not significant.

Line 279: I suppose the expression appears higher, but once again it is not significant.

Figure 6G and line 314: Once again this data is not significant.

Flaws in Experimental Design
Much of the paper hinges on the identification of tissue resident memory cells, however I do not believe the authors when through the proper measures to truly confirm the cells they identify are Trms. For example, in figure 3 the authors only identify Trms by CD69+. Though this is a marker for Trms, it is also a marker for recent T cell activation. Many other liver Trm markers have been identified: CD103, S1PR1, CXCR6 (PMID: 28733665). Further, intravascular staining can be conducted as described by David Masopust’s for further confirmation. Another key flaw is in the gonadectomy experiment. First the authors do not take hormone measurements of the mice to confirm the surgery was successful. Second, they do not perform an experiment to add the hormone back in to confirm the hormone is in fact necessary for the immune response. The authors themselves cite an add back in study on line 381, but do not do it themselves.

Examples of flaws in Experimental Design:

Figure 3b: I am not completely convinced that CD69 alone distinguishes resident memory

Line 177: I want to see more than just those 3 markers to distinguish Trm. Can you do intravascular staining as outlined by Masopust's group?

Line 242: "there are diminished inflammatory signals". To make this claim, it is important to look at these signals at a protein level, not just transcript. In figure 2, the authors investigate cytokine positive cells, but why not measure cytokine abundance following stimulation.

Figure 5A-E: An important validation step is missing. As there is the possibility of errors or incomplete gonadectomies, it is essential to test sex hormone levels in the serum to confirm the success of the surgery. This could potentially aid in explaining why two mice in 5E were not protected.

Supplementary Figure 6A: Why did the authors only look at IFNy? Why not look at granzyme and TNF as they did in figure 2?

Figure 6A-F: The authors should quantify the cytokine in the supernatant following stimulation. That might explain differences rather than changes in the percentage of cell types.

Figure 7A: The rationale for this experiment is not entirely clear or is potentially misguided. The authors spend the entirety of the manuscript attempting to uncover how androgens influence CD8+ Trm cells and are unable to find a concrete mechanism. They the use a more stimulatory vaccine formulation and do not look at influence on CD8 mediated immunity.

Minor Issues:
Line 56: From a mosquito
Line 58: "after replication"
Line 97: Is late stage malaria more common in men?
Figure 2B-C: is this difference in proportions due to the vaccine, or is this common in naïve mice? It would be nice to have unvaccinated controls to determine how the vaccine is influencing immune populations.
Line 233: S5e shows no difference between males and females in IFNa score at the 44 hour timepoint, so this observation is interesting. In figure 4H it appears that both RAS and P&T vaccinated females have significant upregulation in their IFNa response. Was this response in mock females or only in vaccinated females.
Supplementary Table 1: The vom Steeg paper is missing.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I am satisfied with the elaborate response and manuscript modifications. Male murine malaria models are more stringent than female based models for vaccine evaluation. This seems primarily determined by sex hormone conditions during the challenge infection.
I wonder whether differences have been found in males vs females in protection after spz/liver stage vaccines/spz in the controlled human malaria infection model.

Reviewer #3

(Remarks to the Author)
I thank the authors for their well thought out responses to my original comments and those of the other reviewers. The revised manuscript is substantially improved from the original submission and the added experiments, figures, and sections have strengthened the rigor of the the work presented.

One of my original critiques was the classification of TRM cells. I appreciate the authors methodical summary of how their original marker selection was appropriate through the references provided. I also appreciate now that David Masopust's

intravascular staining procedure is not feasible in the liver.

The added experiments with acyline, testosterone proprionate, and DHT have addressed my concerns with regard to enhancing experimental rigor in evaluating the role of androgens aside from gonadectomies alone. Further I appreciate the control of providing serum steroid levels following gonadectomy. Following the the minor correction detailed below, I can recommend the revised manuscript for publication.

Minor correction:
Though experiments have been added utilizing DHT and testosterone proprionate, the methodology for DHT use has been left out of the methods section.

Reply to Reviewers

We thank the reviewers for their comments and have replied in full below. The reviewers raise important points that are addressed in the revision. Line numbers noted herein refer to the Revised Track Changes version of the manuscript.

To address the overarching request to expand on molecular and cellular mechanisms underlying the impact of biological sex on protection outcomes, we conducted of series flow cytometry, immunohistochemistry, and gene expression experiments to demonstrate that androgens directly inhibit $CD8^+$ T recruitment responses. To further add to the impact of this study, we also confirmed sex-divergent protection outcomes in a second pre-clinical malaria mouse model (C57BL/6) and developed an acute and reversible method of reducing and increasing androgen levels to parse through the impact of sex hormones on the vaccine regimen. Note that we will use **black bold to indicate revised manuscript** locations and **red bold to indicate location in original manuscript**. Line number refer to location on tracked changes version

Reviewer #1 (Remarks to the Author):

Next to a reduction in number of vaccine-induced (semi)innate inflammatory mediators as well as CSP-specific CD8 T cell responses, this study shows that sex-hormonal environment at challenge is a major driver in the reduced protection obtained in male compared to female mice. Conducted in a different murine malaria model, reduced protection in male mice has been earlier reported as referenced (ref26) with a major role for testosterone. This study has further dissected the immunological basis for these observations in a different murine malaria model. This elaborate study has been carefully and systematically well- conducted and as such presented and the scientific quality is well acknowledged.

Major Comments.

1. Can the authors comment on the specific choice for this particular malaria model with focus on CD8 mediated protection as there is quite choice of Plasmodium and mouse strain combinations available. In general, Balb/c mice are known for humoral responses, while antibody responses are not reported in this study.

Thanks for highlighting this. While BALB/c mice are commonly used to study antibody responses, they have become essential in malaria research for investigating $CD8^+$ T cell-mediated immunity. PyCSP-specific $CD8^+$ T cell responses in these mice are sufficient to achieve complete sterile protection, making them a valuable model for studying antigen-specific cells associated with protection. To extend the relevance of our findings, we have also confirmed a sex-specific protection outcome in the C57BL/6 mouse model using two additional antigens, PbRPL6 and PbTRAP.

