

Prevention of tuberculosis in cynomolgus macaques by an attenuated *Mycobacterium tuberculosis* vaccine candidate

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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 is a very exciting report describing Enhanced Protective Efficacy of a TB vaccine candidate Mtb Δ sigH, a deletion of a gene encoding a Sigma factor that responds to oxidative stress, administered mucosally in Rhesus Macaques. This vaccination showed significant protection, characterized by the absence of granulomas and the presence of robust antigen-specific T cell responses and hyper-immune trained immunity-like phenotypes in macrophages. Impressively, the comparison of the lungs of animals immunized with Mtb Δ sigH compared to unvaccinated using a recently developed spatial analysis reveals impressive protective immunity. The research suggests Δ sigH as a promising vaccine candidate, demonstrating essential correlates of protection including T cell activation and the recruitment of specialized macrophages.

This report is well-written describing carefully-controlled studies.

Reviewer #2

(Remarks to the Author)

This manuscript presents efficacy data from a candidate TB vaccine – an attenuated M.tb strain, deficient in SigH. This is not a new vaccine candidate but the new data here is testing this vaccine in cynomolgus macaques and is compared to BCG. Both vaccines are given @ the same dose in the lungs. BCG and SigH mutant confer protection but the level of protection as measured by CFU counts is higher with the SigH compared to BCG although there was no difference in pathology score between the two vaccinated groups or CXR scores. Comprehensive interrogation of the induced immune response is presented.

The results and amount of data presented are impressive. But the manuscript is too long, particularly the results section, and I have suggested below some ways it could be made more succinct.

I have some specific comments:

- Abstract line 22 – bacille not bacillar.
- Reference 1 – suggest reference the WHO report rather than a reference referencing it – and correct the numbers – is 1.3m deaths in the latest (2023) report.
- Line 322 The statement: No changes were detected in the frequencies of effector, memory or naïve CD4+ or CD8+ T cells in BAL post Mtb challenge (Fig S2h-m) or in T cell phenotypes in peripheral blood mononuclear cells (PBMCs) post challenge in the three vaccinated groups (not shown) is somewhat surprising – one would have expected this to be higher in the unvaccinated animals with a higher bacillary burden.
- The disconnect between CFU counts and pathology scores should be commented on – usually there is a close correlation between these read outs.
- It is not entirely clear why the authors continue to study this strain – given, as they highlight in the discussion, it only has a single mutation and is not appropriate for moving into clinical trials. They discuss the need to develop a second mutation and see if that still confers protection. As proof of concept in a second strain of macaques, the work presented here is interesting but the vaccine has already been demonstrated to be protective in Rhesus macaques.
- Figure 9 is unnecessary – there are too many figures in this manuscript
- The results section is too long – 26 pages. This could be written much more succinctly without the added interpretation of the results. E.g. line 277: These results clearly show that while mucosal vaccination with BCG offers mild protection against Mtb

challenge in CMs, comparable vaccination with sigH results in a >1000-fold greater protection; and line 309: Together, these results suggest that while the total granulomatous pathology was present at the lowest level in the lungs of sigH vaccinated macaques, greater iBALT responses generated in this group contributed to the higher than baseline lung pathology observed in this group; line 340: B cell follicles are formed in the lung following sigH vaccination and are important for protection from TB; etc. Some of this (there are many other examples) would sit better in the discussion – but also needs to be more succinct and not just transferred en bloc to the discussion.

- Line 389: I do not think the following statement is justified by the data: Because the immune cell dynamic of the lung environment in CMs was drastically altered by sigH vaccination, relative to BCG vaccination; some of the immune readouts show a difference between BCG and delta sig H but not all do and some readouts are comparable. The word drastically is unnecessarily subjective and emotive.
- Some of the language could be simpler – e.g. line 579 – TB vaccines not anti TB vaccines.
- Line 630 – list of protein markers – should be in supp figure not in main text.

Reviewer #3

(Remarks to the Author)

This manuscript tested the efficacy of attenuated *Mycobacterium tuberculosis* vaccine with the absence of sigH, the master regulator of responses to multiple stress conditions such as oxidative and nitrosative stress, phagocytosis, hypoxia and so on, as well as providing mechanistic understandings underlying the protection conferred by Δ sigH vaccination, in the more human-like cynomolgus macaque model of aerosol infection rather than rhesus macaque. Δ sigH vaccination significantly reduced lung and extra-thoracic CFU and pathology in cynomolgus macaques. Furthermore, mucosal vaccination with Δ sigH recruited T cells expressing IFN- γ to the airways and lungs, priming an IFNG-responsive rather than a Type I IFN-responsive phenotype in macrophages. These results might shed light on a potential anti-TB vaccine, although it's in its very early stage. However, there're still several limitations/mistakes across the whole text.

1. The image resolution of some figures (e.g. Figure 5, Figure 8) is too low that the details couldn't be recognized such as gene name and so on.
2. The scRNA-seq data was not well organized that
 - 1) classical markers defining each cell type should be clearly displayed in Fig S7;
 - 2) the makers for each lymphocyte or myeloid cluster should be carefully selected or arranged to ensure that cluster info could be easily received. In addition, violin plots are recommended;
 - 3) There's no need to label colors for different groups in Fig 5b-5g as the group name has been signed. Instead, the expression level of selected genes should be displayed that expression ratio and level could be compared directly;
 - 4) The analysis strategy used in Fig 7 could be also carried out in scRNA-seq data.
3. There're several labeling mistakes. For example, Fig S2p should be Fig S1p (line 277); Fig 1C didn't show the CXR scan result (line 282); line 377, Fig S3b should be Fig S3p? line 483, expressed IFIT3, MX2, IRF7 higher levels of IFIT3.

