nature portfolio

Peer Review File

Poor sleep impairs immune responses and influenza vaccine protection

Corresponding Author: Professor Xiu-Feng Wan

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)
1. Please provide a rationale for selecting 105 male mice for your study. Clarifying how this sample size was determined—whether through power calculations or prior data—will enhance the transparency and statistical validity of your experimental design.
2. You wrote: “The current study was conducted exclusively in young adult male mice to minimize variability from hormonal cycling and because male mice tend to be more active, which better models sleep fragmentation induced by environmental disruption.” While minimizing hormonal variability is a common rationale, the exclusion of female mice raises concerns. Notably, females are known to exhibit stronger adaptive immune responses than males. This suggests that the immunosuppressive effects of chronic sleep fragmentation could be less pronounced in females. Furthermore, chronic sleep disturbances are frequently more prevalent in women than in men, which makes the inclusion of female animals particularly relevant from a translational perspective. Including both sexes would not only strengthen the biological relevance of your findings but also enhance their generalizability to human populations.
3. Your study used 6- to 8-week-old male mice. Can the results be extrapolated to influenza-vulnerable populations, particularly aged individuals? Given that public health vaccination campaigns emphasize the importance of influenza vaccination for older adults due to increased morbidity and mortality risks, it would be valuable to discuss whether your findings are applicable to aged mice, which more closely model the immune responses seen in elderly humans.
4. You state: “During vaccination, 0.2 μg HA of inactivated vaccines or PBS in a volume of 50 μL were injected intramuscularly per mouse.” The timing of vaccination significantly influences the magnitude of the adaptive immune response. Please specify the exact time of day when vaccinations were administered. Additionally, I suggest considering follow-up experiments comparing morning vs. evening vaccination to determine whether the impact of chronic sleep fragmentation on immune responses is modulated by circadian timing.
5. Please specify in each figure legend which statistical tests were used to generate the reported p-values. This is essential for readers to accurately interpret the results and assess the appropriateness of the analysis.
6. In the absence of EEG recordings, did you use alternative methods to quantify rest or sleep? For example, were 24-hour inactivity periods measured as a proxy for sleep? This would help to substantiate that the control and sleep-disrupted groups differ in rest duration or quality.
7. Please detail the housing conditions of the animals in all experimental groups. Were mice housed individually or in groups? Housing conditions can significantly affect stress levels and, consequently, immune responses. Both social isolation and overcrowding can modulate vaccine efficacy via stress-mediated pathways. Including this information is critical for interpreting your findings accurately.

Reviewer #2

(Remarks to the Author)
Poor sleep or chronic sleep fragmentation (CSF) is associated with increased risk of developing end-organ morbidities and alterations in both innate and adaptive immune responses, which are associated with increased risk of infection with influenza virus among other things. The goal of this study was to mechanistically understand the cause of this increased susceptibility using a mouse model of influenza virus vaccination and challenge. The authors first identified a vaccination regime that provided partial protection to a high dose challenge. Next, they performed a single experiment comparing sleep fragmented mice with a control group measuring a very large number of phenotypes from weight loss, mortality, antibody

responses, cellular responses, to detailed single cell transcriptional responses and B cell receptor analysis. Overall, sleep fragmentation reduced vaccine induced antibody responses resulting in higher virus load and a slight increase in mortality after infection. Contrary to expectation, the antigen-specific T and B cell response in these mice were improved suggesting changes in antibody production by these antigen specific B cells. To assess this, the investigators performed single cell sequencing on bulk splenocytes and found altered B cell responses and changes in B cell receptor diversity and composition. Overall, this is an important study that revealed many interesting new aspects of chronic sleep fragmentation on adaptive immunity. However, aside from transcriptional differences in B cells, it falls short in providing a mechanistic understanding of how these changes have occurred, and what factor(s) is driving it. Adoptive transfers experiments, for example, could have revealed a prominent role for T or B cells in antibody responses to vaccination.

Additional comments:
1) The single cell analysis was done on bulk splenocytes and not antigen-specific T or B cells. What was the rationale for this? Further, observing changes at the bulk cell level suggests that sleep fragmentation has a global effect on adaptive immune responses. A recent paper identified a role for BMAL1 and CLOCK (Circadian rhythm genes) and TGFb1 in antibody production after LPS stimulation. Were these genes differentially expressed between the groups? Again, adoptive transfer of congenic B cells from a control mouse into a sleep fragmented animal prior to immunization, could have revealed B cell intrinsic effects.
2) The entire manuscript appears to be one large mouse experiment with 4-5 mice per readout (mortality, virus titer, T and B cell responses etc.). However, some figures only have 2 or 3 data points (2e - T cells, and all of the single cell sequencing studies). Some key experiments should be repeated for scientific rigor.
3) What kind of statistical analysis was performed? Was a multiple comparisons correction applied? For example to Fig 1d.
4) In figure 5, the order of the groups (gSC and gSF) are reversed compared to other figures.
5) The experiments described in the first two paragraphs of the results section appear disconnected from the rest of the manuscript. First, the conclusion is that 0.1ug HA represents the ideal threshold for doing these studies but everything is done with 0.2ug HA. Also, is this the same inactivated whole virus vaccine that was used in the rest of the paper? If so, were antibody responses to other viral proteins measured? NP or NA for example and were similar differences observed?
6) Line 121, it is claimed that higher concentrations of NP are found, but there is no significant difference in 1e

Reviewer #3

(Remarks to the Author)
In this manuscript, the authors examined the effect of chronic sleep fragmentation (CSF) on the efficacy of influenza vaccination. They report that CSF is associated with reduced influenza-specific antibody responses and decreased survival rates. While these findings are of potential interest, much of the data presented are descriptive, with limited mechanistic insight, particularly regarding how CSF influences humoral immunity. Although the authors included single-cell sequencing analyses in the latter part of the manuscript, these results remain primarily descriptive and were not followed by validation experiments. In addition, some of the interpretations of the single-cell data may be somewhat speculative, as they rely largely on correlative associations, and should be interpreted with caution. Some other comments are outlined below:

1. In Figure 1, since the mice were subjected to CSF treatment for 10 weeks, which is a relatively long duration, the study design lacks critical control groups. Specifically, there is no group with CSF treatment alone, and no group with CSF treatment plus infection without vaccination. The absence of the latter is particularly concerning, as it makes it difficult to exclude the possibility that CSF itself affects susceptibility to infection.
2. In Figure 2e, representative wells from the ELISpot assay should be shown to support the quantification. In addition, it would be important to examine whether influenza-specific long-lived plasma cells in the bone marrow are affected.
3. For the cluster annotations in the single-cell sequencing data (Figure 3a and Supplementary Figure 2a), the authors should provide detailed justification for each cluster, including marker gene evidence and relevant references. In addition, it is unclear why so many follicular B cell clusters are defined; these should be consolidated where appropriate. The annotation of cluster 16 as DN T cells is also questionable, as this cluster does not appear to express Cd3d. Similarly, cluster 19 shows minimal expression of the surveyed genes yet is annotated as macrophages, which requires further explanation. Finally, the presence of a cDC1 cluster without a cDC2 cluster is puzzling.
4. Flow cytometric analysis of splenic germinal center B cells, plasma cells, and Tfh cells are important to include. Examining the expression of costimulatory ligands and MHC2 on B cells is warranted, since the authors already found differences in these pathways from their single-cell data.
5. In lines 238 and 247, the authors claim that CSF influences CD8 T cell–B cell signaling. This is unexpected, as direct regulation of antibody production by CD8 T cells is not well established. Such an interpretation appears speculative and may reflect a broader tendency in the manuscript to extend conclusions somewhat beyond the supporting evidence.
6. The TCR and BCR sequencing data presented in Figure 5 do not appear to support any clear conclusions. The authors should provide a more rigorous analysis that can directly addresses the study‘s main questions.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I maintain that the exclusion of female animals and the lack of replication in older animals represent significant limitations of the study. Nonetheless, I appreciate the authors' efforts and their careful consideration of the other points raised, and I have no further comments. My feedback is focused solely on the issues I raised and not on those raised by the other reviewer.

Reviewer #2

(Remarks to the Author)
The authors addressed the reviewers comments and overall improved the manuscript. The additional computational analysis and the flow cytometry experiment have led to a more balanced and tempered message regarding the role of sleep fragmentation on influenza vaccination and immune responses. The inclusion of the human data and the effect of OSA on risk of influenza infection with or without vaccination is a very nice addition to the paper.

