nature portfolio

Peer Review File

The survival of B cells is compromised in kidney disease

Corresponding Author: Dr Partha Biswas

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)
The manuscript by Peroumal et al. investigates the effects of chronic kidney disease (CKD) on humoral immunity. Based on the observation that patients with CKD exhibit weakened humoral immunity against infections, such as SARS-CoV2 or influenza, the study demonstrates in various independent mouse models of CKD that impaired kidney function reduces the germinal center response to antigens. In CKD, B cells display a higher rate of apoptosis in vivo. Subsequently, various uremic toxins are tested, and it is shown that they exacerbate B cell death. The study then focuses on one of the toxins, hippuric acid, which leads to increased apoptosis dependent on the receptor GPR109A including KO mice. In addition to the immune response to an artificial KLH-linked antigen, an influenza model is used, confirming the findings: decreased GC B cells and reduced antibody titers in CKD.

The present study, in my opinion, is excellently written and very comprehensible even for non-immunologists. It guides the reader well through the results and complex figures and provides extensive learning about the medical background, which is comprehensively presented. Therefore, I find this study and the underlying story to be extremely exciting and highly interesting and I have only two minor concerns:

1. It would be interesting to see if there is a correlation between the severity of CKD and its effects on GC B cells. The BUN levels in Suppl. Fig 1 show, as expected, a fairly large variance.

2. The figure legend of Fig S3 is confusing. The title "5/6 Nephrectomy" applies only to panels G and H. Adding histology of these mice would be a nice addition.

Reviewer #2

(Remarks to the Author)
The manuscript offers new information about the observed reduced humoral immunity after vaccinations in patients with kidney disease. By using several mouse models of kidney disease like induction of nephrotoxic kidneys using aristolochic acid I and 5/6 nephrectomy mimicking renal failure, the authors showed a reduced response to T-cell dependent antigens, and this was due to an inhibition of a germinal center (GC) response due to increased induction of apoptosis in B cells. The authors identified hippuric acid as the uremic toxin that induced a loss of mitochondrial membrane potential in GC B cells, and this increased apoptosis was driven by G-protein–coupled receptor109A. Lastly, they show that after influenza virus infection, the GC B cells and antibody titers are reduced in mice with nephrotoxic syndrome and this was accompanied with an higher virus load compared to normal mice.
The results in this manuscript are highly relevant and significant for this research field and may also have huge impact on other related research fields.
The authors use several mouse models to demonstrate the relevance of their findings, in addition to use other knockout models to prove their findings. The amount of data provided is supporting the discussions and conclusions made.
The methodology and analytical approach are very good, and the provided supplementary information is relevant for the understanding of the main results presented.
I have only one minor thing regarding a shortcoming of this extensive research paper: The analyses of the mouse models are performed only on cells isolated from the spleen. It is interesting if the same trend would be observed in lymph nodes, especially in the renal LN?

The manuscript is well written and have a logical organization. Some of the figures could be better organized as some of the panels are not very well aligned and some figures seems squeezed.

Reviewer #3

(Remarks to the Author)
NCOMMS-24-04881
In this manuscript, Peroumal, Jawale, and colleagues investigate why individuals with kidney disease are more susceptible to infections and have poor vaccine-induced antibody responses. Their data suggest that impaired germinal center (GC) responses and plasmablast function contribute to this susceptibility. It is unclear whether B cell activation itself is affected, this point needs clarification. Given the broad effects observed, including on plasmablasts that may not be derived from GCs, it is suggested to change the title to "B cell responses" to avoid misleading readers.

These impairments appear to be driven in part by the uremic toxin hippuric acid, which causes mitochondrial dysfunction and increased apoptosis of GC B cells and plasmablasts via GPR109A activation, ultimately suppressing humoral immunity. The underlying mechanism is not addressed. This work is of significant interest if these mechanisms are proven, but several unknowns need addressing.

Two critical pieces of data include:
1 Figure 3H-M: Using a nephrectomy surgery model.
2 Figure 6Q: Transfer of B cells into a muMT mouse model to determine B cell-specific effects. However, the cell transfer experiments are underdeveloped. Further experiments are needed, including measurements of affinity maturation, apoptosis, proliferation, TMRE, and MitoSOX to differentiate B cell/GC B cell intrinsic vs extrinsic effects of kidney disease.

Overall, the manuscript needs significant improvements, and further effort is required to make figures clear and understandable. Figures should include absolute cell numbers and representative flow data to support all claims. The text should provide detailed explanations of experimental choices and their implications.