Reviewer #4

(Remarks to the Author)

The manuscript by Singh et al evaluates the potential to use a *Mtb* Δ sigH mutant to vaccinate resistant cynomolgus macaques to protect against lethal *Mtb* challenge. Applying a similar approach to one they previously used for rhesus macaques, they first evaluated the clinical, microbiological and pathological outcomes in BCG, Δ sigH, and unvaccinated animals following lethal *Mtb* challenge. A significantly greater protective response was observed in the Δ sigH vaccinated animals in comparison to BCG as demonstrated by reduced bacterial burden, and increased iBALT presence. Next, they used flow cytometry to show that Δ sigH vaccination induced strong T and B cell responses in BAL including enhanced lung homing phenotype indicative of iBALT formation. The authors demonstrated that Δ sigH T cell response was antigen specific through stimulation with CW or EC with significantly higher CD4+ and CD8+ cells expressing IFNG, TNF- α , or both present within the BAL of the Δ sigH vaccinated animals. Post-vaccination B and T cell responses were characterized in BAL using scRNAseq. The data indicate Δ sigH vaccination results in enhanced recruitment of T cells with antigen-specific IFNG expression. This results in stronger cytolytic T cell responses in the lungs due to enhanced T cell – B cell cooperation. They also report signatures of IDO expression in dendritic cells co-expressed with TMEM176A-B+/- required for antigen-presentation to T cells indicating the potential different phagosomal processing of Δ sigH in comparison to *Mtb*. In vitro studies conducted in CDC1551 and Δ sigH infected human macrophages revealed enrichment of ATG genes by Δ sigH and provided some mechanistic insight for the Δ sigH vaccination efficacy. The immune response in excised granulomas from the three arms was evaluated and some enhancements were observed in the Δ sigH over BCG vaccinated granulomas (although some results appear to be overstated). CyCIF labeling and microscopy was performed to further study granuloma phenotypes identified as permissive or protective in unvaccinated and Δ sigH vaccinated respectively. Myeloid cells including IDO+ MDSCs and structural cells were major cell populations present in unvaccinated granulomas whereas Δ sigH vaccinated lesions were primarily iBALT and contained increased B cell numbers indicative of a protective state.

The presented work builds on extensive previous studies by the authors investigating the role of stress response factor SigH in TB pathogenesis in vitro and in NHP models. The significance of the manuscript is high given the urgent need to develop effective TB vaccines for at-risk populations. The reduced bacterial burden in the Δ sigH vaccinated animals in comparison to BCG was clear from the included data. However, significant improvements in pathology (CXR, inflammation, necrosis) were not demonstrated in contrast to the claims made in the results and discussion. Supporting evidence is presented to demonstrate a protective immune phenotype in Δ sigH-vaccinated macaques and superior activation of T cell responses was demonstrated in the Δ sigH vaccinated animals in comparison to BCG-vaccinated and unvaccinated. The proposed mechanism for protection via Δ sigH vaccination is through upregulation of autophagy, cytokine secretion, and antigen presentation/processing and the presented data is generally supportive of this. Moreover, the increased induction of iBALT

in the Δ sigH-vaccinated lungs assigned as protective is an interesting mechanism for the increased Mtb control demonstrated in the Δ sigH-vaccinated animals. However, as the authors do not include data comparing iBALT in granulomas from Δ sigH- versus BCG vaccinated animals it is not possible to assess this.

Main weaknesses of the manuscript are the mechanistic interpretations based on data that is not demonstrated as reaching accepted significance ($p < .05$). This occurs throughout the manuscript (as detailed below). If the authors have a strong rationale for using a higher threshold for significance, then it should be clearly justified. In addition, other claims lack supporting evidence or sufficient explanation. For example, how many animals/granulomas were analyzed to support the claims derived from the CyCIF studies? The significance of the increased protection offered by Δ sigH vaccination is somewhat tempered as for a vaccine to be successful, sustained protection is required and this is not demonstrated in this study, which is conducted relatively shortly post-vaccination. However, the authors identify this limitation and propose to investigate this in future studies.

In summary, I recommend the manuscript may be suitable for acceptance after a thorough review of the significance of the data that 1) supports improved efficacy of Δ sigH in comparison to BCG vaccination and 2) is used to identify proposed mechanisms of protection. Specific concerns to address are detailed below.

The referenced IV BCG vaccination study (Darrah et al PMID: 31894150) showed limited to no improvement in detectable CFU and inflammation in a study arm of Mtb-challenged macaques (measured through Pet CT) vaccinated with a much higher aerosol dose of BCG (5×10^7 CFU) versus unvaccinated. Why was such a low dose (1000 CFU) selected as the challenge dose for this study if the previous higher vaccination doses were already demonstrated to be ineffective?

(48) "Although intravenous delivery of BCG has demonstrated TB prevention in macaques, safety concerns are associated with this vaccination route³" The authors should expand upon this statement. The macaque studies conducted in the referenced paper showed increased markers for inflammation and relatively mild increases in liver-function enzymes, but further evidence or explanation is required that these levels would be unsafe.

(63) "In the absence of SigH, Mtb fails to scavenge host oxidative products, resulting in an inability to survive or induce pathology in lungs¹⁹" Figure 3c of the referenced article (Mehra et al, PMID: 22402035) reveals granulomas present in the lungs of the Mtb: Δ -sigH infected rhesus macaques although far less severe or involved than Mtb CDC1551 infected animals. Therefore, this statement requires further clarification.

Why was 8 weeks selected as the timepoint for the post-vaccination Mtb challenge? This is not explained in the text. As the authors previous publications demonstrate the presence of viable bacilli in the lungs of rhesus macaques at 10+ weeks post Mtb: Δ -sigH vaccination. Is it possible that there was still an active infection with Mtb: Δ -sigH at the time of Mtb CDC1551 challenge?

(280) "These results are supported by the analysis of CXR scans at the endpoint which show significantly lower granulomatous pathology in the Δ sigH vaccinated, relative to the other two groups (Fig 1c)." The CXR data is shown in Fig 1d and there is no significant difference between the BCG vaccinated and Δ sigH vaccinated. Such claims of significance that is not supported by the data occur repeatedly throughout the manuscript.

(305) "The lung lesions observed in the few Δ sigH vaccinated CMs, were non-necrotic, with abundant lymphoid follicles (Fig 1n,q)." While the included histology images do not show necrosis, the morphometric quantification data shown in Fig 1t indicates that some necrosis was present in the Δ sigH vaccinated CMs and the level was not significantly different to the BCG vaccinated CMs. This discrepancy should be explained.

(309) "Together, these results suggest that while the total granulomatous pathology was present at the lowest level in the lungs of Δ sigH vaccinated macaques, greater iBALT responses generated in this group contributed to the higher than baseline lung pathology observed in this group." The authors do not demonstrate that total granulomatous pathology was present at the lowest levels in the lungs of the Δ sigH vaccinated animals as there was no significant differences observed in CXR, lung pathology, % cellular inflammation, or % necrotic area between BCG vaccinated and Δ sigH vaccinated animals.