Reviewer #3

(Remarks to the Author)
I appreciate the authors‘ revisions and their efforts to clarify certain interpretations in the revised manuscript. However, despite these changes, the central conceptual and experimental concerns raised in the initial review remain unresolved. As such, the current manuscript does not substantiate mechanistic conclusions regarding how CSF may influence humoral immunity, and several of the main conclusions continue to extend beyond what is directly supported by the data.
1. The authors acknowledge that additional control groups, specifically mice subjected to CSF alone and mice subjected to CSF combined with infection in the absence of vaccination, were not included and represent a limitation of the study. However, this acknowledgment does not address the core issue. The absence of these controls constitutes a fundamental confound rather than a minor limitation. It remains impossible to distinguish whether the observed reductions in survival and immune responses reflect impaired vaccine-induced protection or, alternatively, a generalized increase in susceptibility to infection caused by CSF itself. Moreover, the authors state that the study was specifically designed to evaluate how CSF alters vaccine-induced immune protection rather than baseline infection outcomes. If this was indeed the intention, then CSF exposure should have been restricted to the vaccination phase rather than applied continuously across the entire experimental timeline. As currently designed, the study cannot disentangle vaccine-specific effects from broader physiological consequences of long-term CSF. Importantly, this issue cannot be resolved through discussion alone. Without the inclusion of appropriate control groups or equivalent experimental evidence, the principal conclusions of the study remain insufficiently supported.
2. The inclusion of human HER (TriNetX) data showing a higher post-vaccination influenza risk among individuals with obstructive sleep apnea provides additional context and is potentially informative. However, similar to the animal data, the current analysis does not clearly distinguish whether this increased risk reflects reduced vaccine efficacy or an increased baseline susceptibility to infection. To address this, the authors could include an analysis comparing the magnitude of protection conferred by vaccination in control individuals (vaccinated versus unvaccinated) with that observed in individuals with obstructive sleep apnea (vaccinated versus unvaccinated).
3. Although the sequencing data add descriptive depth to the phenotypic characterization of immune alterations associated with CSF, several key analyses are either not followed by adequate validation or raise internal inconsistencies that are not sufficiently addressed.
a. The authors still did not analyze costimulatory ligands or MHC class II expression on B cells.
b. Flow cytometric data shown in Supplementary Figure 4 indicate no substantial changes in B cell or Tfh cell populations, raising the question of how the reported alterations in humoral immunity should be interpreted. In addition, the reported proportions of naïve CD4 and CD8 T cells in the spleen (approximately 10% or lower) are unexpectedly low and not consistent with established immunological knowledge. An explanation for these observations, including gating strategies and definitions, is warranted.
c. The TCR and BCR sequencing data presented in Figure 5 remain difficult to interpret and add limited mechanistic insight. Notably, the IgG-related results appear inconsistent with those shown in Figure 2c, in some cases even suggesting opposite trends.
The current presentation mainly describes the analysis results without explaining these inconsistencies or providing a convincing biological interpretation.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
I thank the authors for their efforts in revising the manuscript and for addressing the reviewers' comments. The revisions and additional experiments have improved the clarity of the study. I have no further comments.

Response Letter

EIDTOR COMMENTS

As you will see from the reports copied below, the reviewers raise important concerns. We find that these concerns limit the strength of the study, and therefore we ask you to address them with additional work. Without substantial revisions, we will be unlikely to send the paper back to review. In particular, the revised manuscript will need to validate key results with additional mouse experiments including appropriate controls, include additional mouse experiments to provide insights into the underlying mechanism of action (e.g. passive transfer experiments, functional analyses of immune cell subtypes...), improve and validate the scRNA-Seq analyses, and clarify statistical analyses and study design. Please ensure that the revised manuscript addresses these and all other concerns raised by our reviewers.

Response: We are very grateful for the guidance from the editors and the thoughtful and constructive critiques provided by the three anonymous reviewers. In this revised manuscript, we have undertaken substantial revisions to strengthen the rigor, clarity, and mechanistic depth of the study. These revisions directly address the key concerns raised, including additional experimental validation, improved single-cell analysis, and clearer statistical and study design reporting. Specifically, the following revision have been performed:

- Added flow cytometric quantification of splenic germinal center (GC) B cells, plasma cells, and Tfh cells, together with assessment of surface MHC class II and CD86 expression on total splenic B cells at 35 days post vaccination (dpv) (Supplementary Figure 4). No statistically significant differences were observed between gSF and gSC mice across these B-cell populations or activation markers. Among T-cell populations, gSF mice exhibited a trend toward increased total T-cell frequencies and reduced $CD4^+$ central memory (TCM) frequencies compared to gSC mice ($q = 0.067$ for both comparisons), while all other T-cell subsets were comparable between groups. Although these changes did not meet our predefined threshold for statistical significance ($q < 0.05$), their directionality aligns with the scRNA-seq–derived signatures indicating altered T-cell differentiation and disrupted T–B coordination under chronic sleep fragmentation. Together, these flow cytometric data provide supportive, orthogonal evidence that complements the transcriptomic findings and reinforce a model of subtle but coordinated immune reprogramming rather than gross cellular depletion.
- Provided marker-backed cluster justification and interpretation in the scRNA-seq data, with refined annotations (Fig. 3 and Supplementary Figure 2).
- Expanded repertoire analyses, including Hill diversity and additional clonotype overlap/architecture metrics, and report these alongside the single-cell results (Fig. 2 and Supplementary Figure 2).
- Clarified statistical analyses and specified the exact vaccination time, and housing conditions.
- Noted that a full passive antibody-transfer study and dedicated CSF+infection without vaccination and CSF-only control cohorts would be scientifically valuable; we explicitly acknowledge these as limitations and outline them as priorities for future studies.
- To support translational relevance, we also incorporated human EHR (TriNetX) data showing a higher rate of post-vaccination influenza diagnoses among individuals with obstructive sleep apnea (OSA), a clinical sleep-fragmentation phenotype, after propensity matching (Fig. 6). These findings align with the murine results and support generalizability and clinical significance.

In addition, we have comprehensively revised the manuscript, particularly the Discussion, to strengthen mechanistic interpretation, integrate animal and human findings, and clarify the broader clinical significance of this work. We believe these extensive revisions substantially improve the quality and impact of the manuscript, and we hope the updated version satisfactorily addresses the reviewers' concerns.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

1. Please provide a rationale for selecting 105 male mice for your study. Clarifying how this sample size was determined—whether through power calculations or prior data—will enhance the transparency and statistical validity of your experimental design.

Response: A total of 105 male mice were distributed into four groups: sleep fragmentation (SF), sleep control (SC), mock, and a negative control group. The SF, SC, and mock groups each included 30 mice, while the negative control group included 15 mice. Among the 30 mice per group, 10 were assigned for survival analysis, and 5 mice per group were euthanized at four time points (post-vaccination/pre-challenge, and at 3-, 5-, and 7-days post-infection) for blood and tissue collection. Among the 15 mice in the negative control group, 10 were assigned for survival analysis and 5 were euthanized at the post-vaccination/pre-challenge time point for blood and tissue collection.

Sample sizes were determined based on data from the first of four independent experiments, which demonstrated 100% survival in vaccinated controls compared to approximately 50% survival in SF-vaccinated mice after virus challenge. For survival analyses, power calculations indicated that 7 animals per group would be required to achieve 80% power using a two-sided test at $\alpha = 0.05$ (Cohen's $d \approx 1.57$). Therefore, using 10 mice per group provided enough power to detect biologically meaningful differences. For lung viral titers, power analysis based on the observed effect size (Cohen's $d \approx 1.76$) indicated that 5 mice per group would yield ~70% power ($\alpha = 0.05$) to detect statistically and biologically meaningful differences between the gSF and gSC groups. Because all four experiments were conducted under the conditions with consistent endpoints and group structures, combining these datasets further increased the statistical power. We have now added a statement in the Methods section of the revised manuscript (Line 657-Line 574) clarifying our approach to sample size determination.

2. You wrote: "The current study was conducted exclusively in young adult male mice to minimize variability from hormonal cycling and because male mice tend to be more active, which better models sleep fragmentation induced by environmental disruption." While minimizing hormonal variability is a common rationale, the exclusion of female mice raises concerns. Notably, females are known to exhibit stronger adaptive immune responses than males. This suggests that the immunosuppressive effects of chronic sleep fragmentation could be less pronounced in females. Furthermore, chronic sleep disturbances are frequently more prevalent in women than in men, which makes the inclusion of female animals particularly relevant from a translational perspective. Including both sexes would not only strengthen the biological relevance of your findings but also enhance their generalizability to human populations.

