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Peer Review File

Regulation of Lipoprotein Processing by GPNMB in Foamy Macrophages: Potential Therapeutic Targets for Atherosclerosis

Corresponding Author: Professor Li Tang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
This is a data rich manuscript which however is at times unconvincing in its rigor and the conclusions. For instance, the authors comment on metabolic processes with strong language but never actually assay metabolism such as lipid oxidation. There are other concerns that weaken this reviewer's evaluation, including the points below.

The colocalization in 1E is not convincing.

The correlations in 1D are underwhelming.

The combination of CT with surface fluorescence imaging in Fig. 8F is unconvincing. It remains unclear where the fluorescence signal is coming from. The combination of imaging modalities makes little sense, what would be needed is tomographic fluorescence imaging with appropriate controls.

Animal numbers in Fig 8 are too low for some plots (at least n=6 would be desirable).

The in vivo silencing experiments are lacking rigorous controls and biodistribution analyses. For instance, cellular uptake into target cells remains uncertain and the targeting AB strategy is not supported by isotype controls.

Reviewer #3

(Remarks to the Author)
The authors addressed the function of GPNMB in the formation of atherosclerosis and lipid loaded foam cells. GPNMB has been well known to be expressed in macrophages following lipid loading and by foam cells in atherosclerotic plaque. Some prior mechanisms of GPNMB have been explored, but the influence of GPNMB on cholesterol uptake, storage, and foam cell formation in vivo has not been adequately addressed prior to this study. The authors expand on the detection of GPNMB upregulated in oxLDL stimulated macrophages, human tissue samples, and support a potential role for soluble GPNMB. Whole body and myeloid-specific deletion of GPNMB resulted in reduced foam cell formation and reduced plaque formation in a mouse model of atherosclerosis. Mechanistically, the study explores GPNMD cellular distribution and its influence on macrophage cholesterol uptake, showing that GPNMB plays a direct role in the shuttling of cholesterol, lipid droplet storage, and lipid droplet size. Finally, the authors use an innovative lipid nanoparticle delivery approach to show a therapeutic approach to knockdown GPNMB in atherosclerosis in vivo.

The figures in this paper are very well organized and a lot of attention was paid to their arrangement. In addition, experiments appeared to be performed in a rigorous and thorough manner. Overall, the data support the authors conclusions and provide a significant advancement beyond prior work in the field. This is a very impactful study that emphasized GPNMB as a potential new therapeutic avenue for cardiovascular disease. Nonetheless, addressing some of the comments below may improve the clarity and impact of the work.

-Suggest changing the title to something more active and in line with the conclusions of the study.

-What is the proposed mechanism linking the deletion of GPNMB and the impact on serum cholesterol levels? This should be included in the discussion since it is likely relevant to the outcome of the atherosclerosis studies. In addition, why were only three replicates included in the serum Tg/Chol analysis (Fig 8J) of the siGPNMB in vivo study? Can additional samples be included, since it seems like several of the measurements are not significant due to an underpowered experiment.
-the authors claim enhanced plaque stability in the text, but stability isn't clearly tested in the experiments. Either provide additional analysis of plaque stability measurements or remove the comment.
-The authors should include citations to the original article for GEO data, GEO database, GDS5083 [Line 120], GDS5083 [line 200] when they are introduced in the results.
-Figure 1I is too small. Probably move the individual plots to a supplemental figure so they can be read more clearly.
-The authors show that a few lipid droplet associated genes are influenced by the deletion of Gpnmb (such as Plin 2, Plin 3). Could the authors also include a heatmap for other foam cell or lipid associated macrophage genes (in addition to the nice heat maps already included)? Foam cell genes were outlined in several prior papers (such as Kim, Circulation Research 2018 and Zernecke Circulation Research 2020).

Version 1:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
All of my concerns were addressed.

Reviewer #4

(Remarks to the Author)
The authors have addressed most of the concerns raised during the previous round of review, but several key issues still require further clarification and additional data:

1. Statistical analysis and controls for Western blot data: Several Western blot results lack quantitative statistical analysis, including Figures 2i, 3d, 3e, 3g, S3h, S3j and et al. The authors should provide densitometric quantification with appropriate statistical tests. Additionally, Figure S3j requires a proper negative control (e.g., 0 μg/mL ox-LDL) to validate the specificity of the observed effects.
2. Unexpected biodistribution of CD36+TREM2-LNPs in Figure 8h: The increased renal accumulation of CD36+TREM2-LNPs when compared to control LNPs is unexpected and warrants further discussion. The authors should clarify whether this reflects active targeting or nonspecific clearance. Moreover, the near absence of hepatic fluorescence is surprising, given that liver accumulation remains a major challenge for most LNPs, even those engineered for extrahepatic delivery. The author should explain this observation. This does not look reasonable thus far. Repeated experiments might be necessary to ensure accuracy and reliability.
3. Quantification of in vivo imaging data: The fluorescence intensity data in Figures 8h and 8i should be supplemented with statistical analysis. Regions of interest (ROIs) and normalization methods must be clearly described to ensure reproducibility.