"To confirm that sex-bias in protection was not unique to this mouse and parasite strain, we conducted similar P&T studies using C57BL/6 mice and *P. berghei* (Pb) parasites for trapping (Pb-RAS) and challenge (Pb-WT). DNA encoding PbTRAP and PbRPL6 was used for priming, since these two antigens can be recognized in C57BL/6 (Fig S2a). As expected, P&T vaccinated female C57BL/6 mice showed increased protection over vaccinated male mice (Fig S2b-c), confirming that P&T vaccination induced a sex-bias in protection across two mouse malaria models." Added: Line **182 – 188** and **Fig S2.**

Together, these combinations of mouse strain, parasite strain, and parasite antigen form the leading models for studying $CD8^+$ T cell responses in murine liver stage malaria research. This study does not report antibody responses, as our laboratories' focus is on understanding and optimizing $CD8^+$ T cell responses for long-lasting, tissue-specific immunity. Sex-specific differences in antibody responses are well-documented in mice and humans, as cited in the manuscripts (PMID: 36389797 and 31262583). However, further exploration of these questions from an antibody-based perspective is beyond the scope of this investigation.

2. Fig 1c CD4/CD8 ratio liver and also fig3: total or per gram? Relevant since weights are different (fig 1d). Can CD4/CD8 ratio per total organ be presented similar to parameters in the other figures

To clarify, the data in **Figure 2c** is calculated by taking the ratio of the number of $CD4^+$ T cells in the liver versus the number of $CD8^+$ T cells in the *same* liver. Since this $CD4^+$ to $CD8^+$ T cell ratio is a within-mouse comparison, the addition of a weight constant does not impact the calculated ratio. To present the CD4/CD8 ratio similarly between figures, we added unvaccinated groups to the figure as requested by another reviewer (**Figure 2c**).

3. Fig3: don't understand the justification to calculate statistical significance of F or M versus F/M ratio representing three T-cells subsets. How to interpret this p-value? I can appreciate that the specific TRM liver response is clearly higher in F compared M while proportion of cells doesn't differ. Similar for TCM response in spleen.

Thanks for pointing out that the interpretation of this figure is not clear. The goal of **Figure 3** is to assess whether there was a difference in proportion/skew in T cell memory formation by sex. As another reviewer pointed out, while the trend is there, there is not a significant difference in Trm, Tem, and Tcm cell frequency between male and female mice. Given the new data reported in the revised submission, we condensed the original data in **Figures 2-3** (initial submission) to a single panel in **Figure 2** of this revised submission to focus on the liver data. Additionally, splenic flow cytometry data was moved to the supplements (**Figure S5-S6**).

To clarify on the interpretation of the stacked barplot, this type of plot allows for quick demonstration of p-values across three unique populations and has been previously

employed by published malaria papers that assessed T cell memory subsets (i.e., PMID: 37474653, 30282039). For a detailed breakdown of the values informing **Figure 2k** and **4j**, see breakdowns by memory sub-populations in **Figure S6e** and see below for a full interpretation of these results.

> "P&T vaccinated females and males showed an increased frequency of PyCSP-specific Trm cells in the liver at 28 days post-immunization, though males did not reach significance. Using either definition of PyCSP-specific Trm cells in the liver, there was no significant difference in Trm cell frequency between vaccinated male and female mice as reported as a proportion of $CD8^+$ T cells (Fig 2k-l, S6d-e). In the spleen, most cells had an effector memory phenotype (Tem: $CD44^{hi}CD62L^-CD69^-$), and there were no significant differences in the proportion of PyCSP-specific Tem cells between vaccinated male and female mice (Fig S6d-e)." Line **228 - 288**.

4. Fig4: in my opinion unnecessary data rich with level of detail that surpasses the essential information to show that a number of inflammatory responses in F >M post P&T. Why for instance include RAS-only data while the rest of the paper only analyses P&T? Please restrict this figure to the essential elements.

Thank you for this comment and for the input that the figure is too data rich. The primary goal of this panel was to highlight interesting sex-specific gene expression shifts, while its secondary role was to complement previously reported gene expression data on perturbations following attenuated spz vaccination (PMID: 24703850 and 34731605). The inclusion of the RAS-only group demonstrates that the observed sex differences were not exclusive to P&T vaccination but also evident in a RAS-only vaccine. To streamline the figure, we moved the volcano plots to **Figure S7** and included the summarized GSEA data for 44 hpi in **Figure 3d**.

5. Fig 7: 7b) I assume the specific response is absent in the P&T -/- group but p-values are lacking;

Thank you for bringing this to our attention. We added the requested p-values to the figure. However, after adding a series of new experiments to deconvolute the cellular mechanism underlying protection outcomes, we moved **Figure 7a-e** (first submission) to the supplemental data as **Figure S1**.

7h) An essential control is lacking: ORX56 without P&T vaccination; this allows the assessment of the stringency of the challenge; one cannot exclude that ORX may affect semi-innate immune responses and consequently the Pb liver load. This control should be included in the figure.

Thank you for pointing out this essential control. While we were unable to re-do this experiment with the ORX56 without P&T vaccination, we conducted two independent replicates assessing the impact of ORX and Acyline (a complimentary testosterone

reduction model detailed in lines **615 – 634**) that can now be found in **Figure S11f-g**. We found that at the challenge dose of 1,000 Py-WT sporozoites, there was no detectable impact of Acyline treatment, ORX surgery, and testosterone supplementation on liver burden 44 hours post-infection. Additionally, we had not detected a sex-specific difference in bioluminescent signal, a readout of liver burden, between male and female mice in **Figure 1d**.

> "Notably, there was no difference in luminescence signal at 44 hpi between naïve female and male mice (**Fig 1 c-d**), suggesting that liver stage replication of the parasite was similar for both sexes." Line **162 – 178**.