(359) "Very few CD3+ (Fig S4i), CD4+ (Fig S4j), and CD8+ (Fig S4k) T cells and B cells (Fig S4l) displayed proliferative (Ki67+) phenotype, but in most cases, significantly higher frequencies were observed for the Δ sigH- relative to BCG vaccinated CMs." This may be the case at the early post vaccination time points analyzed, but by the 7 week timepoint there appears to be little difference (the only significant increase in Ki67+ phenotype is in CD20+ cells).

Figure S4d axis states "weeks post Mtb infection", should be post vaccination.

The discussion for the flow cytometry data lacks the temporal aspect of the T cell response (figures S3 and S4). Rarely are the differences between Δ sigH and BCG significant across multiple timepoints.

The inclusion of significance values in the Figures S3 through S6 appears somewhat arbitrary with the inclusions of comparisons that reach significance, some that just miss (just above $p 0.05$), and some much higher (for example, Fig S3m has a comparison with a p value of 0.31). Yet p values are not provided for other comparisons. The authors should be clear regarding the inclusion of p values for some comparisons that are significant and some that are not significant and why p values are not provided at all for many comparisons.

Figure 5, it is difficult to read the gene IDs particularly in 5b and 5e due to poor resolution of the images or compression artifacts.

(464) “The genes significantly induced in the BAL of Δ sigH relative to BCG vaccinated CMs in C6 (Table S4, Fig 5e) included those affected by IFNs, e.g., XAF1, which induces control pathogens via apoptosis and in an IRF1-dependent manner⁵⁴ and ILF2, which promotes T cell proliferation via IL-2.” ILF2 gene expression data appears to be missing from Table S4 and is not legible/present in the axis label for Fig 5e.

(559) “Phagosomal acidification is a critical bacterial control mechanism, but Mtb is known to subvert it in a SigH-dependent manner.” Further evidence or literature citation/s should be supplied to support this statement.

(564) The section title is “Comparison of the phenotype of Δ sigH, relative to BCG and Mtb in human macrophages”. However, the authors don’t present any data from BCG-infected macrophages.

(574) “Thus, the expression of antigen processing genes ATG5 (Fig 6d), ATG7 (Fig 6e), SQSTM1 (Fig 6f) was induced to significantly higher levels in the cells infected with Δ sigH, relative to Mtb or BCG.” This is shown for ATG5 (Fig 6d) and ATG7 (Fig 6e), but SQSTM1 (Fig 6f) is shown to be significantly increased in Mtb-infected cells versus Δ sigH. This discrepancy should be explained. Further, no in vitro data is shown for BCG and no in vitro experiments with BCG are described in the methods. Therefore, it is not clear how the authors can state any comparisons to BCG.

(604) “The granulomas of Δ sigH-vaccinated group harbored a greater frequency of memory CD4+ T cells, relative to both the BCG-vaccinated and the unvaccinated groups (Fig 7c).” There is not a significant difference between the three groups.

(607) “Within the memory CD4+ T cell pool, significantly greater frequency of activated (CD69+) cells were present in the Δ sigH-, relative to the BCG608 vaccinated group (Fig 7d) while their proliferative capacity was significantly lower (Fig 7e).” There is no statistical significance between the groups in fig 7e.

(611) “Within the effector pool, again the frequency of activated (CD69+) CD4+ T cells was significantly higher after Δ sigH, relative to the BCG-vaccination (Fig 7f)” Again, the p value displayed in Fig 7f does not reach significance.

The same issue occurs in additional figure panels (Fig 7h,j,m,n). The authors should review this section and figure 7 carefully to determine which changes in cell phenotypes are significant and accurately describe this in their results and discussion.

Figure 8, It is unclear how the CyCIF analyses were performed. The authors state that “The lung section from the unvaccinated macaque was characterized by granulomas with extensive central necrosis, and higher frequency of myeloid cells in the rim adjoining the necrotic area” and this is shown in one example in panel 8A. However, panel 8B appears to show multiple lung sections without any obvious necrotic (permissive) granulomas. The tissue sections from the Δ -sigH lungs have clear granulomas or iBALT structures (Fig 8e).

(705) “Significantly more B cell populations (B cells, proliferative B cells) were present in the lungs of Δ sigH vaccinated (Fig 8p, S9f), relative to unvaccinated CMs (Fig 8o, S9e). These cells organized in iBALT to a significantly greater extent (Fig 8p, S9f).” It is not clear how significance was calculated here. No numerical or p values are provided in Fig 8 o&p and Fig S9 e&f just show representative microscopy images of cell markers.

(708) “On the contrary, the permissive granulomas from unvaccinated/Mtb infected CMs were characterized by greater influx of myeloid cells (Fig 8a) including IDO+ MDSCs (Fig 8c).” How many granulomas from each condition is this observation based on? Only one representative image is shown for an individual granuloma and no clear granulomas are observed in the whole slide image shown in 8b.

Some figures include actual p values (e.g. Fig 7) whereas others use an asterisk labeling system (Fig 6). The approach should be standardized throughout the manuscript.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors have addressed the reviewers comments

Reviewer #4

(Remarks to the Author)

The revised manuscript is well written and enhanced by it's improved brevity and clarity. The included additional data alleviates concerns regarding the statistical significance of stated improvements in Mtb Δ sigH efficacy in comparison to BCG. Overall, the detailed revisions strongly improve the original manuscript and all previous concerns are addressed. The presented evidence for Δ sigH as a potential vaccine candidate is compelling, even at

this early stage, and the work may have high translational significance for pulmonary TB protection.

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Response to reviewers:

We thank the editors and the reviewers for a thorough review of our work entitled "Prevention of TB in nonhuman primates by a stress-response deficient mutant of Mycobacterium tuberculosis via induction of classical T cell immunity" and for excellent comments which we have responded to and which we believe have made this manuscript stronger and more coherent. We are also thankful to all the four reviewers for their broad support for this work. Here is a point-by-point rebuttal/response document outlining all the edits we have made to the manuscript:

Reviewer #1.