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
The authors have addressed the comments well, I am supporting the acceptance of this work for publication.

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Dear Reviewers,

We sincerely appreciate the opportunity to revise our manuscript titled **"Regulation of Lipoprotein Processing by GPNMB in Foamy Macrophages: Implications for Atherosclerosis" (Manuscript ID: NCOMMS-24-66272-T)**. We extend our heartfelt gratitude for your constructive comments and valuable suggestions, which have significantly improved the quality of our work. We have carefully addressed all the comments point by point and made corresponding revisions to the manuscript. In the revised manuscript and response letter, the newly added data and their corresponding figure legends have been highlighted in yellow for easy reference. In addition to the data modifications mentioned in the following response, we have further supplemented the manuscript by including Table S9 and refining the schematic diagram for enhanced clarity. Below, we provide a detailed response to each comment.

Reviewer #2

This is a data rich manuscript which however is at times unconvincing in its rigor and the conclusions. For instance, the authors comment on metabolic processes with strong language but never actually assay metabolism such as lipid oxidation. There are other concerns that weaken this reviewer's evaluation, including the points below.

Response: We sincerely thank you for your constructive feedback, which has helped us improve the rigor and clarity of our work. You raised concerns about our use of strong language when commenting on metabolic processes without directly assaying metabolism, such as lipid oxidation. We acknowledge this limitation and have meticulously refined the terminology in the manuscript (replacing "lipid metabolism") to ensure the precision of our conclusions (Line 308, 349, and 357, highlighted in bright blue). Below are our point-by-point responses to other comments:

1. The colocalization in 1E is not convincing.

Response: We acknowledge your concern. Given that colocalization analysis was not required for Figure 1E results, we postulate that the immunohistochemical findings presented in Figure 2E may not be convincing. Consequently, we repeated the experiment and replaced the original images with more definitive results that demonstrate the intended colocalization (Figure 2E).

2. The correlations in 1D are underwhelming.

Response: We appreciate your astute observation regarding the correlation strength in Figure 1D. The Spearman correlation coefficient between SCORE risk score and

plasma sGPNMB levels was 0.2352 (*P*=0.0033), indicating a statistically significant but weak positive correlation. Although the correlation strength was modest, the results demonstrated a stepwise elevation in circulating sGPNMB levels across atherosclerosis risk score (Figure 1D, right panel). When integrated with two additional quantitative metrics (Gensini Score and current 10-year ASCVD Risk score), our composite analysis suggests a moderate positive association between sGPNMB and AS progression (Line 127-129, highlighted in bright blue).

We explicitly acknowledge the limited pairwise correlation and have explicitly emphasized the necessity to expand the clinical samples and incorporate additional biomarker parameters (e.g., inflammatory cytokines, advanced lipid profiling). Furthermore, we recommend against the standalone application of sGPNMB for atherosclerosis risk stratification, instead proposing its integration within a multi-modal biomarker panel. All aforementioned modifications have been incorporated into the revised manuscript (Lines 142-146, highlighted in yellow).

3. The combination of CT with surface fluorescence imaging in Fig. 8F is unconvincing. It remains unclear where the fluorescence signal is coming from. The combination of imaging modalities makes little sense, what would be needed is tomographic fluorescence imaging with appropriate controls.

Response: We appreciate your feedback regarding the combination of imaging modalities in Figure 8F. To clarify the origin of the fluorescence signal and improve the interpretation, we designed LNPs conjugated with an isotype antibody for anti-CD36 antibody and an isotype antibody for anti-TREM2 antibody (Control-LNPs) as the controls. Furthermore, we performed additional experimental validation (*in vivo* small animal live-cell imaging) beyond photoacoustic and *in vivo* fluorescence imaging (Figure 8F-G and Figure S12k-m). These observations displayed a significantly increased distribution of CD36+TREM2-LNPs in atherosclerotic plaques and kidneys, accompanied by a marked reduction in hepatic accumulation, while no significant differences were observed in other major organs, compared to Control-LNPs. All aforementioned modifications have been incorporated into the revised manuscript (Lines 521-540, highlighted in yellow).