This indicates that biological sex was not significantly shifting parasite liver burden in an unvaccinated mouse at the challenge dose of 1,000 spz. Together, these findings provide evidence that a semi-innate immune response is not a main contributing factor for the dramatic protection differences observed in the ORX56 treated group.

6. Although there are clear differences in protection between M and F after P&T vaccination, there are large standard deviations within and substantial overlaps in the indicated immune responses. How to reconcile with the impressive difference in protective efficacy between F (100%) and M (15%). In search for immune correlates of protection, study of post challenge immune responses in protected/unprotected M and F mice, might be an option for future studies?

Thank you for your comment. In our initial submission, we identified that the sex hormone environment at the time of challenge was important for protection. However, we did not observe a significant difference in the $CD8^+$ T cell repertoire at the memory timepoint. This evidence suggests that androgens may influence $CD8^+$ T cell activity during the recall phase of vaccine-induced memory T cells.

We thoroughly explored this point with respect to correlates of protection in **Figures 6** and **7** (revised submission) and in the new result section titled "Androgens inhibit $CD8^+$ T cell recruitment response".

> "The mechanisms by which vaccine-induced $CD8^+$ T cells enact their effector mechanisms at time of challenge involve three mechanisms: cytolytic pathways, cytokine pathways[33], and "sensing and alarm" to facilitate recruitment of other immune cells[51]. To study the direct impact of androgens on $CD8^+$ T cells cytolytic enzyme and cytokine production, we used an *ex vivo* stimulation assay where we exogenously supply dihydrotestosterone (DHT), the metabolically active form of the testosterone, and evaluated the impact on $CD8^+$ T cells in an otherwise hormone-free media environment. Intact male and female mice, and ORX male mice were P&T vaccinated (**Fig 6a**) and lymphocytes were isolated from the liver and stimulated with $PyCSP^{280\text{-}288}$ peptide in the presence or in the absence of DHT (**Fig 6b**). $CD8^+$ T cells from ORX male mice showed a reduction in IFN-g and Granzyme B, but not TNF-a and CD107a, in the presence of DHT (**Fig 6c-d**). In the presence of DHT, there was no reduction in proportion of a putative

functional $CD8^+$ T cells expressing IFN-g, TNF-a and CD107a (**Fig 6e**). However, in female mice Granzyme B^+ functional $CD8^+$ T cells were reduced (**Fig 6f**). Thus, overall, the presence of DHT reduced the activity of $CD8^+$ T cells via inhibition of IFN-g^+ and Granzyme B^+, however, this fold reduction was quite slight.

Next, we evaluated the impact of androgens on the "sensing and alarm" function of Trm cells, which involves antigen-specific Trm cells serving as a local sensor and initiating an innate-like alarm signal to recruit more $CD8^+$ T cells and other immune cells. Previous work identified a positive association between number of $CD8^+$ T cells per infected hepatocyte and *Py* parasite clearance[52]. Thus, we used immunohistochemistry to quantify inflammatory foci and $CD8^+$ cells[53] in P&T vaccinated and unvaccinated male, ORX, and female mice challenged with Py-WT spz. For this experiment, we intentionally selected a high challenge dose of $1x10^5$ spz to ensure sufficient immune foci for spatial quantification (**Fig 7a-b**). As expected, we observed a higher number of inflammatory foci and $CD8^+$ cells at 38 - 44hpi in P&T vaccinated mice compared to unvaccinated mice challenged with Py-WT spz and mock-injected control mice (**Fig 7c**). Within the vaccinated mice, female and ORX male mice had more and larger inflammatory foci compared to male mice (**Fig 7d-e**). Furthermore, within each inflammatory focus, there was a higher total number and density of $CD8^+$ cells in female and ORX male mice compared to intact male mice (**Fig f- h)**. Thus, the presence of androgens reduced the number and size of inflammatory foci and impaired the recruitment of $CD8^+$ cells to these foci in vaccinated male mice compared to female or ORX male mice.

Next, having determined that androgens impact $CD8^+$ cell recruitment to immune foci, we sought to confirm there was reduced inflammation in male mice. From the same set of mice, we evaluated the gene expression of key pro-inflammatory markers *Cxcl9*, *Cxcl10,* and *Ifng* in the liver at time of challenge by gene specific RT-PCR. Previous studies have shown that production of chemokines, CXCL9 and CXCL10, and the cytokine IFN-γ correlate with attracting and enhancing T cell responses to inflammatory foci[33,54-58]. We found significantly higher expression of *Cxcl9*, but not *Cxcl10* and *Ifng,* in female and ORX male mice compared to male mice at the time of challenge (**Fig 7i**). Taken together, this data demonstrates that the presence of androgens at time of challenge slightly reduces memory $CD8^+$ T cell cytolytic capacity and cytokine production and reduces inflammation connected with *Cxcl9* expression, linking to subsequent impaired recruitment of additional immune cells in male mice." Lines **637 – 678**.

Thus, we also found that androgens suppress $CD8^+$ T cell recruitment to inflammatory foci by limiting inflammatory responses. Additional discussion has been added to the text to evaluate the molecular and cellular mechanisms underlying this effect, as detailed below.

“The alternative explanation was that immune effects of androgens at the “recall phase” were driving sex-bias in protection outcomes from P&T vaccination. Remarkably, when we reduced androgens levels *after* P&T vaccination but *prior* to spz challenge, high levels of protection in males were achieved, confirming this was the key driver of the sex-specific outcomes in our model. To effectively protect mice during this recall phase, Trm cells must first detect and migrate toward infected hepatocytes[13]. This involves inflammatory signaling, and under inflammatory conditions, the speed and motility of local $CD8^+$ T cells has been shown to increase[54,67]. Thereafter, Trm cells employ cytolytic and cytokine-mediated effector mechanisms to eliminate infection[4,33,68,69]. Importantly, cytokines produced by Trm cells also activate and recruitment more immune cells toward infected hepatocytes, an innate-like process often referred to as ‘sensing and alarm’[38,51,52]. Thus, having shown that effects of androgens at the recall phase were driving sex-bias in protection outcomes, we next sought to separately and more discretely probe cytolytic and cytokine effector function versus cell recruitment and propagated inflammation.