Other Major Comments

FIGURE 1
Provide information on using AAII as a chemical control for AAI.

Does AAI impact B cell populations in the spleen and bone marrow?

Absolute cell numbers should be measured for accuracy. Also for data presented in other figures.

Figure 1D: Improve the quality of immunofluorescence images (IFs). The statement "There was no difference in the number and size of GC between control or AAII-injected spleens" (lines 162-163) is unsupported by the provided data. The immunofluorescence image shows a GC with reduced size compared to controls, but no quantification of GC clusters is provided. Therefore, the concluding statement about compromised GC formation in kidney disease is not yet supported by the presented data.

Figure 1H: Clarify why different NP conjugation ratio reagents were used to measure antibody affinity at day 12 (NP4) vs day 21 (NP7) after immunization.

Explain why the absolute amount (μg/ml) of NP7-specific serum IgG1 is higher than NP27-specific serum IgG1 at day 21, suggesting that high-affinity antibodies are more concentrated than total anti-NP IgG1 antibodies.

Figure 1I: Specifically describe whether the ELISPOT assay for plasma cells producing NP4-specific IgG1 was performed with cells from the spleen or bone marrow. Similar clarification is needed for data in Figure 2F.

FIGURE 2
The figure is complex, with messages about TFH and secondary immune responses. Restructure the presentation for better readability. Include a representative flow stain for TFH to assess how the authors define this population.

Figure 2B: Clarify the meaning of LPS re-stimulation. If referring to LPS re-stimulation of GC B cells, show that these are the cells expressing IL6. The correlation between reduced IL6 production and reduced TFH in the AAI condition is not strong enough to be a main figure.

Data showing no IL6 induction upon AAI treatment suggests a profound impairment in B cell activation beyond GC B cells. Can the authors please elaborate?

FIGURE 4
Figure 4G: The authors observe increased apoptosis in NP+ GC B cells measured by active caspase 3 stain. Explain why increased apoptosis is only seen in NP+ GC B cells and not in NP- GC B cells. The impact on LZ/DZ distribution seems equally affected in kidney disease, how do the authors interpret differences between NP-binding and non-binding GC B cells?

FIGURE 5
Figure 5D: Correct the statement that it is not feasible to generate GC under in vitro conditions. GC-like cells can be generated using a well-established in vitro culture system PMID: 21897376 DOI: 10.1038/ncomms1475.

Provide representative flow plots for population gating. Clarify the impact on B cell populations in these cultures.

FIGURE 6
The figure is confusing due to mixing in vitro and in vivo experiments and multiple conditions without much guidance. Organize it more clearly.

Explain the mechanism of GPR109A activation inducing mitochondrial dysfunction.

Is cell death of plasmablasts/B cells rescued by inhibiting or quenching ROS production in in vitro assays?

Figure 6J-K and P: Like Figure 1H, explain why the absolute amount (µg/ml) of NP27-specific IgG1 is lower than NP7-specific IgG1. NP27 ELISA should detect all NP-specific IgG1 antibodies, including those with higher affinity to NP.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
All my concerns have been addressed by the authors. Thanks! I fully support publication of this manuscript.

Reviewer #2

(Remarks to the Author)
The authors have answered all concerns, but its seems like the change in the title is not made.

Reviewer #3

(Remarks to the Author)
The reviewer appreciates the authors' thorough response to the comments and the additional work undertaken to address the main concerns raised. The responses and revisions satisfactorily resolve most issues. However, the reviewer has one remaining comment regarding Figure 4G. The reviewer does not believe that data should be removed from the manuscript solely because an explanation for the difference between NP+ and NP- GC B cells is currently unavailable. Instead, the reviewer suggests retaining this data in the main manuscript or, alternatively, adding it as supplementary material while noting the observed discrepancy in the main text. This will enable readers to assess the observed difference between NP+ and NP- GC B cells and form their own interpretations.