Comment/query 1: This is a very exciting report describing Enhanced Protective Efficacy of a TB vaccine candidate Mtb Δ sigH, a deletion of a gene encoding a Sigma factor that responds to oxidative stress, administered mucosally in Rhesus Macaques. This vaccination showed significant protection, characterized by the absence of granulomas and the presence of robust antigen-specific T cell responses and hyper-immune trained immunity-like phenotypes in macrophages. Impressively, the comparison of the lungs of animals immunized with Mtb Δ sigH compared to unvaccinated using a recently developed spatial analysis reveals impressive protective immunity. The research suggests Δ sigH as a promising vaccine candidate, demonstrating essential correlates of protection including T cell activation and the recruitment of specialized macrophages.

This report is well-written describing carefully controlled studies.

Response: We thank reviewer 1 for their encouraging comments supporting the merit of our work.

Reviewer #2.

Comment/query 1: This manuscript presents efficacy data from a candidate TB vaccine – an attenuated M.tb strain, deficient in SigH. This is not a new vaccine candidate but the new data here is testing this vaccine in cynomolgus macaques and is compared to BCG. Both vaccines are given @ the same dose in the lungs. BCG and SigH mutant confer protection but the level of protection as measured by CFU counts is higher with the SigH compared to BCG although there was no difference in pathology score between the two vaccinated groups or CXR scores.

Response: We thank the reviewer for their insightful comments and their recognition of the novel data in this manuscript. At least two distinct factors dictated the lack of difference in lung pathology between the BCG and the Δ sigH groups. We briefly discussed this in the initial version but have expanded the discussion in the revision in response to the reviewer comments. The first is that the cynomolgus is a significantly more resistant NHP model relative to the rhesus, as has been borne out by the work of Dr. Flynn's group at the University of Pittsburgh. As a result, the amount of lung pathology generated after equivalent Mtb infection is significantly lower in cynomolgus than in rhesus exposed to low virulence Mtb CDC1551 strain. Furthermore, lung pathology, as calculated by our board-certified veterinary pathologist collaborators, that are authors on the paper (Drs Shivanna and Dick, Jr), is a sum of different pathological attributes, including area occupied by granuloma, necrosis, pneumonia, edema, hyperplasia, etc., and includes lymphoid follicles/aggregates. As we had shown in the original Fig 1x, the extent of lymphoid aggregates (B cell follicles or iBALT) occupied significantly greater lung area in Δ sigH relative to BCG vaccinated macaques. Lymphocytic aggregates are also considered a pathological sign as normal healthy lungs are devoid of such structures. Immunological profiling revealed higher antigen specific T and B cells in lungs of Δ sigH relative to BCG vaccinated macaques. Furthermore, Δ sigH is cleared more efficiently relative to BCG by macrophages. We believe these key immunological differences between Δ sigH and BCG vaccination drives higher antigenic clearance in the Δ sigH group.

Comment/query 2: Comprehensive interrogation of the induced immune response is presented.

The results and amount of data presented are impressive. But the manuscript is too long, particularly the results section, and I have suggested below some ways it could be made more succinct.

Response: We have significantly shortened the manuscript in response specifically to the comments by Rev 2. Our initial submission spanned 16640 words and 1524 lines, while the revised version is 14401 words and 1354 lines long, i.e., shortened by ~ 20% despite the addition of other data.

Comment/query 3: specific comments:

- Abstract line 22 – bacille not bacillar.

Response: We regret this error. The typographical error has been corrected in the revised manuscript (line 22).

Comment/query 4: specific comments:

- Reference 1 – suggest reference the WHO report rather than a reference referencing it – and correct the numbers – is 1.3m deaths in the latest (2023) report.

Response: Please see [lines 40, 1002-1005](#) in the revised manuscript. We have updated the reference.

Comment/query 5: specific comments: • Line 322 The statement: No changes were detected in the frequencies of effector, memory or naïve CD4+ or CD8+ T cells in BAL post Mtb challenge (Fig S2h-m) or in T cell phenotypes in peripheral blood mononuclear cells (PBMCs) post challenge in the three vaccinated groups (not shown) is somewhat surprising – one would have expected this to be higher in the unvaccinated animals with a higher bacillary burden.

Response: We agree with the reviewer that the frequencies of effector T cells strongly correlate with antigenic burden and associated pathology. However, secondary immune responses are elicited significantly more rapidly after a prior vaccination, compared to primary responses. In the unvaccinated group the responses are primary in nature and driven by higher pathogenic burden while in the vaccinated group/s these responses are secondary in nature. These immunological differences are however more pronounced in the lung compartment compared to peripheral blood, especially due to the aerosol vaccination nature of our experiment. Vaccinated animals usually confer a faster recall response which can be observed in early phases in the BAL and observing these changes in the periphery is challenging with limited subjects (n) per group.

Comment/query 6: specific comments: • The disconnect between CFU counts and pathology scores should be commented on – usually there is a close correlation between these read outs.

Response: We have now discussed this at length in response to query 1 and 5 above.

Comment/query 7: specific comments: • It is not entirely clear why the authors continue to study this strain – given, as they highlight in the discussion, it only has a single mutation and is not appropriate for moving into clinical trials. They discuss the need to develop a second mutation and see if that still confers protection. As proof of concept in a second strain of macaques, the work presented here is interesting but the vaccine has already been demonstrated to be protective in Rhesus macaques.

Response: The reviewer's point is well taken. We have now developed multiple double and triple (DKO, TKO) knock-out mutants in Mtb based on *ΔsigH*. These mutants are rationally designed in that the other genes in which deletions have been made are unrelated to SigH but result in attenuation. Furthermore, these deletions have been generated precisely using high-efficiency phage technology, resulting in the subsequent excision of antibiotic resistance markers. The multiple candidate *ΔsigH* based DKO/TKO vaccine strains are therefore human-ready. We have tested these strains for safety in immunocompetent and immunosuppressed (SIV co-infected) macaques (Arora G et al, manuscript in preparation) and are currently seeking to obtain funding from the NIH to test their immunogenicity and efficacy. We eventually do see the path forward with *ΔsigH* based DKO/TKO strains for human testing. At this point, however, we do not have immunogenicity/efficacy data with DKO/TKO strains. The current study however provides in-depth understanding of very early immune responses engendered by *ΔsigH* and how such responses shape protective adaptive responses. Such knowledge, we content, is critical not only for the development of *ΔsigH*-based, but other vaccines, against TB.

• Figure 9 is unnecessary – there are too many figures in this manuscript

Response: We have excluded this figure from the revised version of the manuscript.