To mimic the direct impact of androgens on $CD8^+$ T cell cytolytic and cytokine function, we repeated *ex vivo* stimulation in hormone-free conditions and exogenously added DHT into the media. There was a small, but significant, reduction in granzyme B expression across all groups and IFN-g in ORX male mice. This supports prior literature that had identified androgen receptor (AR) interaction with the open chromatin regions associated with *Ifng* and *Gzmb* in effector memory $CD8^+$ T cells and regulate rapid production of IFN-g and granzyme B upon TCR stimulation[45]. However, in the same assay, we were unable to detect a difference in CD107a expression, a marker of degranulation, indicating that we were unable to connect granzyme B expression to functional readout of cytolytic capacity. Whether this is a technical issue or a true biological phenomenon remains to be determined. Additionally, previous work had identified a non-essential role of granzyme B and other cytolytic mechanisms in mediating protection in female mice[68]. Thus, while were able to find evidence for direct effects of androgens suppressing $CD8^+$ T cell effector functions, these effects were minor under conditions of acute antigen stimulation, differing from the increased function of $CD8^+$ T cells observed during androgen deprivation with chronic antigen stimulation models[44-46].

To assess how androgens influence the sense and alarm function of Trm cells, and their ability to recruit immune cells to the site of infection, we evaluated the spatial distribution of hepatic $CD8^+$ cells during *Plasmodium* spz challenge. Previous work has identified that multiple $CD8^+$ T cells per infected hepatocyte are important to clear infection in the *Py*/BALB/cJ model[52]. Here we confirmed that male mice have fewer inflammatory foci with $CD8^+$ cells, and within these foci there are fewer $CD8^+$ cells compared to female and ORX male mice. This parallels previously observed testosterone-mediated suppression of T cell recruitment observed in the hepatic infection model of cholangitis[70]. As this assay marks $CD8^+$ cells, it is important to note it captures both antigen-specific $CD8^+$ T

cells, but also nonspecific activated $CD8^+$ cells attracted to foci. Given we were unable to determine a definitive direct effect of androgens on $CD8^+$ T cell cytokine production in our other assays, this difference in foci formation is likely also a byproduct of other androgen-mediated immune cell functions. One possible explanation could be reduced MHC Class I presentation on infected hepatocytes in male mice. This is supported by a recent study that identified AR signaling represses MHC Class I expression in a tumor microenvironment as a mechanism in which hormones modulate escape from $CD8^+$ T cell killing[71].

A second explanation for reduced recruitment of $CD8^+$ cell to immune foci in male mice could be $CD8^+$ T cell extrinsic and related to general inflammation and other immune cells. Androgens are also known to have an immunosuppressive effect on inflammation and innate immune cells[60,66,72]. We show restricted expression of CXCL9, an IFN-γ inducible chemokine, in male mice following attenuated and virulent spz infection. During viral infections, CXCL9-CXCR3 interactions are important for clustering of antigen-specific effector $CD8^+$ T cells around infected cells[73]. As androgens did not affect CXCR3 expression on antigen-specific and nonspecific CD8 T cells (**Fig S9h**), future work should examine their impact on CXCL9-producing cells. Two potential culprits are hepatic neutrophil and monocyte populations, which are known to be affected by testosterone in the context of other hepatotropic infections[74-76]. Further investigations into how the sex-specific inflammatory milieu might alter the innate or adaptive immune cell recruitment toward infected hepatocytes at time of challenge will thus be a key focus of future work." Lines **755 – 819**.

7. Discussion 457: Capturing sex differences may not only be relevant in pre-clinical but also in clinical as such differences in immunogenicity to vaccines have been also observed in humans (e.g. doi: 10.1038/s41577-020-0338-x)

Excellent point, we revised the sentence to read:

"Beyond malaria liver stage vaccines, capturing sex differences in pre-clinical and clinical studies is a National Institute of Health mandate and an important step towards understanding intrinsic biases in vaccine development." (Line **889 – 892**).

Reviewer #2 (Remarks to the Author):

In this manuscript, Duncombe et al addressed the role of androgens in the CD8+ T cell mediated protective immunity in malaria mouse model. The authors reported the striking difference in protection between male and female mice under their prime and trap vaccination strategy. By performing extensive animal experiments, immunological and transcriptome analyses the authors attempted to explain the difference observed in protection model. However, the experimental data and their interpretation are not as

concrete and clear as the protection difference results between the gender. For example, the transcriptome difference at the level whole liver organ (Fig. 4) is not reflected at the level of cellular analysis that the authors focused (Fig. 2 and 3). Moreover, the whole liver organ size difference between the genders may have uniformity or difference in cell counts but not in frequency or proportion of any parental cell population – this is not addressed holistically in the manuscript. It is also unclear why the authors tried to boost immunity in male mice in Fig 7, before fully explaining the improved protection they observe in male mice by gonad removal in Fig 5. While the study presents interesting findings based on gender, it does not comprehensively provide mechanistic details that explain the difference.

For example, the transcriptome difference at the level whole liver organ (Fig. 4) is not reflected at the level of cellular analysis that the authors focused (Fig. 2 and 3).

Thank you for point out this important point. This is true -- we did not conduct a cellular analysis to complement the transcriptomic data. Because the data in the paper pointed out the importance of hormone environment at time of challenge, further evaluations during the post-RAS timepoint were considered less important for investigating the impact of sex on protection. While there are limitations in bulk RNAseq, the goal of this figure was to provide a broad sense of the different environments in which $CD8^+$ T cell memory responses are established. We now acknowledge this limitation in the discussion section:

> "Additionally, the gene expression data is limited by measuring the RNA levels and no protein level analysis was conducted." Line **879 – 880**.

Moreover, the whole liver organ size difference between the genders may have uniformity or difference in cell counts but not in frequency or proportion of any parental cell population – this is not addressed holistically in the manuscript.