Editor, *Nature Communications*
Re: Submission of a revised manuscript

Date: October 8, 2024

Dear Editor and Reviewers,

We truly appreciate your time and effort in reviewing our manuscript, "*The survival of B cells is compromised in kidney disease."* We have considered all the reviewers' suggestions and have attempted to address their concerns, as outlined below. With the incorporation of the suggested changes, we believe that this manuscript has been strengthened significantly and hope it will be considered acceptable for publication in *Nature Communications*. Major changes in the manuscript are highlighted in yellow. Specific point-by-point responses follow below:

Reviewer 1:

1. It would be interesting to see if there is a correlation between the severity of CKD and its effects on GC B cells. The BUN levels in Suppl. Fig 1 shows, as expected, a fairly large variance.
We have provided data showing a negative correlation between the severity of chronic kidney disease (CKD) (as measured by serum BUN) and the percentage of GC B cells in the NP-KLH in alum immunized mice (***Suppl. Fig S1E***). (*Results: page 7)*

2. The figure legend of Fig S3 is confusing. The title "5/6 Nephrectomy" applies only to panels G and H. Adding histology of these mice would be a nice addition.
We thank the reviewer for pointing out this omission. We have corrected the mistake and added the kidney histology images of 5/6 nephrectomized mice (***Suppl. Fig S3F***). (*Results: page 9; Supplementary Material: page 6*)

Reviewer 2:

I have only one minor thing regarding a shortcoming of this extensive research paper: The analyses of the mouse models are performed only on cells isolated from the spleen. It is interesting if the same trend would be observed in lymph nodes, especially in the renal LN.
We concur with the reviewer on this issue. The spleens are commonly analyzed to measure B cell response following intra-peritoneal injection of NP-KLH in alum or 2.5% SRBC, since this route of immunization induces a robust humoral immune response in the spleen compared to renal lymph nodes. Moreover, it is technically challenging to obtain enough cells from renal lymph nodes to assess B cell response by flow cytometry. So, we decided

to measure B cell response in the spleen of uremic and control mice after immunization with T-dependent antigen throughout the study.

As suggested by the reviewer, we performed new experiments to show that percentage of total GC B cells in renal lymph nodes of AAN mice is reduced compared to PBS or AAII-injected mice at day 12 post-NP-KLH immunization (***Fig 1***: rebuttal). Since we do not have renal lymph node data for all the experiments, we prefer not to include this result in the manuscript.

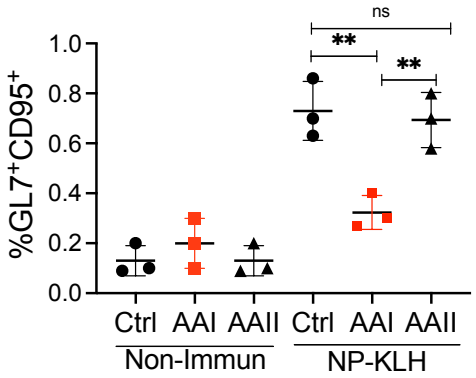

Fig 1: Diminished GC formation in the renal lymph nodes of immunized AAN mice. *C57BL/6 (WT) mice (n=3) were i.p. injected with a single dose of AAI, AAII (7.5 mg/kg b.wt) or PBS (Ctrl). Mice were either immunized with NP-KLH in alum (n=6-12) or left non-immunized (non-immun) (n=3-4) 4 days post-AAI injection. At 12 days post-immunization, renal lymph nodes were assessed for total GC B cells (liveB220$^+$GL7$^+$CD95$^+$) by flow cytometry. Each dot represents individual mice. Data expressed as Mean ± SD. Statistical analyses by One-way ANOVA.*

Reviewer 3:

General comments: It is suggested to change the title to "B cell responses" to avoid misleading readers.

As the reviewer suggested, we have changed the title to "The survival of B cells is compromised in kidney disease."

Figure 6Q: Transfer of B cells into a muMT mouse model to determine B cell-specific effects. However, the cell transfer experiments are underdeveloped. Further experiments are needed, including measurements of affinity maturation, apoptosis, proliferation, TMRE, and MitoSOX to differentiate B cell/GC B cell-intrinsic vs. extrinsic effects of kidney disease.

We thank the reviewer for raising this important point. To address the role of B/GC B cell-intrinsic GPR109A expression in limiting B cell response in kidney disease, we have provided new data showing GC formation, antibody affinity maturation, proliferation, loss of mitochondrial membrane potential, mitochondrial ROS production, and apoptosis in B/GC B cells lacking GPR109A expression using an adoptive transfer approach (***Fig 7E-L and Suppl. Fig S7B-F***). (*Results: page 15; Supplementary Material: page 12*)

FIGURE 1

Provide information on using AAII as a chemical control for AAI.

As suggested, we included the information on using AAII as a chemical control for AAI. (*Results: page 6*)

Does AAI impact B cell populations in the spleen and bone marrow?