Response: Thank you and we fully acknowledge that females often generate stronger adaptive immune responses than males and that inclusion of both sexes would enhance the generalizability of our findings. Our decision to focus on young adult male mice in this study was driven by the intension to reduce experimental variability associated with hormonal cycling and to ensure reproducibility across multiple behavioral and immunological endpoints. Male mice are more active during the dark cycle thus allow us to robustly model SF induced by environmental disruption. We respectfully note that, while chronic insomnia is indeed more prevalent among females, other common sleep disorders characterized by disrupted sleep architecture, such as obstructive sleep apnea (OSA), are significantly more prevalent in males, particularly prior to menopause. As such, both sexes experience substantial burdens of sleep disruption, and we agree that understanding sex-specific immune consequences is crucial for translational relevance. In the manuscript, we did address this limitation the Discussion (Line 433-Line 434). In the added human EHR analysis, we also evaluate outcomes stratified by sex (Line 314-Line 346).

3. Your study used 6- to 8-week-old male mice. Can the results be extrapolated to influenza-vulnerable populations, particularly aged individuals? Given that public health vaccination campaigns emphasize the importance of influenza vaccination for older adults due to increased morbidity and mortality risks, it would

be valuable to discuss whether your findings are applicable to aged mice, which more closely model the immune responses seen in elderly humans.
Response: Thank you for this great comment. Indeed, aged individuals represent a key target population for influenza vaccination, and it is critical to consider how our findings may translate to this group. We anticipate that the impact of SF on the vaccine efficacy could be even more pronounced in aged hosts, given their baseline reductions in immune responsiveness. In the revised manuscript, we have specified paragraph in the Discussion section (Line 432-434), highlighting this limitation. In the added human EHR analysis, we also evaluate outcomes stratified by age along with sex (Line 314-346).

4. You state: "During vaccination, 0.2 μg HA of inactivated vaccines or PBS in a volume of 50 μL were injected intramuscularly per mouse." The timing of vaccination significantly influences the magnitude of the adaptive immune response. Please specify the exact time of day when vaccinations were administered. Additionally, I suggest considering follow-up experiments comparing morning vs. evening vaccination to determine whether the impact of chronic sleep fragmentation on immune responses is modulated by circadian timing.
Response: We thank the reviewer for this critical comment. All vaccinations were indeed performed early morning between 8:30 and 10:30 a.m. during the light (inactive) phase to ensure consistency across experiments. This has been clarified in the updated manuscript (Line 557). We agree that circadian timing can influence vaccine-induced immune responses, and we have now added a brief note in the Discussion (Line 434) highlighting morning versus evening vaccination as a relevant area for future investigation.

5. Please specify in each figure legend which statistical tests were used to generate the reported p-values. This is essential for readers to accurately interpret the results and assess the appropriateness of the analysis.
Response: We have now revised all figure legends to clearly indicate the specific statistical tests used to generate the reported p-values.

6. In the absence of EEG recordings, did you use alternative methods to quantify rest or sleep? For example, were 24-hour inactivity periods measured as a proxy for sleep? This would help to substantiate that the control and sleep-disrupted groups differ in rest duration or quality.
Response: We thank the reviewer for this important comment. While we did not perform EEG/EMG recordings in this study, the SF protocol used here has been extensively validated in our prior publications. These studies employing this same SF model have confirmed, via EEG recordings, that it induces frequent brief arousals during the rest phase without significantly altering total sleep time or circadian architecture, but with clear impairment in sleep continuity and quality (e.g., Kaushal et al., PLoS One 2012; Hakim et al., Sleep 2015; Puech et al., Sleep 2023). For example, in Ramesh et al. (J Neuroinflammation, 2012), EEG data clearly showed successful induction of episodic awakenings in mice subjected to SF, with no significant change in sleep stage architecture but a significant reduction in sleep latency and continuity ($P < 0.0001$).

- Kaushal N, Ramesh V, Gozal D. TNF-α and temporal changes in sleep architecture in mice exposed to sleep fragmentation. PLoS One. 2012;7(9):e45610. doi: 10.1371/journal.pone.0045610. Epub 2012 Sep 21. PMID: 23029133; PMCID: PMC3448632.
- Hakim F, Wang Y, Carreras A, Hirotsu C, Zhang J, Peris E, Gozal D. Chronic sleep fragmentation during the sleep period induces hypothalamic endoplasmic reticulum stress and PTP1b-mediated leptin resistance in male mice. Sleep. 2015 Jan 1;38(1):31-40. doi: 10.5665/sleep.4320. PMID: 25325461; PMCID: PMC4262953.
- Puech C, Badran M, Barrow MB, Runion AR, Gozal D. Solriamfetol improves chronic sleep fragmentation-induced increases in sleep propensity and ameliorates explicit memory in male mice. Sleep. 2023 May 10;46(5):zsad057. doi: 10.1093/sleep/zsad057. PMID: 36866452; PMCID: PMC10413435.

- Ramesh V, Nair D, Zhang SX, Hakim F, Kaushal N, Kayali F, Wang Y, Li RC, Carreras A, Gozal D. Disrupted sleep without sleep curtailment induces sleepiness and cognitive dysfunction via the tumor necrosis factor-α pathway. J Neuroinflammation. 2012 May 11;9:91. doi: 10.1186/1742-2094-9-91. PMID: 22578011; PMCID: PMC3411474.

7. Please detail the housing conditions of the animals in all experimental groups. Were mice housed individually or in groups? Housing conditions can significantly affect stress levels and, consequently, immune responses. Both social isolation and overcrowding can modulate vaccine efficacy via stress-mediated pathways. Including this information is critical for interpreting your findings accurately.
Response: Thank you for this critical comment. All mice were housed in groups of 4–5 animals per cage, maintaining the same group composition as when they arrived from the commercial vendor. We aimed to avoid introducing social isolation stress and to preserve the established social environments since weaning for these animals. No mice were housed individually at any point during the study. Housing conditions were consistent across all experimental groups and remained unchanged throughout the study. We have now added this information to the revised Methods section (Line 574).

Reviewer #2 (Remarks to the Author):
Poor sleep or chronic sleep fragmentation (CSF) is associated with increased risk of developing end-organ morbidities and alterations in both innate and adaptive immune responses, which are associated with increased risk of infection with influenza virus among other things. The goal of this study was to mechanistically understand the cause of this increased susceptibility using a mouse model of influenza virus vaccination and challenge. The authors first identified a vaccination regime that provided partial protection to a high dose challenge. Next, they performed a single experiment comparing sleep fragmented mice with a control group measuring a very large number of phenotypes from weight loss, mortality, antibody responses, cellular responses, to detailed single cell transcriptional responses and B cell receptor analysis. Overall, sleep fragmentation reduced vaccine induced antibody responses resulting in higher virus load and a slight increase in mortality after infection. Contrary to expectation, the antigen-specific T and B cell response in these mice were improved suggesting changes in antibody production by these antigen specific B cells. To assess this, the investigators performed single cell sequencing on bulk splenocytes and found altered B cell responses and changes in B cell receptor diversity and composition. Overall, this is an important study that revealed many interesting new aspects of chronic sleep fragmentation on adaptive immunity. However, aside from transcriptional differences in B cells, it falls short in providing a mechanistic understanding of how these changes have occurred, and what factor(s) is driving it. Adoptive transfers experiments, for example, could have revealed a prominent role for T or B cells in antibody responses to vaccination.
Response: We sincerely thank the reviewer for the careful evaluation and the positive assessment of the significance and rigor of our study. The primary objective of this work was to define, in a controlled and mechanistically informative murine model, how chronic sleep fragmentation (CSF) reshapes vaccine-induced immunity across multiple layers, including serology, cellular composition, transcriptional programming, and immune receptor architecture. While our approach is inherently correlative, the integrated evidence consistently points to disrupted B-cell maturation, altered plasma-cell stress responses, and reconfigured T–B coordination as central features of CSF-mediated immune dysregulation. To strengthen mechanistic support in this revision, we have added flow cytometric validation of splenic germinal center B cells, plasma cells, and Tfh cells, together with assessment of MHC class II and CD86 expression on B cells (Supplementary Fig. 4), and refined the scRNA-seq and repertoire analyses with improved annotation and expanded diversity metrics (Supplementary Fig. 2). We agree that adoptive transfer experiments would be a powerful strategy to define causal, cell-intrinsic contributions of T and B cells to vaccine-induced protection under CSF. However, such studies require dedicated cohorts and specialized lineage-transfer designs and therefore fall beyond the feasible scope of this revision. We explicitly acknowledge this as a limitation and identify adoptive transfer studies as a priority for future work in the Discusison (Line 421-424). To enhance translational relevance, we incorporated a large-scale

human analysis using de-identified electronic health records from the TriNetX platform, demonstrating that vaccinated individuals with obstructive sleep apnea, a clinical phenotype of sleep fragmentation, exhibit significantly higher post-vaccination influenza risk compared with matched controls (Line 314-346). These human data reinforce the clinical relevance of our murine findings. We believe the revised manuscript now provides a strengthened mechanistic framework defining how CSF impairs adaptive immunity while appropriately delineating areas requiring further causal validation.