Thank you for pointing this out. We now included an evaluation of liver size, cell proportion, and cell number for each of our main comparisons – male vs female (**Figure 2e - g**) and male SHAM vs male ORX (**Figure 4d - g**). Throughout the paper, the parental cell population was selected based on the outcome intended to measure, with the most common parental population being $CD8^+$ T cells due to the targeted nature of our questions.

It is also unclear why the authors tried to boost immunity in male mice in Fig 7, before fully explaining the improved protection they observe in male mice by gonad removal in Fig 5. While the study presents interesting findings based on gender, it does not comprehensively provide mechanistic details that explain the difference.

Thank you for pointing this out. Please refer to our response to Reviewer #1, Question 6, for a detailed mechanistic explanation. Additionally, we have moved **Figure 7** to **Supplemental Figure S1** to better emphasize the new mechanistic insights in the manuscript.

Reviewer #3 (Remarks to the Author):

In the submitted manuscript, Duncombe et al utilize a mouse model to investigate how sex hormones influence CD8+ memory responses to vaccination and subsequent challenge with the malaria causing parasite Plasmodium yoelii. Prior studies from other groups have found a female bias for mounting efficacious CD8+ immune responses against malaria, and Duncombe et al seek to build from these studies and better define how sex hormones affect CD8+ function and memory phenotype. Currently, sex differences in immune responses to infectious disease is a critically understudied topic. This is especially true for immune responses to malaria. Though the hypothesis is novel, unfortunately much of the data in the manuscript is nonsignificant and inconclusive. No significant difference was found in Trm, Tem, or Tcm cell population when comparing male and female mice and gonadectomized mice. It is noteworthy that there are observable trends toward significance, but this makes it perplexing as to why the authors did not increase the sample size to potentially gain significance. Upon concluding, the manuscript does not offer any added clarity on how sex hormones influence the CD8+ T cell response in male mice. As such, the results provided do not add to what has already been published by vom Steeg et al in 2019 (PMID 31262583). A lack of novelty coupled with some flaws in experimental design (detailed below) lead to my recommendation of rejection.

Thank you for your thoughtful review and for highlighting ways to improve our manuscript. The work by vom Steeg et al. indeed laid the foundation for our research, as it was the first to demonstrate a direct sex difference in a repeated RAS vaccine strategy that provided *partial* protection against *Plasmodium berghei* in C3H mice. Their study served as an important basis for our research, allowing us to expand and build upon their findings significantly.

In response to the reviewer's comments regarding the vom Steeg paper, we would like to highlight several key differences between our study and theirs.

Our first major difference from vom Steeg's work is that our model employs a *completely* protective vaccine as compared to the *partially* protective vaccine used in that earlier paper. The benefit of using our completely protective P&T vaccine as a model to study sex differences is that we were able to isolate the sex-specific contribution to sterile immunity in male mice.

Our second major difference from the vom Steeg paper is that the paper only analyzed IFNγ-secreting CD8$^+$ T cells via ELISPOT and flow cytometry. Our report brings more immunology tools to bear on this question, such as immunohistochemistry, RNAseq, and multiple panels of multi-parameter flow cytometry. While vom Steeg et al. attributed hormone environment differences to IFNγ CD8$^+$ T cell numbers in the liver, we were unable to confirm that finding via ELISPOT, ICS, or surface stain flow cytometry in the BALB/cJ and C57BL/6 mouse model. This discrepancy may be related to differences in sterile protection and/or the specific mouse model and parasite strain.

Our third major difference between the vom Steeg paper and our paper is that we identified the time point during which hormones markedly influence protection outcomes. All of the hormone manipulations in the vom Steeg paper were prior to vaccination. In our paper, we confirmed with both a surgical approach (ORX) and a chemical approach (Acyline) that the hormone environment *at time of challenge* was the major contributing factor to protection outcomes. We additionally employed an acute testosterone add-back experiment that further corroborated the importance of hormone environment at time of challenge (**Figure 5d**).

With the revisions, you will also see the fourth major difference in that we have added a spatial dimension and identified that gonadally-intact male experience restricted $CD8^+$ cell recruitment toward inflammatory foci, which is connected to restricted inflammatory responses. Additionally, we identified a slight reduction in cytolytic and cytokine capacity of $CD8^+$ T cells in the presence of DHT with IFNγ and Granzyme B with stimulation under a carefully controlled hormone environment *ex vivo*. We have thus posited a cellular and molecular mechanism that explains protection outcomes, which is beyond what was reported by the vom Steeg paper.

All in all, in our paper, we conducted several major analyses that distinguish the work from the vom Steeg publication: 1) in-depth review of the T cell repertoire by sex; 2) evaluation of T cell function with 12 different markers of fate and function; 3) transcriptomic studies to define differences in inflammatory environments during vaccination; 4) demonstration of a mechanistic rationale for why androgens inhibit protection in the liver microenvironment; and 5) demonstration that these sex-specific difference are evident in two additional malaria P&T mouse models. We hope that the reviewer will reconsider the merits of this manuscript based on this summary and the added studies done to arrive at the revised manuscript.

Major Issues:

Lack of significant findings:

The vast majority of the paper details figures that depict trends but are ultimately nonsignificant. The major basis of this paper was to further investigate how the cause/mechanism by which CD8+ T cell responses in vaccinated male mice failed to protect while the CD8+ cells in females did protect. While there is some promising transcript data in figure 4. The authors fail to show differences in Trm, Tcm, and Tem populations in vaccinated males. The key positive data in the paper was demonstrating that vaccinated female mice have more TNF+, IFNy+, 107a+ cells and that gonadectomies resulted in male protection upon challenge. Both of these take aways have already been demonstrated in the vom Steeg et al paper referenced earlier (PMID 31262583).

We thank the reviewer for pushing us to dig deeper. We would like to note that we did not fail to show differences but rather demonstrated that there are no differences by T

cell subsets, which is a useful finding that in and of itself has not been previously reported. The main point of initial flow cytometry data was to determine whether or not there were detectable differences in $CD8^+$ T cell fate and function at a memory timepoint. We also note that we have older data supporting this finding with a slightly altered flow cytometry surface stain panel that identified T cell memory subsets, but decided not to include this older data in the paper since the findings were essentially the same across both older and newer flow panels. As previously discussed, this finding helps to distinguish our work from the vom Steeg paper, which quantified antigen-specific $IFN\gamma^+$ producing-cells.