Our data demonstrate that AAN and control mice showed comparable number of B cells in the spleen and bone marrow at day 12 post-immunization (***Fig 1B and Suppl. Fig S1C***). (*Results: page 6; Supplementary Material: page 2*)

Absolute cell numbers should be measured for accuracy. Also, for data presented in other figures.
As suggested by the reviewer, we have provided absolute cell numbers for total B cells in the spleen and bone marrow (***Fig 1B and Fig S1C***); total GC B cells in the spleen (***Fig 1C***); total NP-specific IgG1$^+$ B cells (***Fig 2A***) and T follicular helper cells (***Fig 2F***). Since absolute cell numbers of B and T cells mirrored percentage data and there was a comparable number of total B cells between AAI injected and control (AAII and PBS injected) spleens (***Fig 1B***), we opted to present the results in percentage of total splenic B cells rather than absolute cell numbers for subsequent figures (except the adoptive transfer experiment in ***Fig 7***). (*Results: pages 6-8*)

Figure 1D: Improve the quality of immunofluorescence images (IFs). The statement "There was no difference in the number and size of GC between control or AAII-injected spleens" (lines 162-163) is unsupported by the provided data. The immunofluorescence image shows a GC with reduced size compared to controls, but no quantification of GC clusters is provided. Therefore, the concluding statement about compromised GC formation in kidney disease is not yet supported by the presented data.
We have improved the quality of immunofluorescence images and corrected the statement made in connection to the number and size of GC between the groups (***Fig 1E***). (*Results: pages 6*)

Figure 1H: Clarify why different NP conjugation ratio reagents were used to measure antibody affinity at day 12 (NP4) vs day 21 (NP7) after immunization.
The quantification of antibody affinity maturation at day 21 post-immunization was performed almost two years after the initial day 12 measurement using a different batch of NP-KLH and hapten conjugation ratio. Since the conjugation ratio for any given immunochemical varies with each production batch and companies do not offer customized loading ratios, we had to rely on available reagents with different conjugation ratios to measure antibody affinity maturation at day 12 (NP4) *vs* day 21 (NP7) post-immunization (both used for the quantification of serum high-affinity antibodies in NP-KLH immunized mice).

Explain why the absolute amount (µg/ml) of NP7-specific serum IgG1 is higher than NP27-specific serum IgG1 at day 21, suggesting that high-affinity antibodies are more concentrated than total anti-NP IgG1 antibodies.
The reviewer is right, and we sincerely apologize for the inadvertent overlook. We went back to our original workbook and figured out that there was some calculation errors made in connection to the NP7-specific serum IgG1 dilution factors. We redid the entire analysis and present the new results in ***Fig 2D***.

Figure 1I: Specifically describe whether the ELISPOT assay for plasma cells producing NP4-specific IgG1 was performed with cells from the spleen or bone marrow. Similar clarification is needed for data in Figure 2F.
We confirm that the ELISPOT assays for plasma cells producing NP4-specific IgG1 were performed with cells from the spleen in ***Fig 1I*** and ***2F*** (***Fig 2E*** and ***2I*** in the revised manuscript). (*Results: pages 7 and 8*)

FIGURE 2

The figure is complex, with messages about TFH and secondary immune responses. Restructure the presentation for better readability. Include a representative flow stain for TFH to assess how the authors define this population.

We have restructured and simplified the presentation of ***Fig 2*** for better readability. We have now included a representative flow cytometry staining for T follicular helper cells (TFh) as ***Suppl. Fig S2F***. (*Results: page 8; Supplementary Material: page 4*)

Figure 2B: Clarify the meaning of LPS re-stimulation. If referring to LPS re-stimulation of GC B cells, show that these are the cells expressing IL6. The correlation between reduced IL6 production and reduced TFH in the AAI condition is not strong enough to be a main figure.

We have re-phrased the sentence made in connection to "LPS re-stimulation" of total splenic B cells isolated from NP-KLH immunized AAI-injected and control mice. As per the suggestion of the reviewer, we have removed the figure showing reduced IL-6 production by B cells in the AAN spleen in the Supplementary Figure (***Suppl. Fig S2H***). (*Results: page 8; Supplementary Material: page 4*)

Data showing no IL6 induction upon AAI treatment suggests a profound impairment in B cell activation beyond GC B cells. Can the authors please elaborate?