Comment/query 8: • The results section is too long – 26 pages. This could be written much more succinctly without the added interpretation of the results. E.g. line 277: These results clearly show that while mucosal vaccination with BCG offers mild protection against Mtb challenge in CMs, comparable vaccination with *ΔsigH* results in a >1000-fold greater protection; and line 309: Together, these results suggest that while the total granulomatous pathology was present at the lowest level in the lungs of *ΔsigH* vaccinated macaques, greater iBALT responses generated in this group contributed to the higher than baseline lung pathology observed in this group; line 340: B cell follicles are formed in the lung following *ΔsigH* vaccination and are important for protection from TB; etc. Some of this (there are many other examples) would sit better in the discussion – but also needs to be more succinct and not just transferred en bloc to the discussion.

Response: We have now shortened the entire manuscript, and particularly the results section, considerably. This includes the examples cited by the Reviewer.

Comment/query 9: • Line 389: I do not think the following statement is justified by the data: Because the immune cell dynamic of the lung environment in CMs was drastically altered by *ΔsigH* vaccination, relative to BCG vaccination; some of the immune readouts show a difference between BCG and delta sig H but not all do and some readouts are comparable. The word drastically is unnecessarily subjective and emotive.

Response: We have altered the text to temper our results and conclusions so as not to be subjective.

Comment/query 10: • Some of the language could be simpler – e.g. line 579 – TB vaccines not anti TB vaccines.

Response: Edited as recommended.

Comment/query 11: • Line 630 – list of protein markers – should be in supp figure not in main text.

Response: We have made this change.

Reviewer #3.

Comment/query 1: This manuscript tested the efficacy of attenuated *Mycobacterium tuberculosis* vaccine with the absence of sigH, the master regulator of responses to multiple stress conditions such as oxidative and nitrosative stress, phagocytosis, hypoxia and so on, as well as providing mechanistic understandings underlying the protection conferred by Δ sigH vaccination, in the more human-like cynomolgus macaque model of aerosol infection rather than rhesus macaque. Δ sigH vaccination significantly reduced lung and extra-thoracic CFU and pathology in cynomolgus macaques. Furthermore, mucosal vaccination with Δ sigH recruited T cells expressing IFN- γ to the airways and lungs, priming an IFNG-responsive rather than a Type I IFN-responsive phenotype in macrophages. These results might shed light on a potential anti-TB vaccine, although it's in its very early stage.

Response: We thank the reviewer for positive comments. We agree that the results are early stage and point this out in the revised discussion (line 701).

Comment/query 2: However, there're still several limitations/mistakes across the whole text.

The image resolution of some figures (e.g. Figure 5, Figure 8) is too low that the details couldn't be recognized such as gene name and so on.

Response: We regret these mistakes and have taken care to correct these throughout in the revised version. The resolution on submitted images was lost while rendering the figures on the journal submission website. We are attaching a high-resolution PDF of the updated figures with this submission for the reviewer's perusal.

Comment/query 3: The scRNA-seq data was not well organized that

- 1) classical markers defining each cell type should be clearly displayed in Fig S7.

Response: We have updated figure S7 as requested.

- 2) the makers for each lymphocyte or myeloid cluster should be carefully selected or arranged to ensure that cluster info could be easily received. In addition, violin plots are recommended;

Response: We have updated figure 4b and 6c and included the violin plots in figure 4c and 6c.

- 3) There's no need to label colors for different groups in Fig 5b-5g as the group name has been signed. Instead, the expression level of selected genes should be displayed that expression ratio and level could be compared directly;

Response: We have updated figures 5 b-g and 6d-e to reflect the same.

- 4) The analysis strategy used in Fig 7 could be also carried out in scRNA-seq data.

Response: Figure 4c-I depicts scRNAseq cell fractions and has been presented in a similar manner as Fig 7.

- 5) There're several labeling mistakes. For example, Fig S2p should be Fig S1p (line 277); Fig 1C didn't show the CXR scan result (line 282); line 377, Fig S3b should be Fig S3p? line 483, expressed IFIT3, MX2, IRF7 higher levels of IFIT3.

Response: These errors has been corrected in the updated manuscript. Please see lines 225, 227, 269 and 383 in the revised manuscript.

Reviewer #4.

Comment/query 1: The presented work builds on extensive previous studies by the authors investigating the role of stress response factor SigH in TB pathogenesis in vitro and in NHP models. The significance of the manuscript is high given the urgent need to develop effective TB vaccines for at-risk populations. The reduced bacterial burden in the Δ sigH vaccinated animals in comparison to BCG was clear from the included data. However, significant improvements in pathology (CXR, inflammation, necrosis) were not demonstrated in contrast to the claims made in the results and discussion.

Response: We have updated the results to accurately reflect the aforementioned comments. This has also been pointed out by R2 and have been discussed in length in Query 1 and 5 above.

Comment/query 2: Supporting evidence is presented to demonstrate a protective immune phenotype in Δ sigH-vaccinated macaques and superior activation of T cell responses was demonstrated in the Δ sigH vaccinated animals in comparison to BCG-vaccinated and unvaccinated. The proposed mechanism for protection via Δ sigH vaccination is through upregulation of autophagy, cytokine secretion, and antigen presentation/processing and the presented data is generally supportive of this. Moreover, the increased induction of iBALT in the Δ sigH-vaccinated lungs assigned as protective is an interesting mechanism for the increased Mtb control demonstrated in the the Δ sigH-vaccinated animals. However, as the authors do not include data comparing iBALT in granulomas from Δ sigH- versus BCG vaccinated animals it is not possible to assess this.

Response: We have now included this data in Fig o-r and lines 507-511.

Comment/query 3: Main weaknesses of the manuscript are the mechanistic interpretations based on data that is not demonstrated as reaching significance (p<.05) throughout the manuscript. If the authors have a strong rationale for using a higher threshold for significance, then it should be clearly justified.

Response: We have updated the results and discussion to accurately reflect the data reaching significance.

Comment/query 4: In addition, other claims lack supporting evidence or sufficient explanation. For example, how many animals/granulomas were analyzed to support the claims derived from the CyCIF studies?

Response: One section containing randomly sampled lung biopsies from the same lung lobe were analyzed from each group. Figure 8b has been updated to reflect the section containing granuloma shown in figure 8a. The samples could not be randomly selected for the *ΔsigH* group due to scarcity of well-formed granulomas in 8 out of 9 animals.