Examples of lack of significance:

Figure 2: The title of this figure is misleading. Only subfigure "e" in the entirety of this figure demonstrates a significance between vaccinated male and female mice. There is a difference in subfigures C and D, however without naïve controls for C, I am unsure if this difference is due to the vaccine or not.

Thank you for drawing our attention to this point. We now revised the initial figure by adding in the naïve groups (**Figure 2c**). Indeed, you will see that this was an effect of vaccination interestingly on the T cell skew, since the difference by sex is only apparent post-vaccination.

Line 172: The difference is modest, but not significant when compared males or even unvaccinated for that matter.

Thank you for drawing our attention to this point. Since this figure was assessing CSP-$tetramer^+$, $CD44^{hi}$, $CD8^+$ cells, which was not the dominant question for the panel, we revised the section and moved the referenced figure to **Supplemental Figure S6c**. As a replacement, we condensed **Figures 2** and **3** to emphasize the new mechanistic and cellular mechanisms details (**Figure 2** – revised submission).

Figure 3D: I don't understand how this figure is demonstrating anything different from 3C. Both are the % of Tetramer+, CD44hi, CD8+ cells. 3D just breaks down the proportions of each memory phenotype, but the total of the % positive should add up to what is in 3C. Somehow it does not and is now significant in 3D where it was not in 3C.

To clarify, **Figure 3d** (first submission) was the composite of three separate graphs depicted in the supplements (**Figure S5d-e**; first submission). The purpose of this figure was to identify if there was a difference in proportion of T cell memory subsets. To enhance clarity, we removed the graph that was redundant in terms of analysis of the frequency and proportion of the cell populations (**Figure 3e** – first submission).

Regarding the math of **Figure 3d** (first submission), there is one memory quadrant population not included in the graph, $CD62L^+CD69^+$. Since we are focused on the

known Tem, Trm, and Tcm memory cell subsets, inclusion of the fourth population ($CD69^{+}CD62L^{+}$) was not deemed to be necessary.

In the first submission, **Figure 3d** was a composite of three separate graphs from the supplements (**Figure S5d-e**) designed to assess differences in the proportions of T cell memory subsets. To improve clarity, we removed a redundant graph that analyzed the frequency and proportion of these populations (**Figure 3e**). Additionally, one memory quadrant population (CD62L+CD69+) was not included in the now **Figure 2k**, as our focus was on the established Tem, Trm, and Tcm memory subsets, making the inclusion of this fourth quadrant population unnecessary.

Figure 3: Nowhere in the figure is there a significant sex difference despite the figure title. Not only that but the authors fail to achieve a significant difference between vaccinated and unvaccinated controls in 3C. At the very least there is an observable trend, but I am perplexed as to why the authors did not increase the number of mice in their study to achieve statistical significance.

We have revised this section to clarify that no significant sex differences were observed and to ensure that the figure title accurately reflects the data. Additionally, while we acknowledge the observable trend in **Figure 3C**, the sample size was determined based on prior studies. Increasing the number of mice was not deemed critical for understanding the underlying mechanistic and cellular mechanisms driving sex differences in protection given other factors have since been identified; however, the reported trends may warrant further investigation in future studies in other mouse models. Refer to **Figure 2** (second submission) and **Supplemental Figures S6c-e** for details on these revisions.

Figure 5E: Once again not significant.

The data in **Figure 5e** (first submission) was redundant, as it presented the same information as **Figure 5d** but broken down by Plasmodium 18S rRNA value. To enhance clarity, we revised the figure to only include **Figure 5d**, which more accurately reflects the binary distinction between protection and no protection, rather than gradations of the outcome. This binary distinction is crucial, as a single infected hepatocyte can initiate a blood-stage infection, which is the key challenge for achieving sterile liver-stage vaccine protection. **Figure 5e** (old submission) has been moved to **Supplemental Figure S8b** (new submission).

Line 279: I suppose the expression appears higher, but once again it is not significant.

Thank you for your feedback. We decided to remove this figure from the paper as it did not contribute significantly to the overall conclusion. Given that the result was not statistically significant and more critical data is presented later, we felt it was unnecessary to include.

Figure 6G and line 314: Once again this data is not significant.

Thank you for pointing this out. While it is interesting that no significant difference was observed based on this 28-day timepoint, we agree that TCF1 is not an important marker to differentiate within the central findings of the paper. We therefore condensed interpretation of TCF1 expression data as follows:

> "As expected, higher expression of TCF1 was observed on central memory $CD8^+$ T cells (Tcm) in the liver than on Tem and Trm cells (**Fig S9g**). However, importantly, there was no difference in TCF1, PD-1, and LAG-3 expression on tissue resident $CD8^+$ T cells (defined as CSP-tetramer$^+$CD62L$^-$CD69$^+$CD44^{hi} $CD8^+$ T cells) in ORX compared to SHAM vaccinated mice (**Fig 4k-m**).
>
> In all, there was no detectable difference between the frequency, density, functionality profiles, or exhaustion/dysfunction status, of antigen-specific $CD8^+$ T cells in male mouse livers at 28 days post-immunization following ORX or SHAM surgeries." Line **444 – 599**.

Flaws in Experimental Design
Much of the paper hinges on the identification of tissue resident memory cells, however I do not believe the authors when through the proper measures to truly confirm the cells they identify are Trms. For example, in figure 3 the authors only identify Trms by CD69+. Though this is a marker for Trms, it is also a marker for recent T cell activation. Many other liver Trm markers have been identified: CD103, S1PR1, CXCR6 (PMID: 28733665). Further, intravascular staining can be conducted as described by David Masopust's for further confirmation. Another key flaw is in the gonadectomy experiment. First the authors do not take hormone measurements of the mice to confirm the surgery was successful. Second, they do not perform an experiment to add the hormone back in to confirm the hormone is in fact necessary for the immune response. The authors themselves cite an add back in study on line 381, but do not do it themselves.