The reviewer is right in pointing out the negative impact of uremia (induced by AAI injection) on B cell activation, as evidenced by diminished IL-6 production. However, the mechanisms of impaired IL-6 production from activated B cells in uremic conditions are unknown. Previous study has shown the critical role of BCR, anti-CD40, and TLR signaling in IL-6 production from activated B cells (PMID: 28899868). It is unclear how these B cell-intrinsic cell signaling pathways are compromised in uremia. Additionally, B cell-extrinsic signals drive IL-6 production from activated B cells. One such B cell-extrinsic factor is IFNγ, previously shown to induce IL-6 in conjunction with BCR, anti-CD40, and TLR signaling (PMID: 28899868). Hence, it is possible that uremia suppresses IFNγ production in the spleen, leading to reduced IL-6 production from activated B cells. Future studies should focus on understanding the impact of various uremic toxin(s) on B cell intrinsic and extrinsic factors in limiting IL-6 production during immunization. We have included these lines in the Discussion section of the manuscript. (*Discussion: page 18*)

FIGURE 4

Figure 4G: The authors observe increased apoptosis in NP+ GC B cells measured by active caspase three stain. Explain why increased apoptosis is only seen in NP+ GC B cells and not in NP- GC B cells. The impact on LZ/DZ distribution seems equally affected in kidney disease, how do the authors interpret differences between NP-binding and non-binding GC B cells?

Currently, the reasons for the discrepancy between the apoptosis of NP binding and non-binding GC B cells is poorly understood. However, it is possible that apoptosis and abnormal LZ/DZ distribution in mice with kidney disease are regulated independently by one or more uremic toxin(s) by distinct mechanisms. In such a case, the difference in the apoptosis of NP binding and non-binding GC B cells cannot be attributed to abnormal LZ/DZ distribution during uremia. Moreover, the possibility of increased GPR109A expression in NP-binding GC B cells than NP-non-binding GCs in uremia cannot be ruled

out and awaits further confirmation. This is an intriguing issue that warrants careful investigation in the future. Since we do not have any evidence to explain this discrepancy, we have removed the NP non-binding GC B cell apoptosis data in ***Fig 4G***.

FIGURE 5

Figure 5D: Correct the statement that it is not feasible to generate GC under in vitro conditions. GC-like cells can be generated using a well-established in vitro culture system PMID: 21897376 DOI: 10.1038/ncomms1475.

As the reviewer suggested, we have rephrased the sentence made in context to the *in vitr*o generation of GC-like cells. (*Results: page 12*)

Provide representative flow plots for population gating.

We have provided representative flow plots for population gating as ***Suppl. Fig S5A, B, E, and G***. (*Results: pages 11 and 12; Supplementary Material: page 9*)

FIGURE 6

The figure is confusing due to mixing in vitro and in vivo experiments and multiple conditions without much guidance. Organize it more clearly.

As per the suggestion of the reviewer, we have reorganized the original ***Fig 6*** into two separate figures, ***Fig 6*** and *7*. ***Fig 6*** mainly shows all the *in vitro* experiments, while ***Fig 7*** contains all the *in vivo* results. As a result, the original ***Fig 7*** is now ***Fig 8***.

Explain the mechanism of GPR109A activation inducing mitochondrial dysfunction.

We have discussed the mechanisms of GPR109A activation in inducing mitochondrial dysfunction and apoptosis in the Discussion section of the manuscript (*Discussion: page 21*)

Is cell death of plasmablasts/B cells rescued by inhibiting or quenching ROS production in in vitro assays?

This is an interesting point raised by the reviewer. To test this issue, resting splenic B cells were stimulated with αIgM + αCD40 + IL-21 in the presence or absence of N-acetylcysteine (NAC) (ROS scavenger/inhibitor: dose 0.15 mM to 1.5 mM). The dose of NAC is selected based on prior study on human B cells (PMID: 12165081). The cells were assessed for apoptosis by Annexin-V/7-AAD staining 24 h later. In contrast to human B cells, mouse splenic B cells showed increased sensitivity to NAC treatment, as evidenced by increased late apoptosis in a dose-dependent manner (***Fig 2***: rebuttal). This data indicates that NAC treatment activates apoptosis in mouse B cells, thus making it technically difficult to test whether apoptosis of B cells in the presence of hippuric acid or uremic serum can be rescued by inhibiting or quenching ROS production.