Comment/query 5: The significance of the increased protection offered by *ΔsigH* vaccination is somewhat tempered as for a vaccine to be successful, sustained protection is required and this is not demonstrated in this study, which is conducted relatively shortly post-vaccination. However, the authors identify this limitation and propose to investigate this in future studies.

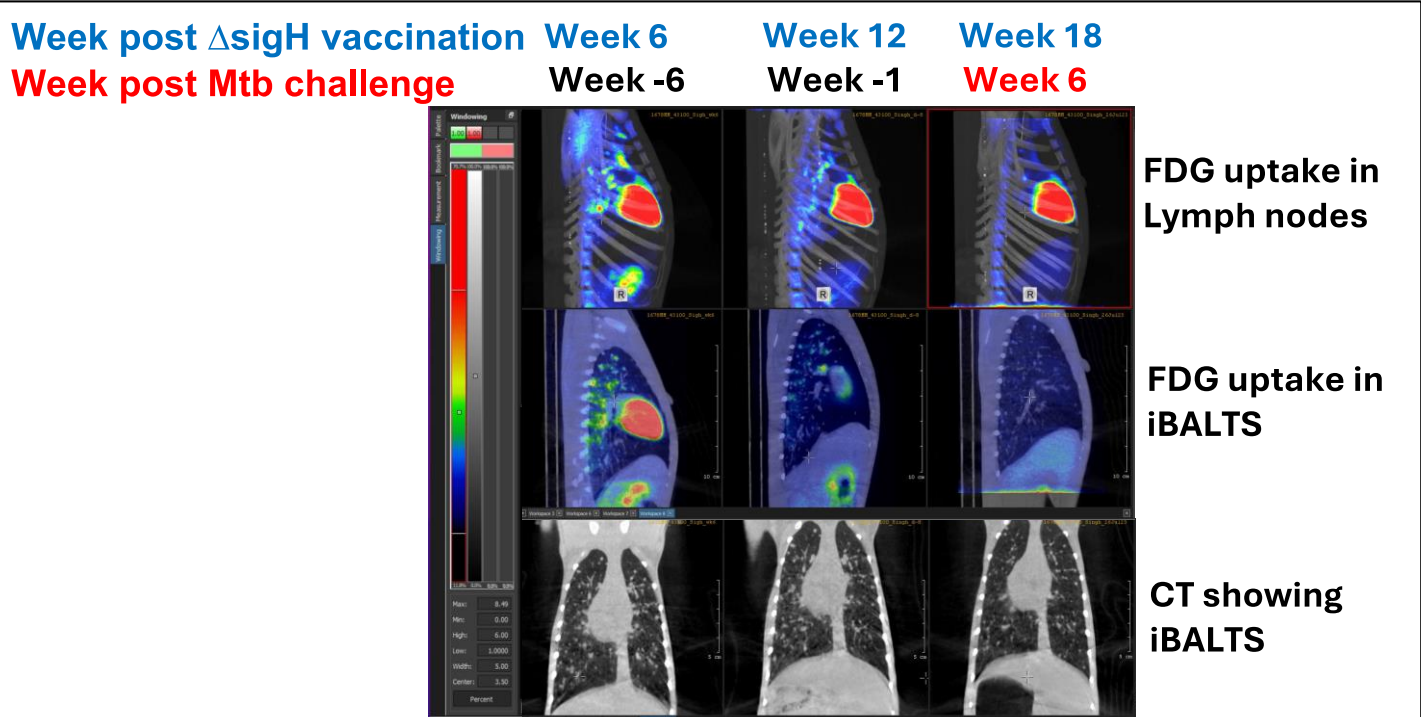
Response: We appreciate the reviewer’s insight and agree that induction of sustained protection is an essential feature of a vaccine candidate. We have upcoming studies planned to interrogate the durability of *ΔsigH* induced protection.

Comment/query 6: In summary, I recommend the manuscript may be suitable for acceptance after a thorough review of the significance of the data that 1) supports improved efficacy of *ΔsigH* in comparison to BCG vaccination and 2) is used to identify proposed mechanisms of protection. Specific concerns to address are detailed below.

Response: We thank the reviewer for their detailed and important comments. We have significantly revised the manuscript with respect to the significance of the data and revised statements unacceptable to the reviewer. Please see specific responses below.

Comment/query 7: The referenced IV BCG vaccination study (Darrah et al PMID: 31894150) showed limited to no improvement in detectable CFU and inflammation in a study arm of Mtb-challenged macaques (measured through Pet CT) vaccinated with a much higher aerosol dose of BCG (5 × 10⁷ CFU) versus unvaccinated. Why was such a low dose (1000 CFU) selected as the challenge dose for this study if the previous higher vaccination doses were already demonstrated to be ineffective?

Response: Darrah et al have conclusively shown limited efficacy of both intradermal and intramucosal BCG at a much higher dose. However, our premise of using the low dose of 1000 CFU of BCG was to match it with the delivery dose of *ΔsigH* and our studies also recorded a modest protection in the aerosol BCG group. However,



our overarching aim in this experiment was to establish superiority of *ΔsigH* strain over BCG. We have performed follow up studies with comparatively higher doses of *ΔsigH* going up to 10000 CFU and recorded dose dependent induction of iBALTs observed in temporal CT scans. (Figure provided for reviewer’s perusal).

Comment/query 8: (48) “Although intravenous delivery of BCG has demonstrated TB prevention in macaques, safety concerns are associated with this vaccination route³” The authors should expand upon this statement.

The macaque studies conducted in the referenced paper showed increased markers for inflammation and relatively mild increases in liver-function enzymes, but further evidence or explanation is required that these levels would be unsafe.

Response: Intravenous BCG vaccination induces very strong and durable immune responses. However, it is uniformly believed that delivery of high doses of BCG intravenously is unlikely to be approved for clinical administration in humans. This is particularly due to the impact of persisting BCG in HIV co-infected people and children. We have now referenced these statements (lines 597-599). BCG is known to persist in the host for extended duration and may cause BCGitis/BCGiosis (e.g., PMC7785948) Intravesicular BCG is used in bladder cancer and several reports have attributed systemic inflammatory BCGitis/BCGosis (e.g., PMC9096469) as a potential risk.