Figure 3b: I am not completely convinced that CD69 alone distinguishes resident memory

There is extensive literature supporting CD69 as a marker for Trm cells in the liver. While multiple markers are associated with liver Trm cells, $CD69^+$ expression is widely used to distinguish them from other memory T cell subsets (PMID: 19305395, 30282039, 25957682). Additionally, we stain for CXCR3, which is another established Trm marker and is known to be lower on Tem cells (PMID: 31618655, 27939671). The use of CD69 and CXCR3 as markers of liver tissue residence has been validated in multiple studies (PMID: 31618655, 31477704, 34731605) and has been recommended in at least one expert review (PMID: 27867517).

While CD103 is commonly associated with skin-resident memory T cells, previous studies have shown that it does not effectively distinguish Trm cells in the liver. Furthermore, CD69 signaling is required to downregulate S1PR1, preventing egress from the liver, making additional assessment unnecessary (PMID: 25624457). Finally, our data in **Supplemental Figure S6d** show very few $CD69^+$ cells in the spleen, further supporting CD69 as a distinguishing marker between Tem and Trm cells in the liver.

Line 177: I want to see more than just those 3 markers to distinguish Trm. Can you do intravascular staining as outlined by Masopust's group?

While we would love to use the Masopust method to define tissue residents, the unique position of the Trm cells in the liver prevent the Masopust method from differentiating a Trm cell from a circulating cell. Trm cells in the liver patrol the liver sinusoids and come into direct contact with the circulatory system (PMID: 15799695, 25526304), therefore making hepatic Trm cells unsuitable to staining by the Masopust method for identification (PMID: 31618655). To address the point on the third marker, we have included CXCR3 as an additional marker of Trm cells, which is expressed on the 95-100% of CSP-tetramer$^+$CD62L$^-$CD69$^+$ Trm cells.

Line 242: "there are diminished inflammatory signals". To make this claim, it is important to look at these signals at a protein level, not just transcript. In figure 2, the authors investigate cytokine positive cells, but why not measure cytokine abundance following stimulation.

Thank you for point out this important point. This is true -- we did not conduct a protein analysis to complement the transcriptomic data. Because the data in the paper pointed out the importance of hormone environment at time of challenge, further evaluations during the post-RAS timepoint were considered less important for investigating the impact of sex on protection. While there are limitations in bulk RNAseq, the goal of this figure was to provide a broad sense of the different environments in which $CD8^+$ T cell memory responses are established, which we hope has been accomplished. We now acknowledge this limitation in the discussion section:

> "Additionally, the gene expression data is limited by measuring the RNA levels and no protein level analysis was conducted." Line **879 – 880**.

To clarify the interpretation of the RNA-seq data in the manuscript, we revised the following line to better emphasize that we are capturing the response to inflammatory signals.

> "Overall, this suggests that while male mice can form chemokine cues important for $CD8^+$ T cells, there is a diminished response to inflammatory signals, like interferons, when compared to female mice." Line **374 – 376**.

By emphasizing the response aspect, we are not implicating a protein level assessment. There are limitations in bulk RNAseq, but the point of this figure is to get a broad sense of the different environments in which $CD8^+$ T cell memory responses are established.

Figure 5A-E: An important validation step is missing. As there is the possibility of errors or incomplete gonadectomies, it is essential to test sex hormone levels in the serum to confirm the success of the surgery. This could potentially aid in explaining why two mice in 5E were not protected.

Thanks for pointing out this important step. All surgeries in these experiments were conducted by a board-certified veterinarian. Mice were visually confirmed for castration based on shifts in behavior and hair growth. While serum was not collected from every mouse following surgery, we included a subset of the testosterone ELISA data in the supplemental figures and confirmed that hormones levels in castrated mice were significantly different from SHAM operated mice (**Figure S8c**, **S10b**, **S11**).

Supplementary Figure 6A: Why did the authors only look at IFNy? Why not look at granzyme and TNF as they did in figure 2?

The ELISPOT in **Supplemental Figure S6A** (first submission) is a complementary assay conducted during a routine challenge experiment to enumerate preliminary antigen-specific cells in the spleen. Since our initial flow cytometry data did not identify differences for TNF and Granzyme B in the splenic repertoire, we decided not to further pursue these markers with a preliminary ELISPOT. For more in-depth data on Granzyme B and TNF, please look to **Figure 2**, **5**, and **6**.

Figure 6A-F: The authors should quantify the cytokine in the supernatant following stimulation. That might explain differences rather than changes in the percentage of cell types.

Thank you for this suggestion. While we would have liked to co-measure cytokine production, the low abundance of lymphocytes in the liver led us to prioritize the single-cell resolution analysis provided by flow cytometry. Accurately quantifying cytokines in the supernatant would require a complete repetition of all studies. This is because ICS stimulation requires BFA addition, which blocks cytokine secretion, thus measuring cytokines in the supernatant during a traditional ICS assay would be inaccurate. Future studies could explore cytokine quantification; however, now that we have established the impact of the hormonal environment on $CD8^+$ T cell recruitment, alternative spatial approaches capturing the cytokine and chemokine milieu would be more suitable.

Figure 7A: The rationale for this experiment is not entirely clear or is potentially misguided. The authors spend the entirety of the manuscript attempting to uncover how androgens influence CD8+ Trm cells and are unable to find a concrete mechanism. They the use a more stimulatory vaccine formulation and do not look at influence on CD8 mediated immunity.

Thank you for pushing us to further define the mechanism behind the difference in protection outcomes. Please see response to Reviewer #1, question 6 for detailed mechanistic response to this point. We have also revised and moved **Figure 7** to supplemental **Figure S1**, to make space to emphasize the new mechanistic insights in the manuscript.

Minor Issues:
Line 56: From a mosquito

Revised as suggested in **line 65**.

Line 58: "after replication"

Revised as suggested in **line 67**.