Additional comments:
1) The single cell analysis was done on bulk splenocytes and not antigen-specific T or B cells. What was the rationale for this? Further, observing changes at the bulk cell level suggests that sleep fragmentation has a global effect on adaptive immune responses. A recent paper identified a role for BMAL1 and CLOCK (Circadian rhythm genes) and TGFb1 in antibody production after LPS stimulation. Were these genes differentially expressed between the groups? Again, adoptive transfer of congenic B cells from a control mouse into a sleep fragmented animal prior to immunization, could have revealed B cell intrinsic effects.
Response: We thank the reviewer for these constructive comments. Our use of single-cell RNA sequencing and immune receptor profiling on bulk splenocytes was intended to capture the broad immune impact of sleep fragmentation on responses to influenza vaccination. This approach allowed us to assess changes in B cell maturation, plasma cell stress responses, T–B cell coordination, and broader lymphoid and myeloid networks. While we agree that antigen-specific analyses can reveal clonal or cell-intrinsic effects, the low frequency of influenza-specific B- and T-cells post-vaccination limits feasibility without enrichment. Future studies using tetramer-based sorting or antigen-specific enrichment are planned to explore these mechanisms in greater depth (Line 428-Line 431). Regarding the reviewer's query on circadian regulators, we examined Bmal1 (Arntl), Clock, and Tgfb1 expression in B cells and found no significant differences between SF-treated and control mice. However, we observed modest alterations in downstream stress- and metabolism-related pathways in plasma cells, which may reflect indirect circadian influence (Fig. 4a and Supplementary Fig. 2d). We also appreciate the suggestion to use adoptive transfer models. While such experiments may not change the overall findings of this study, we are actively exploring them to define B cell–intrinsic consequences of CSF (also please see the response to the earlier comment). A brief discussion of these points has been added to the revised manuscript (Line 421-Line 424).

2) The entire manuscript appears to be one large mouse experiment with 4-5 mice per readout (mortality, virus titer, T and B cell responses etc.). However, some figures only have 2 or 3 data points (2e - T cells, and all of the single cell sequencing studies). Some key experiments should be repeated for scientific rigor.
Response: We appreciate the reviewer's comment regarding sample size. The primary outcome measures (survival and virological endpoints) were guided by a priori power analyses (see response to Reviewer #1), confirming that the group sizes used were sufficient to detect biologically meaningful differences. For transcriptional analyses, we performed paired scRNA-seq and scTCR/BCR sequencing using three biological replicates per group (gSF and gSC), consistent with standard practice in single-cell studies to balance biological replication with sequencing depth and ensure robust detection of reproducible, condition-associated transcriptional and repertoire-level differences. The observed patterns were concordant with independent experiments (Supplementary Fig. 1) and supported by orthogonal flow cytometry and serological data. We have clarified the rationale for sample size and study design in the revised manuscript (Line 567-574).

3) What kind of statistical analysis was performed? Was a multiple comparisons correction applied? For example to Fig 1d.
Response: The data were originally analyzed using unpaired t-test. Both analyses and figure legend have been updated. $TCID_{50}$ titers and RNA copies were analyzed on the $\log_{10}$ scale with A one-way ANOVA, followed by Holm–Šídák–adjusted post-hoc pairwise comparisons among the six contrasts (single family; familywise $\alpha=0.05$); adjusted $P<0.05$ was considered significant. This has been updated in Figure legend as well as statistical method section (Line 760-767).

4) In figure 5, the order of the groups (gSC and gSF) are reversed compared to other figures.
Response: This has been updated.

5) The experiments described in the first two paragraphs of the results section appear disconnected from the rest of the manuscript. First, the conclusion is that 0.1ug HA represents the ideal threshold for doing these studies but everything is done with 0.2ug HA. Also, is this the same inactivated whole virus vaccine that was used in the rest of the paper? If so, were antibody responses to other viral proteins measured? NP or NA for example and were similar differences observed?
Response: We thank the reviewer for this insightful comment. While 0.1 μg HA represented the minimal dose capable of eliciting a detectable immune response, we selected 0.2 μg HA for the main study to ensure more reliable quantification of antibody and cellular responses using conventional serological and immunological assays. We have made clarification in the results (Line 108-112). The same inactivated whole-virus vaccine preparation was used throughout the study. We focused on HA-specific responses as the primary correlate of protection; antibody responses to NP or NA were not measured in this study, but represent valuable directions for future investigation (Line 431-434).

6) Line 121, it is claimed that higher concentrations of NP are found, but there is no significant difference in 1e
Response: We appreciate the reviewer's comment. The statement on line 121 was intended to describe a qualitative observation rather than a statistically significant difference. Our wording ("revealed higher concentrations of NP") reflects the visual trend observed in the immunohistochemistry images, consistent with the data shown in Figure 1e. We have clarified this in the revised text to avoid any possible misunderstanding (Line 131).

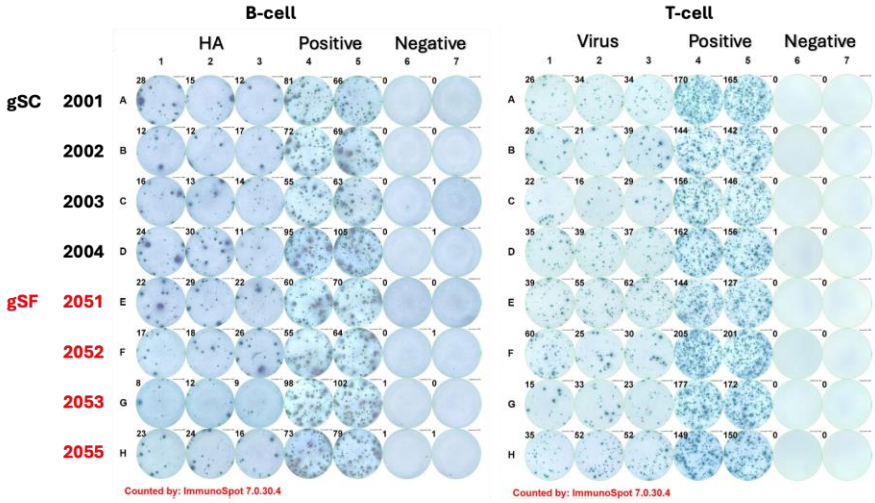
Reviewer #3 (Remarks to the Author):
In this manuscript, the authors examined the effect of chronic sleep fragmentation (CSF) on the efficacy of influenza vaccination. They report that CSF is associated with reduced influenza-specific antibody responses and decreased survival rates. While these findings are of potential interest, much of the data presented are descriptive, with limited mechanistic insight, particularly regarding how CSF influences humoral immunity. Although the authors included single-cell sequencing analyses in the latter part of the manuscript, these results remain primarily descriptive and were not followed by validation experiments. In addition, some of the interpretations of the single-cell data may be somewhat speculative, as they rely largely on correlative associations, and should be interpreted with caution. Some other comments are outlined below:
Response: We thank the reviewer for the thoughtful critique. We agree that deeper mechanistic validation is important. In this revision, we strengthened the mechanistic foundation by adding orthogonal flow cytometric validation of key single-cell inferences, including splenic GC B cells, plasma cells, Tfh cells, and B-cell MHC-II/CD86 expression (Supplementary Figure 4), and by refining scRNA-seq annotations with marker-backed justification and tempered, explicitly correlative interpretation (Supplementary Figure 2). We also expanded TCR/BCR repertoire analyses to provide greater contextual insight (Line 289-306). Together, these data support a coherent pattern of disrupted B-cell maturation, plasma-cell stress responses, and altered T–B coordination under CSF. To enhance translational relevance, we further incorporated complementary human EHR (TriNetX) data demonstrating higher post-vaccination influenza risk among individuals with obstructive sleep apnea, a clinical correlate of sleep fragmentation (Line 314-346). We believe these revisions substantially strengthen the manuscript while maintaining appropriate caution in causal interpretation.

1. In Figure 1, since the mice were subjected to CSF treatment for 10 weeks, which is a relatively long duration, the study design lacks critical control groups. Specifically, there is no group with CSF treatment alone, and no group with CSF treatment plus infection without vaccination. The absence of the latter is particularly concerning, as it makes it difficult to exclude the possibility that CSF itself affects susceptibility to infection.