Comment/query 9: (63) “In the absence of SigH, Mtb fails to scavenge host oxidative products, resulting in an inability to survive or induce pathology in lungs¹⁹” Figure 3c of the referenced article (Mehra et al, PMID: 22402035) reveals granulomas present in the lungs of the Mtb:Δ-sigH infected rhesus macaques although far less severe or involved than Mtb CDC1551 infected animals. Therefore, this statement requires further clarification.

Response: We have updated the text in [redacted] to reflect “In the absence of SigH, *Mtb* is susceptible to host stress with significantly reduced survival and pathogenicity in lungs¹⁸, even after immunosuppression due to SIV co-infection¹⁹.”

Comment/query 10: Why was 8 weeks selected as the timepoint for the post-vaccination Mtb challenge? This is not explained in the text. As the authors previous publications demonstrate the presence of viable bacilli in the lungs of rhesus macaques at 10+ weeks post Mtb:Δ-sigH vaccination. Is it possible that there was still an active infection with Mtb:Δ-sigH at the time of Mtb CDC1551 challenge?

Response: The animal experiments included in this manuscript closely followed our previous work (Kaushal et al Nature Communications 2015) where aerosol ΔsigH vaccination was shown to be highly efficacious in the rhesus macaque challenge model. In that experiment we chose to challenge 8-9 weeks post vaccination, since very little to no culturable vaccine strains (BCG or the mutant) could be detected in the lung compartment (BAL) of high-dose vaccinated macaques. Furthermore, we observed that the T cell responses induced by ΔsigH vaccination initiated by week 3, peaked at weeks 5-7, and began contracting by week 7 indicating antigenic clearance from the host.

Comment/query 11: (280) “These results are supported by the analysis of CXR scans at the endpoint which show significantly lower granulomatous pathology in the ΔsigH vaccinated, relative to the other two groups (Fig 1c).” The CXR data is shown in Fig 1d and there is no significant difference between the BCG vaccinated and ΔsigH vaccinated. Such claims of significance that is not supported by the data occur repeatedly throughout the manuscript.

Response: We have updated this and other text to more accurately reflect the data.

Comment/query 12: (305) “The lung lesions observed in the few ΔsigH vaccinated CMs, were non-necrotic, with abundant lymphoid follicles (Fig 1n,q).” While the included histology images do not show necrosis, the morphometric quantification data shown in Fig 1t indicates that some necrosis was present in the ΔsigH vaccinated CMs and the level was not significantly different to the BCG vaccinated CMs. This discrepancy should be explained.

Response: Only one of the (~ 1000 cfu) ΔsigH vaccinated animals showed the presence of granulomas, and while some necrosis was present, it was significantly lower than in the unvaccinated animals, i.e., after ~ 100 cfu Mtb infection. We agree with the reviewer that the level of necrosis after ΔsigH vaccination was statistically indistinguishable from the BCG group, and we allude to this in results (lines 238-240) and discussion (lines 651-654).

Comment/query 13: (309) “Together, these results suggest that while the total granulomatous pathology was present at the lowest level in the lungs of ΔsigH vaccinated macaques, greater iBALT responses generated in this group contributed to the higher than baseline lung pathology observed in this group.” The authors do not demonstrate that total granulomatous pathology was present at the lowest levels in the lungs of the ΔsigH vaccinated animals as there was no significant differences observed in CXR, lung pathology, % cellular inflammation, or % necrotic area between BCG vaccinated and ΔsigH vaccinated animals.

Response: In response to this important comment by the reviewer, we have revised the interpretation of our results in this section and edited the text to remove the statement pointed out. Please see lines 229-253 for the revised pathology results. We have previously shown, that in the susceptible rhesus macaque model of Mtb CDC1551 challenge, aerosol vaccination with ΔsigH results in significantly lower pathology relative to not only unvaccinated, but also comparably BCG-vaccinated animals. Due to the highly resistant nature of the model (cynomolgus) used here, we believe that it has not possible to demonstrate statistical difference in lung

pathology scores between the BCG and the *ΔsigH* groups. We discuss this shortcoming in the Discussion (lines 651-654).

Comment/query 14: (359) “Very few CD3+ (Fig S4i), CD4+ (Fig S4j), and CD8+ (Fig S4k) T cells and B cells (Fig S4l) displayed proliferative (Ki67+) phenotype, but in most cases, significantly higher frequencies were observed for the *ΔsigH*- relative to BCG vaccinated CMs.” This may be the case at the early post vaccination time points analyzed, but by the 7 week timepoint there appears to be little difference (the only significant increase in Ki67+ phenotype is in CD20+ cells).

Response: We agree with the reviewer that the cellular immune response show an expansion during week 3 and 5 post vaccination and contraction through week 7 indicating antigenic clearance. This is likely also due to the model used in the current study. Cynomolgus are known to be more resistant to *Mtb* infection relative to rhesus, which we have previously utilized in our work.

Comment/query 15: Figure S4d axis states “weeks post *Mtb* infection”, should be post vaccination.

Response: We regret the error and thank the reviewer for pointing it out. This axis label has been corrected.

Comment/query 16: The discussion for the flow cytometry data lacks the temporal aspect of the T cell response (figures S3 and S4). Rarely are the differences between *ΔsigH* and BCG significant across multiple timepoints.

Response: The temporal aspect of flow cytometry data was minimally discussed to reduce the length of the manuscript. Key temporal trends have now been briefly discussed in lines: 278-312.

Comment/query 17: The inclusion of significance values in the Figures S3 through S6 appears somewhat arbitrary with the inclusions of comparisons that reach significance, some that just miss (just above p 0.05), and some much higher (for example, Fig S3m has a comparison with a p value of 0.31). Yet p values are not provided for other comparisons. The authors should be clear regarding the inclusion of p values for some comparisons that are significant and some that are not significant and why p values are not provided at all for many comparisons.

Response: In the revised version of the manuscript, we have only included p-values that are significant or approaching significance in the supplement.

Comment/query 18: Figure 5, it is difficult to read the gene IDs particularly in 5b and 5e due to poor resolution of the images or compression artifacts.

Response: A high-resolution PDF for figures has been provided with this submission.

Comment/query 19: (464) “The genes significantly induced in the BAL of *ΔsigH* relative to BCG vaccinated CMs in C6 (Table S4, Fig 5e) included those affected by IFNs, e.g., XAF1, which induces control pathogens via apoptosis and in an IRF1-dependent manner⁵⁴ and ILF2, which promotes T cell proliferation via IL-2.” ILF2 gene expression data appears to be missing from Table S4 and is not legible/present in the axis label for Fig 5e.