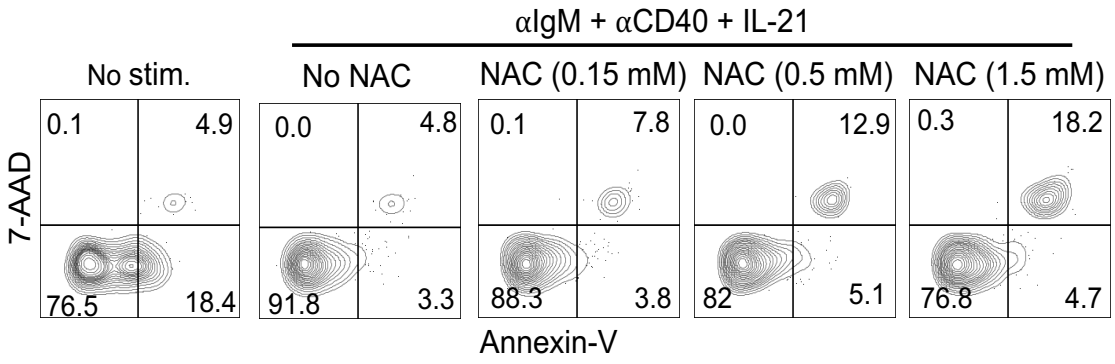

Fig 2: Treatment of in vitro stimulated B cells with ROS quencher. *WT resting splenic B cells were stimulated with αIgM + αCD40 + IL-21 in the presence or absence of NAC at the indicated doses. The number of early and late apoptotic $B220^{+}CD138^{+}$ cells was determined by Annexin-V/7-AAD staining followed by flow cytometry at 24 h. Dot plot represents 1 of 3 independent experiments with similar results. The number in each quadrant indicate percentage of cells.*

Figure 6J-K and P: Like Figure 1H, explain why the absolute amount (μg/ml) of NP27-specific IgG1 is lower than NP7-specific IgG1. NP27 ELISA should detect all NP-specific IgG1 antibodies, including those with higher affinity to NP.

Please see the response to *Rev 3 – Fig 1, point 6*.

As the same faulty Excel template has been used to calculate the concentration of serum NP7-specific IgG1 in ***Figs 6J-K*** and ***P***, the error is unknowingly carried to these analyses as well. We have reanalyzed the results and presented the new data in ***Fig 6I*** and ***Fig 7D***.

Sincerely,

Dr. Partha S. Biswas, DVM, PhD
Professor of Microbiology and Immunology
Dept. of Microbiology and Immunology
Renaissance School of Medicine
Stony Brook University
Stony Brook, NY

Editor, *Nature Communications*
Re: Submission of a revised manuscript

Date: November 18, 2024

Dear Editor and Reviewers,

We truly appreciate your time and effort in reviewing our manuscript, "*The survival of B cells is compromised in kidney disease".* We have considered all the reviewers' suggestions and have attempted to address their concerns, as outlined below. With the incorporation of the suggested changes, we believe that this manuscript has been strengthened significantly and hope it will be considered acceptable for publication in *Nature Communications*. Specific point-by-point responses follow below:

Reviewer #1 (Remarks to the Author):

All my concerns have been addressed by the authors. Thanks! I fully support publication of this manuscript.
No comments.

Reviewer #2 (Remarks to the Author):

The authors have answered all concerns, but its seems like the change in the title is not made.
Please note that the change in title is made and the new title is "The survival of B cells is compromised in kidney disease".

Reviewer #3 (Remarks to the Author):

The reviewer appreciates the authors' thorough response to the comments and the additional work undertaken to address the main concerns raised. The responses and revisions satisfactorily resolve most issues. However, the reviewer has one remaining comment regarding Figure 4G. The reviewer does not believe that data should be removed from the manuscript solely because an explanation for the difference between NP+ and NP- GC B cells is currently unavailable. Instead, the reviewer suggests retaining this data in the main manuscript or, alternatively, adding it as supplementary material while noting the observed discrepancy in the main text. This will enable readers to assess the observed difference between NP+ and NP- GC B cells and form their own interpretations.
As the reviewer suggested, we have retained the data describing Caspase3 activation in the NP^+ and NP^- GC B cells following NP-KLH in alum immunization in mouse spleen (***Fig 4G***).

Sincerely,

Dr. Partha S. Biswas, DVM, PhD
Professor of Microbiology and Immunologyv
Dept. of Microbiology and Immunology
Renaissance School of Medicine
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