Response: We thank the reviewer for this insightful comment. We agree that including additional control groups, such as mice subjected to SF treatment alone and SF treatment plus infection without vaccination, would strengthen interpretation of SF independent effects on infection susceptibility. The current study was specifically designed to evaluate how SF alters *vaccine-induced* immune protection rather than baseline infection outcomes. We have now explicitly acknowledged this as a limitation in the revised manuscript and clarified that future studies will incorporate these groups to directly assess the impact of CSF on infection susceptibility and host resistance in the absence of vaccination (Line 427-429).

2. In Figure 2e, representative wells from the ELISpot assay should be shown to support the quantification. In addition, it would be important to examine whether influenza-specific long-lived plasma cells in the bone marrow are affected.

Response: We thank the reviewer for the constructive suggestions. The ELISpot wells have been added to Source Data (see below) to support the quantitative results. We agree that evaluating influenza-specific long-lived plasma cells in the bone marrow would provide important insight into the durability of antibody responses. Although these analyses were not included in the present study, we have now acknowledged this as a limitation and highlighted it as a key direction for future work in the revised Discussion (Line 421-Line 424).



3. For the cluster annotations in the single-cell sequencing data (Figure 3a and Supplementary Figure 2a), the authors should provide detailed justification for each cluster, including marker gene evidence and relevant references. In addition, it is unclear why so many follicular B cell clusters are defined; these should be consolidated where appropriate. The annotation of cluster 16 as DN T cells is also questionable, as this cluster does not appear to express Cd3d. Similarly, cluster 19 shows minimal expression of the surveyed genes yet is annotated as macrophages, which requires further explanation. Finally, the presence of a cDC1 cluster without a cDC2 cluster is puzzling.

Response: We thank the reviewer for these insightful comments and suggestions regarding the scRNA-seq cluster annotations. Clusters 2-7 were positioned closely together in the UMAP space, indicating high similar transcriptional profiles. We originally designated these cells as follicular B (FoB) cells, based on elevate expression of known FoB markers, including *Fcer2a* (CD23), *Cd24a* (CD24), and *Ighd*. To strengthen these annotation, we computed FoB gene-set modulte scores for each cluster. As shown in the revised Supplementary Fig. 2a-b, all six slusters displayed positive FoB module scores, with modest but

reproducible variation, consistent with related FoB subsets of different activation or maturation states. Notably, cluster 4 exhibited the lowest FoB module score, which correspons to the early branchpoint of the prseudotime trajectory (Fig. 3e), futher supporting its interpretation as an earlier-stage FoB population.

We agree with the reviewer that the *Cd3d* expression was lower in cluster 16 compared to other T cell clusters. However, these cells showed high expression of *Cd247* (encoding CD3ζ), and paired αβ TCR contigs were detected (Fig. 5a), indicating that they were bona fide T cells. In addition, these cells showed strong expression of TCR signaling–associated genes *Itk* and *Prkcq* (encoding PKCθ), but relatively low expression of *Zap70* and *Lck*. This mixed pattern suggests cluster 16 may represent a noncanonical or developmentally distinct T cell state, potentially reflecting altered activation or maturation rathter than a separate lineage.

We further subclustered the original cluster 19 into clusters 19 and 20, and the original cluster 21 into clusters 22–24, and refined their annotations accordingly. The revised cluster 19 was identified as macrophages based on high expression of canonical macrophage markers (*C1qa, C1qb,* and *C1qc*). Cluster 20 showed strong expression of multiple *S100a* family members (*S100a4, S100a6,* and *S100a11*) together with MHC class II genes (*H2-Aa* and *H2-Ab1*), consistent with a monocyte identity (Wang et al. 2024). Cluster 22 expressed *Sirpa* (encoding CD172a) and *Itgax* (encoding CD11c), supporting its annotation as cDC2. Cluster 23 was characterized by expression of *Ccr2* and *Adgre1* (encoding F4/80), together with low MHC class II expression, consistent with monocyte-derived dendritic cells (DCs) (MoDCs). Finally, cluster 25 exhibited *Cacnb3* expression, a defined feature of migratory DCs (MigDCs) (Woo et al. 2023).

We have revised the manuscript (Line 184-197), figures, and supplementary materials to provide detailed marker-based justification for each cluster, together with supporting references.

Fig. 2a

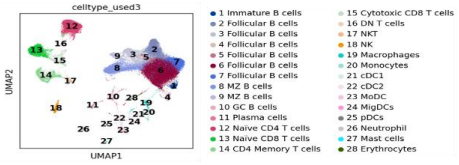


Fig. 2c

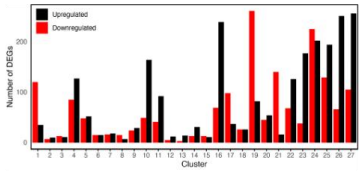


Fig. 2e

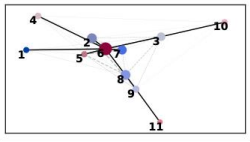
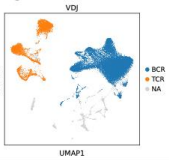
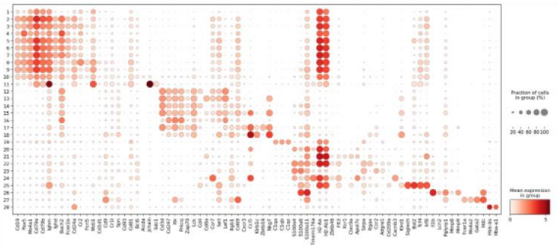


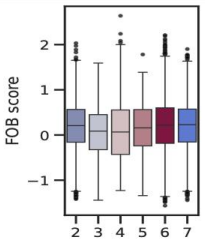
Fig. 5a



Supplementary Fig. 2a



Supplementary Fig. 2b



- Wang YF, Zhang WL, Li ZX, Liu Y, Tan J, Yin HZ, Zhang ZC, Piao XJ, Ruan MH, Dai ZH, Wang SJ, Mu CY, Yuan JH, Sun SH, Liu H, Yang F. METTL14 downregulation drives S100A4^{+} monocyte-derived macrophages via MyD88/NF-κB pathway to promote MAFLD progression. Signal Transduct Target Ther. 2024 Apr 17;9(1):91. doi: 10.1038/s41392-024-01797-1. PMID: 38627387; PMCID: PMC11021505.

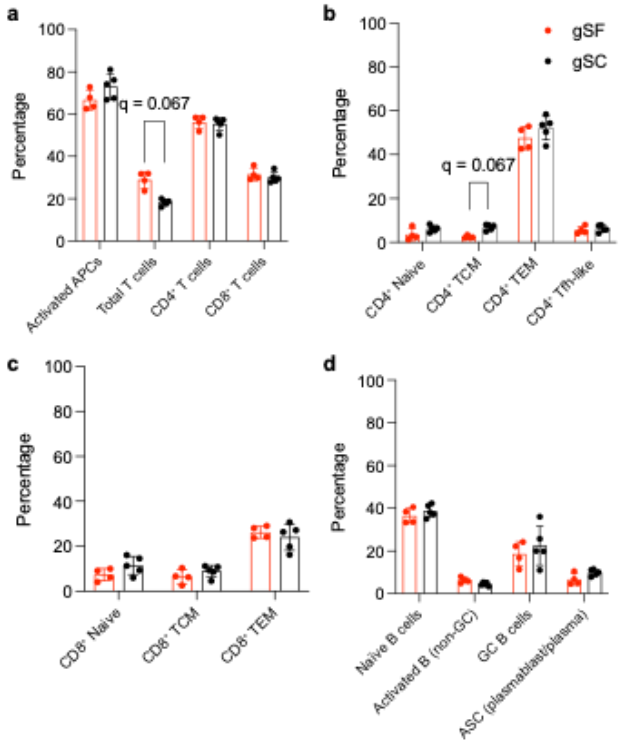
- Woo MS, Ufer F, Sonner JK, Belkacemi A, Tintelnot J, Sáez PJ, Krieg PF, Mayer C, Binkle-Ladisch L, Engler JB, Bauer S, Kursawe N, Vieira V, Mannebach S, Freichel M, Flockerzi V, Vargas P, Friese MA. Calcium channel β3 subunit regulates ATP-dependent migration of dendritic cells. Sci Adv. 2023 Sep 22;9(38):eadh1653. doi: 10.1126/sciadv.adh1653. Epub 2023 Sep 20. PMID: 37729408; PMCID: PMC10511199.