Line 97: Is late stage malaria more common in men?

If by late-stage malaria, you are referring to RBC-stage malarial disease, then the literature shows that there is higher prevalence of blood stage infection in adult males compared to adult females. Interestingly, this trend is not observed for younger children and infants, implicating a role for sex hormones in modifying prevalence of blood stage infection (Reviewed in PMID: 37695429).

Figure 2B-C: is this difference in proportions due to the vaccine, or is this common in naïve mice? It would be nice to have unvaccinated controls to determine how the vaccine is influencing immune populations.

Thank you for your insightful suggestion. We have now included data from naïve (unvaccinated) mice to better assess the impact of vaccination on immune cell populations. By incorporating these controls, we were able to demonstrate that the observed differences are indeed influenced by vaccination status. The revised **Figure 2b-c** in the new submission clearly reflects these updates, providing a more comprehensive understanding of how the vaccine shapes immune cell proportions. We appreciate your feedback, as it has strengthened the clarity and interpretation of our findings.

Line 233: S5e shows no difference between males and females in IFNa score at the 44 hour timepoint, so this observation is interesting. In figure 4H it appears that both RAS and P&T vaccinated females have significant upregulation in their IFNa response. Was this response in mock females or only in vaccinated females.

In revised **Figure 3d**, we included the GSEA evaluation for both the 44-hpi and 6-day timepoints. Interestingly, induction of interferon-alpha in the RAS-only timepoint is only evident at 44 hpi, which is reflected in the statistics in **Figure S7e**. This value was calculated relative to the respective mock-injected samples by sex and accounts for baseline differences by sex. See the following for the interpretation in the text:

> "During the initial innate immune response phase at 44 hrs post-RAS immunization, male and female mice responded similarly for several pathways, including IFN-a response regardless of vaccination status (Fig 3d). Further evaluation of differentially expressed genes identified a gene signature shared between male and female mice related to liver inflammation (*Saa1, Saa3*) (Fig S7c)*."* Line **345 – 349**.

Supplementary Table 1: The vom Steeg paper is missing.

This table was meant to summarize a review that did not include the vom Steeg paper. We have added clarity to the manuscript on this in line **112 – 115**:

> "For example, in a review by Nganou-Makamdop and Sauerwein, of the 17 reviewed papers on spz immunization in mice, none reported using male mice, 12/17 reported using exclusively female mice, and 5/17 reported no sex information[25] (Table ST1)."

Additionally, we do clearly credit Vom Steeg for talking about the first male and female in lines **118 – 122**:

> "The limited data we do have on the impact of sex on immune responses to liver stage malaria vaccines come from a single recent pre-clinical study[26]. Following repeated RAS vaccination, female mice were found to have more hepatic IFN-g-producing $CD8^+$ T cells and greater protection against *P. berghei* spz challenge than male mice. This reduced protection in males could be overcome by removal of testicular hormones prior to vaccination[26]."

Re-calculation comment: In addition to the aforementioned changes in response to reviewer comments, we determined that there was a minor misallocation of naïve mice in Figures 2, 4 and S1. We conducted a re-review of the data and recalculated statistics including p-values. Recalculated p-values differed slightly from the values listed in the original submission, but do not alter the conclusions of the experiments or the overall study.

Overall conclusion:
We thank all reviewers and editors for encouraging us to further dig into cellular and molecular mechanisms underlying the sex-based difference in protection outcomes. This revised submission now includes identification of the inhibitory effect of androgens on $CD8^+$ T cell recruitment toward inflammatory foci via a restricted inflammatory response. We hope this has fulfilled the criteria of the major revision and look forward to hearing back further on our manuscript.

Reply to Reviewers

We thank the reviewers for their comments and re-review of the manuscript. We have replied in full below.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I am satisfied with the elaborate response and manuscript modifications. Male murine malaria models are more stringent than female based models for vaccine evaluation. This seems primarily determined by sex hormone conditions during the challenge infection.
I wonder whether differences have been found in males vs females in protection after spz/liver stage vaccines/spz in the controlled human malaria infection model.

Thank you for the review. We have discussed this point in the manuscript within the discussion section in the paragraph on lines 516 through 525. Additionally, we have recently reported a sex-based difference in liver stage infection in the placebo arm of controlled human malaria infection (CHMI) models (preprint link: https://papers.ssrn.com/sol3/papers.cfm?abstract_id=5087394). This work contributed to the case that vaccines targeting the *Plasmodium* liver stage are impacted by host biological sex.

Reviewer #2 Comments mediated by reviewer #1

The authors made a serious effort to address the comments of reviewer 2 and made changes in the manuscript accordingly. It is difficult to judge on behalf of reviewer 2 whether this is satisfactory, but at least sufficient in my opinion.
I hope that this response will adequately meet your is standards in order to draw final conclusions. In case you need more/other feedback, please let me know.

Thank you for addressing the comments on behalf of reviewer 2.

Reviewer #3 (Remarks to the Author):

I thank the authors for their well thought out responses to my original comments and those of the other reviewers. The revised manuscript is substantially improved from the original submission and the added experiments, figures, and sections have strengthened the rigor of the the work presented.

One of my original critiques was the classification of TRM cells. I appreciate the authors

methodical summary of how their original marker selection was appropriate through the references provided. I also appreciate now that David Masopust's intravascular staining procedure is not feasible in the liver.

The added experiments with acyline, testosterone proprionate, and DHT have addressed my concerns with regard to enhancing experimental rigor in evaluating the role of androgens aside from gonadectomies alone. Further I appreciate the control of providing serum steroid levels following gonadectomy. Following the the minor correction detailed below, I can recommend the revised manuscript for publication.

Minor correction:
Though experiments have been added utilizing DHT and testosterone proprionate, the methodology for DHT use has been left out of the methods section.

Thank you for the re-review of the manuscript. We added information related to DHT supplementation in the flow cytometry experiments. Please see the addition in the highlighted section of line 679 of the methods section.

Once again we would like to thanks the reviewers for their thoughtful comments and hope these revisions address their recommendations.