Response: We have now significantly reduced the results section for scRNAseq to only focus on the most important results.

Comment/query 20: (559) “Phagosomal acidification is a critical bacterial control mechanism, but *Mtb* is known to subvert it in a *SigH*-dependent manner.” Further evidence or literature citation/s should be supplied to support this statement.

Response: Please see edits (lines 439-441).

Comment/query 21: (564) The section title is “Comparison of the phenotype of *ΔsigH*, relative to BCG and *Mtb* in human macrophages”. However, the authors don’t present any data from BCG-infected macrophages.

Response: Since no data has been presented for BCG, the text in this section has been updated to reflect comparison of *ΔsigH*, relative to wild type *Mtb* CDC1551.

Comment/query 22: (574) “Thus, the expression of antigen processing genes ATG5 (Fig 6d), ATG7 (Fig 6e), SQSTM1 (Fig 6f) was induced to significantly higher levels in the cells infected with *ΔsigH*, relative to *Mtb* or BCG.” This is shown for ATG5 (Fig 6d) and ATG7 (Fig 6e), but SQSTM1 (Fig 6f) is shown to be significantly increased in *Mtb*-infected cells versus *ΔsigH*. This discrepancy should be explained. Further, no in vitro data is shown for BCG and no in vitro experiments with BCG are described in the methods. Therefore, it is not clear how the authors can state any comparisons to BCG.

Response: Since no data has been presented for BCG, the text in this section has been updated to reflect comparison of *ΔsigH*, relative to wild type *Mtb* CDC1551. Regarding SQSTM1, it has roles beyond autophagy and we do not well understand changes in its expression pattern. We have therefore removed the expression of SQSTM1 from the paper, as ATG5 and 7 data adequately explain our point.

Comment/query 23: (604) “The granulomas of Δ sigH-vaccinated group harbored a greater frequency of memory CD4+ T cells, relative to both the BCG-vaccinated and the unvaccinated groups (Fig 7c).” There is not a significant difference between the three groups.

Response: Additional granulomas have been analyzed and the updated dataset has been provided in Fig 7. The results and discussion are updated accordingly.

Line 484: “The granulomas of Δ sigH-vaccinated group harbored a greater frequency of memory CD4+ T cells, relative to the BCG-vaccinated group (Fig 8c).”

Comment/query 24: (607) “Within the memory CD4+ T cell pool, significantly greater frequency of activated (CD69+) cells were present in the Δ sigH-, relative to the BCG608 vaccinated group (Fig 7d) while their proliferative capacity was significantly lower (Fig 7e).” There is no statistical significance between the groups in fig 7e.

Response: Additional granulomas have been analyzed and the updated dataset has been provided in figure 7. The results and discussion are updated accordingly.

Line 488: “The proliferative capacity was comparable across the vaccinated groups but significantly lower than unvaccinated groups (Fig 8e),”

Comment/query 25: (611) “Within the effector pool, again the frequency of activated (CD69+) CD4+ T cells was significantly higher after Δ sigH, relative to the BCG-vaccination (Fig 7f)” Again, the p value displayed in Fig 7f does not reach significance.

Response: Additional granulomas have been analyzed and the updated dataset has been provided in figure 8. For this section no update in text was needed.

Comment/query 26: The same issue occurs in additional figure panels (Fig 7h,j,m,n). The authors should review this section and figure 7 carefully to determine which changes in cell phenotypes are significant and accurately describe this in their results and discussion.

Response: Additional granulomas have been analyzed and the updated dataset has been provided in figure 7. The results and discussion sections have been updated accordingly.

Line 501: “Within this fraction, significantly higher frequencies of activated (Fig 8l) and comparatively higher but statistically non-significant lung homing (CCR5+) (Fig 8m) cells were also present after Δ sigH-, relative to BCG-vaccination.”

Comment/query 27: Figure 8, It is unclear how the CyCIF analyses were performed. The authors state that “The lung section from the unvaccinated macaque was characterized by granulomas with extensive central necrosis, and higher frequency of myeloid cells in the rim adjoining the necrotic area” and this is shown in one example in panel 8A. However, panel 8B appears to show multiple lung sections without any obvious necrotic (permissive) granulomas. The tissue sections from the Δ -sigH lungs have clear granulomas or iBALT structures (Fig 8e).

Response: Fig 9b has been updated to reflect the section containing granuloma shown in Fig 9a.

Comment/query 28: (705) “Significantly more B cell populations (B cells, proliferative B cells) were present in the lungs of Δ sigH vaccinated (Fig 8p, S9f), relative to unvaccinated CMs (Fig 8o, S9e). These cells organized in iBALT to a significantly greater extent (Fig 8p, S9f).” It is not clear how significance was calculated here. No numerical or p values are provided in Fig 8 o&p and Fig S9 e&f just show representative microscopy images of cell markers.

Response: We have updated the statement to reflect “Relatively more B cell populations (B cells, proliferative B cells) were present in the lungs of Δ sigH vaccinated (Fig 9p, S9f), relative to unvaccinated CMs (Fig 9o, S9e). These cells organized in iBALT to a greater extent (Fig 9p, S9f).” Statistical significance couldn’t be calculated due to non-randomized granuloma targeted sampling of lung lobes and n=1 per group.

Comment/query 29: (708) “On the contrary, the permissive granulomas from unvaccinated/Mtb infected CMs were characterized by greater influx of myeloid cells (Fig 8a) including IDO+ MDSCs (Fig 8c).” How many granulomas from each condition is this observation based on? Only one representative image is shown for an individual granuloma and no clear granulomas are observed in the whole slide image shown in 8b.

Response: One section containing randomly sampled lung biopsies from the same lung lobe were analyzed from each group. Figure 8b has been updated to reflect the section containing granuloma shown in figure 9a. The samples could not be randomly selected for the Δ sigH group due to scarcity of well-formed granulomas in 8 out of 9 animals.

Comment/query 30: Some figures include actual p values (e.g. Fig 7) whereas others use an asterisk labeling system (Fig 6). The approach should be standardized throughout the manuscript.

Response: We regret this oversight. We now present a revised version of this manuscript where one standard approach has been used in all the figures/supplemental data section.