4. Flow cytometric analysis of splenic germinal center B cells, plasma cells, and Tfh cells are important to include. Examining the expression of costimulatory ligands and MHC2 on B cells is warranted, since the authors already found differences in these pathways from their single-cell data.

Response: We appreciate the reviewer's suggestion to validate B-cell activation pathways highlighted in our single-cell analyses. In response, we performed flow-cytometric quantification of four splenic B-cell subsets, including naïve B cells ($GL7^{-}CD86^{-}IgM^{+}$), activated non-GC B cells ($GL7^{-}CD86^{+}MHC\text{-}II^{+}$), germinal center (GC) B cells ($GL7^{+}$), and plasmablast/plasma cells ($CD138^{+}$), along with 10 T-cell subsets (total $CD3^{+}$ T cells; $CD4^{+}$ and $CD8^{+}$ T cells; CD4 naïve, central memory, and effector memory; CD8 naïve, central memory, and effector memory; and Tfh-like cells) and one non–T-cell subset ($CD3^{-}CD40^{+}$ activated non-T lymphocytes) (Supplementary Fig. 4).

These B-cell subsets encompass the major phenotypic states that differ in co-stimulatory ligand and MHC-II expression, with activated non-GC and GC B cells representing the principal B-cell antigen-presenting compartments. Across all four B-cell subsets, we did not observe significant compositional differences between gSF and gSC mice. Although our panel did not quantify expression-level distributions of MHC-II or CD86, these markers were included and used to phenotype activated non-GC and GC B-cell populations. Thus, CSF does not alter the abundance of B-cell subsets in which costimulatory and antigen-presentation pathways are typically regulated, consistent with our scRNA-seq findings that CSF primarily affects transcriptional activation states rather than population frequencies. Within the T-cell compartment, gSF mice showed a trend toward higher total T-cell frequencies and lower $CD4^{+}$ effector-memory T cells ($q = 0.067$), with all other subsets comparable between groups. These patterns align with our single-cell signatures of altered T-cell differentiation and T–B coordination under CSF. Together, these flow-cytometric data provide orthogonal support for our transcriptomic observations.

Supplementary Figure 4



5. In lines 238 and 247, the authors claim that CSF influences CD8 T cell–B cell signaling. This is unexpected, as direct regulation of antibody production by CD8 T cells is not well established. Such an interpretation appears speculative and may reflect a broader tendency in the manuscript to extend conclusions somewhat beyond the supporting evidence.

Response: We thank the reviewer for this important point. We agree that a direct role for CD8 T cells in regulating B-cell antibody production is not well established, and our original wording may have implied a mechanistic interaction beyond the available evidence. Our intention was to indicate that the ligand–receptor analyses revealed altered signaling involving CD8 T cells and B cells, rather than suggesting a direct regulatory pathway. We have revised the text accordingly to clarify that these findings are correlative rather than mechanistic (Line 250-Line 251).

6. The TCR and BCR sequencing data presented in Figure 5 do not appear to support any clear conclusions. The authors should provide a more rigorous analysis that can directly addresses the study's main questions.

Response: We thank the reviewer for this helpful comment. We agree that our earlier description may not have fully conveyed the depth of the repertoire analysis. Figure 5 includes multiple quantitative layers, UMAP-based lineage assignment, clonotype expansion profiles, CDR3-length distributions, V- and J-gene usage, and Hill diversity indices (q = 0–4), which together provide a multidimensional assessment of how chronic sleep fragmentation reshapes adaptive immune repertoire architecture. To improve clarity, we have expanded the accompanying text to explicitly link these metrics to the study's main questions. The TCR data align with our ELISpot findings, showing increased clonal expansion of virus-reactive T cells in gSF mice (Lines 304–Line 306). In contrast, the BCR analyses reveal class-switching and subtly altered CDR3 architecture in gSF mice (Line 289- Line 300). These subtle features align with the broader transcriptomic signatures observed in CSF-exposed mice and may reflect modest perturbations in lymphocyte maturation under CSF, which could contribute to reduced vaccine-induced protection. We have updated this section accordingly (Line 282- Line 312).

Response Letter

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I maintain that the exclusion of female animals and the lack of replication in older animals represent significant limitations of the study. Nonetheless, I appreciate the authors' efforts and their careful consideration of the other points raised, and I have no further comments. My feedback is focused solely on the issues I raised and not on those raised by the other reviewer.

Response: We thank the reviewer for their thoughtful evaluation. We agree that the exclusion of female animals and the absence of aged cohorts are important limitations. We have added an explicit limitation statement in the revised Discussion, noting that sex- and age-dependent differences in immune responses may influence susceptibility and vaccine responses, and that our findings should be interpreted within the context of young adult male mice (Line 474-485).

Reviewer #2 (Remarks to the Author):

The authors addressed the reviewers comments and overall improved the manuscript. The additional computational analysis and the flow cytometry experiment have led to a more balanced and tempered message regarding the role of sleep fragmentation on influenza vaccination and immune responses. The inclusion of the human data and the effect of OSA on risk of influenza infection with or without vaccination is a very nice addition to the paper.

Response: We sincerely thank the reviewer for their positive and encouraging assessment of our revised manuscript. We thank the reviewer for their constructive feedback throughout the review process, which has substantially improved the clarity, rigor, and impact of the manuscript.

Reviewer #3 (Remarks to the Author):

I appreciate the authors' revisions and their efforts to clarify certain interpretations in the revised manuscript. However, despite these changes, the central conceptual and experimental concerns raised in the initial review remain unresolved. As such, the current manuscript does not substantiate mechanistic conclusions regarding how CSF may influence humoral immunity, and several of the main conclusions continue to extend beyond what is directly supported by the data.

Response: We thank the reviewer for their careful re-evaluation and for clearly articulating the remaining conceptual concerns. In response, we added infection control cohorts to address the central confound, expanded the human EHR analysis to compare vaccination-associated protection between OSA and non-OSA populations, and strengthened the scRNA-seq interpretation with additional validation and clearer annotation support. We also revised the Results and Discussion to ensure that conclusions are limited to what is directly supported by the data.

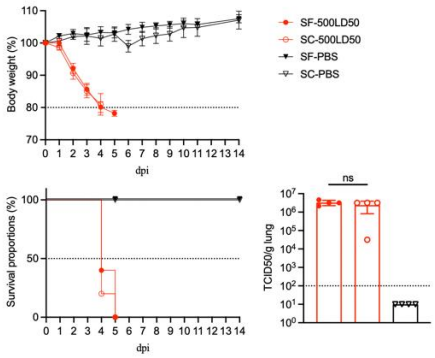
1. The authors acknowledge that additional control groups, specifically mice subjected to CSF alone and mice subjected to CSF combined with infection in the absence of vaccination, were not included and represent a limitation of the study. However, this acknowledgment does not address the core issue. The absence of these controls constitutes a fundamental confound rather than a minor limitation. It remains impossible to distinguish whether the observed reductions in survival and immune responses reflect impaired vaccine-induced protection or, alternatively, a generalized increase in susceptibility to infection caused by CSF itself. Moreover, the authors state that the study was specifically designed to evaluate how CSF alters vaccine-induced immune protection rather than baseline infection outcomes. If this was indeed

the intention, then CSF exposure should have been restricted to the vaccination phase rather than applied continuously across the entire experimental timeline. As currently designed, the study cannot disentangle vaccine-specific effects from broader physiological consequences of long-term CSF. Importantly, this issue cannot be resolved through discussion alone. Without the inclusion of appropriate control groups or equivalent experimental evidence, the principal conclusions of the study remain insufficiently supported.

Response: We agree that the absence of infection control cohorts in the original design limited our ability to distinguish reduced vaccine-mediated protection from increased baseline susceptibility to infection caused by CSF. To address this directly, we added infection control cohorts in which mice underwent the same CSF exposure paradigm but were not vaccinated, followed by influenza challenge using the same dose and outcome assessments.

Specifically, we added: 1) CSF exposed mice receiving PBS instead of vaccination and then challenged with influenza; 2) sleep control mice receiving PBS instead of vaccination and then challenged identically. Across these cohorts, we did not detect differences between CSF and sleep control mice in survival, body weight loss, or lung viral loads under our experimental conditions, indicating that the reduced protection observed in vaccinated CSF mice is not explained solely by a generalized increase in baseline susceptibility (see Response Figure 1, corresponding to Supplementary Figure S1d). The new data are included in Supplementary Figure S1d and are described in the Results (Line 135-139).

Regarding the timing of CSF exposure, our study was designed to model CSF as a persistent physiological state, consistent with human conditions such as OSA, rather than an isolated peri-vaccination perturbation. Continuous CSF exposure therefore reflects the clinically relevant scenario in which vaccination, immune priming, and infection occur in the context of ongoing sleep disruption (please see the rationale for mice experiment, Line 84-91). Importantly, the inclusion of infection-only control cohorts now allows us to dissociate vaccine-associated effects from baseline susceptibility under this chronic exposure paradigm.



Response Fig. 1. Infection-only control cohorts used to assess baseline susceptibility to influenza following CSF. Body weight change (top left), survival (bottom left), and lung viral titers at day 3 post-infection (right) are shown for CSF and sleep control (SC) mice challenged with influenza virus in the absence of vaccination. Under the experimental conditions used, CSF exposure alone did not result in detectable differences in weight loss, survival, or lung viral burden compared with SC mice, supporting interpretation of vaccine-specific effects in the main experiments.

2. The inclusion of human EHR (TriNetX) data showing a higher post-vaccination influenza risk among individuals with obstructive sleep apnea provides additional context and is potentially informative. However, similar to the animal data, the current analysis does not clearly distinguish whether this increased risk reflects reduced vaccine efficacy or an increased baseline susceptibility to infection. To address this, the authors could include an analysis comparing the magnitude of protection conferred by

vaccination in control individuals (vaccinated versus unvaccinated) with that observed in individuals with obstructive sleep apnea (vaccinated versus unvaccinated).

Response: We agree that directly comparing the magnitude of vaccine-associated protection within OSA and non-OSA populations is necessary to distinguish reduced vaccine efficacy from increased baseline susceptibility. We therefore added analyses comparing vaccinated versus unvaccinated individuals within each population.

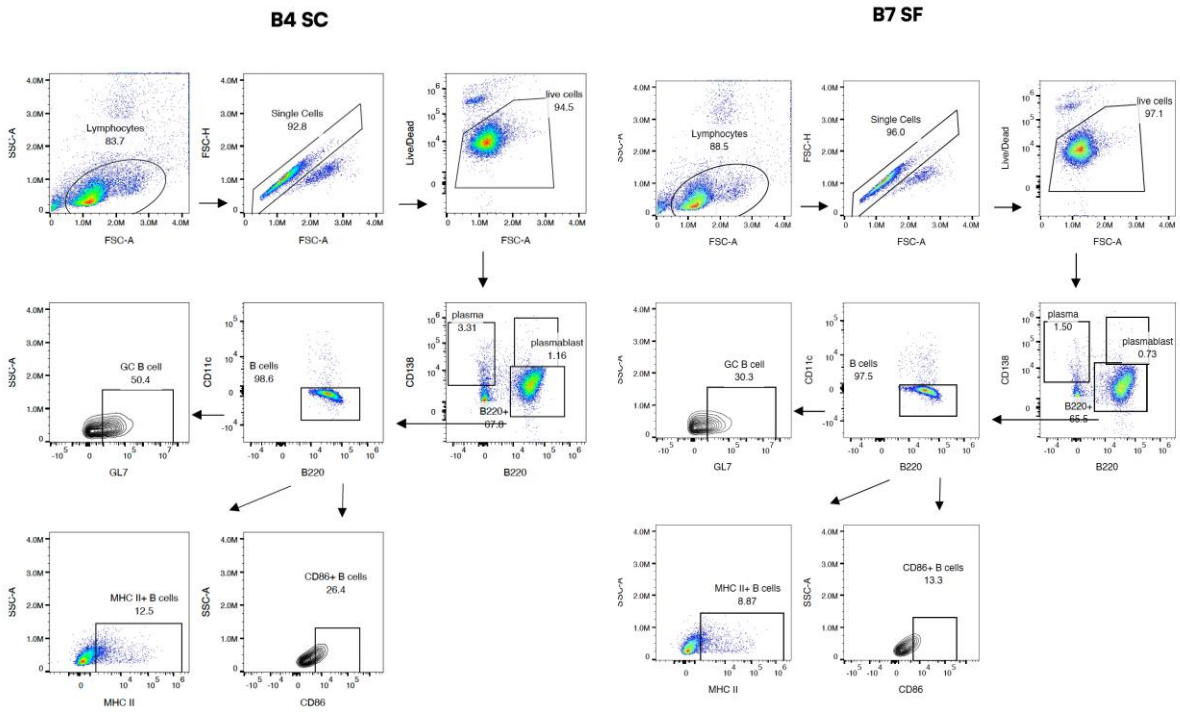
We first assessed laboratory-confirmed influenza; however, we observed patterns consistent with differential healthcare utilization and testing between vaccinated and unvaccinated cohorts, which can bias test-positivity outcomes in EHR data. To reduce ascertainment bias, we added an analysis using influenza-associated pneumonia as a more specific severe outcome and applied propensity score matching on demographics and major metabolic, pulmonary, and cardiovascular comorbidities.

Using influenza-associated pneumonia as the outcome, vaccination was associated with reduced risk in both populations. Odds ratios were calculated comparing unvaccinated versus vaccinated individuals. In the OSA cohort ($n \approx 300{,}000$), the incidence was 0.27% in unvaccinated individuals versus 0.22% in vaccinated individuals (OR = 1.22). In the non-OSA cohort ($n \approx 1{,}400{,}000$), the incidence was 0.10% in unvaccinated individuals versus 0.07% in vaccinated individuals (OR = 1.43).

Comparison of log odds ratios demonstrated that the magnitude of vaccination-associated protection was significantly lower in individuals with OSA than in those without OSA ($Z = 2.43$, $p = 0.015$). Importantly, baseline risk in the unvaccinated groups was also higher in the OSA cohort, indicating that OSA is associated with both increased baseline susceptibility and attenuated vaccination-associated protection. These analyses have been incorporated into the revised manuscript (Line 358-367).

3. Although the sequencing data add descriptive depth to the phenotypic characterization of immune alterations associated with CSF, several key analyses are either not followed by adequate validation or raise internal inconsistencies that are not sufficiently addressed.
a. The authors still did not analyze costimulatory ligands or MHC class II expression on B cells.

Response: We agree and have now quantified MHC class II and costimulatory ligand expression on splenic B cells by flow cytometry, including MHC-II and the B7-family costimulatory ligand CD86 (Supplementary Fig. 4). The corresponding Methods and Results text have been updated, and full quantitative data are provided in the Source Data.



Response Fig. 2. Representative flow cytometry gating strategy for splenic B-cell phenotyping in sleep control group (gSC) and chronic sleep fragmentation group (gSF) mice. Representative flow cytometry plots from one mouse in the gSC group and one mouse in the gSF group illustrating the gating strategy used for splenic B-cell analysis. Cells were sequentially gated on lymphocytes (FSC-A vs SSC-A), singlets (FSC-A vs FSC-H), and live cells. Total B cells were identified as $B220^+$ cells. Plasma cells ($B220^-CD138^+$) and plasmablasts ($B220^+CD138^+$) were delineated within the B-cell compartment. Conventional B cells ($B220^+CD138^-CD11c^-$) were further gated to identify germinal center (GC) B cells defined as $GL7^+$. Expression of MHC class II and the costimulatory molecule CD86 was assessed on splenic B cells to evaluate activation status. Percentages indicate the frequency of cells within the parent gate. Complete gating plots for all mice are provided in the Source Data.

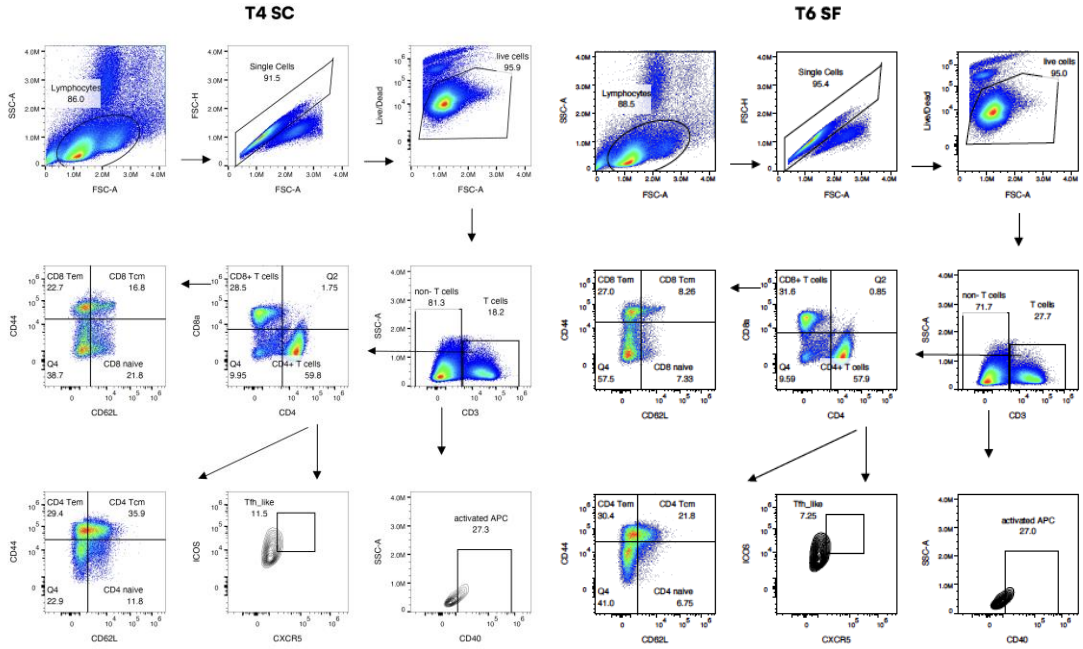
b. Flow cytometric data shown in Supplementary Figure 4 indicate no substantial changes in B cell or Tfh cell populations, raising the question of how the reported alterations in humoral immunity should be interpreted. In addition, the reported proportions of naïve CD4 and CD8 T cells in the spleen (approximately 10% or lower) are unexpectedly low and not consistent with established immunological knowledge. An explanation for these observations, including gating strategies and definitions, is warranted.

Response: We agree that we did not observe large differences in frequencies of total B cells or T follicular helper (Tfh) cells at the analyzed timepoint. Our conclusions regarding impaired humoral protection are instead supported by functional outputs (antigen-specific antibody titers, viral control after challenge) and qualitative immune features (e.g., antigen-specific responses and repertoire/ transcriptional analyses). The absence of major frequency shifts in B or Tfh cells suggests that CSF exposure does not broadly deplete these compartments, but rather alters the quality and output of the humoral response, such as functional antibody production and clonal selection, without substantially changing population size.

Regarding naïve T cell frequencies, we re-analyzed the data with updated compensation and gating. Naïve T cells were defined as CD44^lo CD62L^hi cells and quantified within the splenic T cell compartment. Splenic cells were harvested from mice at 16 weeks of age following a prime-boost vaccination regimen. In the updated analysis, naïve $CD4^+$ T cells ranged from 7.66%–11.8%, while naïve $CD8^+$ T cells ranged from 10.1%–21.8% (Response Fig. 3; Supplementary Figure 4, and Source Data).

Although these proportions are lower than those typically reported in unvaccinated mice, they are consistent with established immunological knowledge for antigen-experienced animals. Prime–boost vaccination induces robust expansion of CD44^hi effector and memory $CD4^+$ and $CD8^+$ T cells, which proportionally reduces the naïve (CD44^lo CD62L^hi) compartment with secondary lymphoid tissues This phenomenon is well documented for prime–boost vaccination strategies, which preferentially expand antigen-experienced T cell populations (Palgen et al., Front. Immunol., 2021; Nolz & Harty, Adv. Exp. Med. Biol., 2011). Detailed gating strategies, marker definitions, and representative plots are provided in the Source Data.

- Palgen, Jean-Louis et al. "Optimize Prime/Boost Vaccine Strategies: Trained Immunity as a New Player in the Game." Frontiers in immunology vol. 12 612747. 8 Mar. 2021, doi:10.3389/fimmu.2021.612747
- Nolz JC, Harty JT. Strategies and implications for prime-boost vaccination to generate memory CD8 T cells. Adv Exp Med Biol. 2011;780:69-83. doi: 10.1007/978-1-4419-5632-3_7. PMID: 21842366.



Response Fig. 3. Representative T cell gating strategy in gSC and gSF mice. Splenocytes were first gated on lymphocytes by forward and side scatter, followed by singlet discrimination and exclusion of dead cells using a fixable viability dye. Live singlet cells were separated into $CD3^+$ T cells and $CD3^-$ non-T cells. $CD3^+$ T cells were further subdivided into $CD4^+$ and $CD8^+$ populations. Within each lineage, naïve ($CD44^-CD62L^+$), central memory ($CD44^+CD62L^+$), and effector memory ($CD44^+CD62L^-$) subsets were defined. T follicular helper–like (Tfh-like) cells were identified within the $CD4^+$ compartment as $CXCR5^+ICOS^+$ cells. A $CD3^-CD40^+$ population was delineated as activated non-T lymphocytes and used as a comparator population. Percentages indicate the frequency of cells within the parent gate. Representative plots from one SC mouse and one SF mouse are shown; all gates were applied identically across samples. Complete gating plots for all mice are provided in the Source Data.

c. The TCR and BCR sequencing data presented in Figure 5 remain difficult to interpret and add limited mechanistic insight. Notably, the IgG-related results appear inconsistent with those shown in Figure 2c, in some cases even suggesting opposite trends.
The current presentation mainly describes the analysis results without explaining these inconsistencies or providing a convincing biological interpretation.

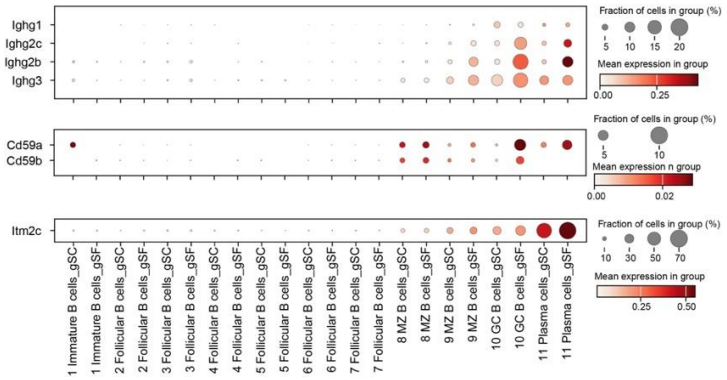
Response: We thank the reviewer and agree that the repertoire and transcriptomic analyses required clearer framing and interpretation. We have revised the text to explicitly distinguish the biological readouts represented by Figures 2c and 5 to avoid overinterpretation. Figure 2c measures antigen-specific circulating IgG, which reflects functional humoral output against the influenza antigen. In contrast, Figure 5 summarizes immunoglobulin constant-region transcript usage and B cell state composition derived from scRNA-seq of total B cells; this readout captures transcriptional programs and class-switch signatures across the sampled B cell populations, but does not measure antigen specificity, antibody secretion efficiency, or antibody quality.

Because these assays interrogate distinct aspects of humoral immunity, their results can diverge. For example, increased IgG constant-region transcripts within splenic B cell states can occur alongside reduced antigen-specific serum IgG if CSF impairs (1) the differentiation or maintenance of antigen-specific long-lived plasma cells, (2) antibody secretion efficiency, (3) affinity maturation and effective antigen targeting, or (4) the relative contribution of vaccine-specific versus non-vaccine-specific IgG-expressing B cell subsets. Discriminating among these non-mutually exclusive possibilities would require lineage-resolved or antigen-specific single-cell approaches and is beyond the scope of the current study.

To strengthen and clarify the BCR-related findings, we performed additional gene expression analyses. These analyses demonstrate increased expression of multiple IgG constant-region genes (Ighg1, Ighg2c, Ighg2b, and Ighg3) in the gSF group, along with elevated expression of genes associated with IgG-producing or activated B cells, including *Cd59a*, *Cd59b*, and *Itm2c* (see Response Figure 4, corresponding to Supplementary Figure S3b), consistent with previously reported markers of antibody-secreting cell states (e.g., Cheng et al., Nat Commun 2023). These data are now described in the Results (Line 307-310) and integrated into the Discussion (Lines 444–454).

Together, these revisions clarify that the sequencing data reflect qualitative changes in B cell activation and differentiation programs, rather than direct measures of antigen-specific or protective antibody function. We have revised the manuscript accordingly to ensure that conclusions drawn from the repertoire and transcriptomic analyses remain appropriately bounded by the data.

- Cheng, R.YH., de Rutte, J., Ito, C.E.K. et al. SEC-seq: association of molecular signatures with antibody secretion in thousands of single human plasma cells. Nat Commun 14, 3567 (2023). https://doi.org/10.1038/s41467-023-39367-8



Response Fig. 4 Dot plot showing the mean expression of selected genes in B cell clusters across treatment groups.

Response Letter

Reviewer #3 (Remarks to the Author)

I thank the authors for their efforts in revising the manuscript and for addressing the reviewers' comments. The revisions and additional experiments have improved the clarity of the study. I have no further comments.

Response: We thank the reviewer for their positive assessment of our revisions and for their constructive feedback throughout the review process. We are pleased that the revisions and additional experiments have improved the clarity of the study.