4. Animal numbers in Fig 8 are too low for some plots (at least n=6 would be desirable).

Response: We understand your concern about the low number of animals used in some experiments. To address this, we have increased the sample size for key experiments, including Figure 8K, Figure 8M, and Figure S12o. This will help us achieve more robust statistical power and reliable conclusions. All aforementioned modifications have been incorporated into the revised manuscript (Lines 549-555 and 558-560, highlighted in yellow).

5. The *in vivo* silencing experiments are lacking rigorous controls and biodistribution analyses. For instance, cellular uptake into target cells remains uncertain and the targeting AB strategy is not supported by isotype controls.

Response: We agree with your suggestion to include stricter controls and a detailed

analysis of biodistribution in our *in vivo* silencing experiments. We have added the isotype controls for the targeting antibody (Control-LNPs). Moreover, *in vivo* small animal live-cell imaging displayed a significantly increased distribution of CD36+TREM2-LNPs in atherosclerotic plaques and kidneys, accompanied by a marked reduction in hepatic accumulation, while no significant differences were observed in other major organs, compared to Control-LNPs. These observations indicated that CD36+TREM2-Cy5.5-LNPs exhibit significantly enhanced targeted accumulation in atherosclerotic plaques compared to Control-LNPs.

Additional *in vivo* small animal live-cell imaging showed obvious deposition of CD36+TREM2-Cy5.5-LNPs in plaques (Figure 8G). Flow cytometry analysis confirmed the presence of CD36+TREM2-Cy5.5-LNPs^{+} cells in plaque-containing aortas (Figure S12s), and immunofluorescence staining further verified the targeting of CD36+TREM2-Cy5.5-LNPs to plaque macrophages and foam cells (Figure 8J). All aforementioned modifications have been incorporated into the revised manuscript (Lines 519-566, highlighted in yellow).

Thank you again for your constructive feedback. We look forward to addressing these points thoroughly and hope that our revisions will meet your expectations.

Reviewer #3

The authors addressed the function of GPNMB in the formation of atherosclerosis and lipid loaded foam cells. GPNMB has been well known to be expressed in macrophages following lipid loading and by foam cells in atherosclerotic plaque. Some prior mechanisms of GPNMB have been explored, but the influence of GPNMB on cholesterol uptake, storage, and foam cell formation in vivo has not been adequately addressed prior to this study. The authors expand on the detection of GPNMB upregulated in OxLDL stimulated macrophages, human tissue samples, and support a potential role for soluble GPNMB. Whole body and myeloid-specific deletion of GPNMB resulted in reduced foam cell formation and reduced plaque formation in a mouse model of atherosclerosis. Mechanistically, the study explores GPNMB cellular distribution and its influence on macrophage cholesterol uptake, showing that GPNMB plays a direct role in the shuttling of cholesterol, lipid droplet storage, and lipid size. Finally, the authors use an innovative lipid nanoparticle delivery approach to show a therapeutic approach to knockdown GPNMB in atherosclerosis in vivo.
The figures in this paper are very well organized and a lot of attention was paid to their arrangement. In addition, experiments appeared to be performed in a rigorous and thorough manner. Overall, the data support the authors conclusions and provide a significant advancement beyond prior work in the field. This is a very impactful study that emphasized GPNMB as a potential new therapeutic avenue for cardiovascular disease. Nonetheless, addressing some of the comments below may improve the clarity and impact of the work.

Response: We are deeply honored by your recognition of our work and truly excited to share these findings with the scientific community. Thank you for your insightful comments and suggestions. Your feedback has been very helpful in identifying areas where we can improve the manuscript. Below are our point-by-point responses to other comments:

1. Suggest changing the title to something more active and in line with the conclusions of the study.

Response: We appreciate your suggestion to make the title more specific and relevant. We have revised the title to better reflect the active mechanism uncovered in our study: "Regulation of Lipoprotein Processing by GPNMB in Foamy Macrophages: Potential Therapeutic Targets for Atherosclerosis".

2. What is the proposed mechanism linking the deletion of GPNMB and the impact on serum cholesterol levels? This should be included in the discussion since it is likely relevant to the outcome of the atherosclerosis studies. In addition, why were only three replicates included in the serum Tg/Chol analysis (Fig 8J) of the si*Gpnmb in vivo* study? Can additional samples be included, since it seems like several of the measurements are not significant due to an underpowered experiment.

Response: We agree that it is important to include a discussion on the mechanism by which GPNMB deletion influences cholesterol levels. We have added a paragraph in the Discussion where we hypothesize potential mechanisms (Line 621-645, highlighted in yellow).

We understand your concern about the low number of animals used in some experiments. To address this, we have increased the sample size for key experiments, including Figure 8K (Figure 8J in the initial manuscript submission) and Figure S12o. This will help us achieve more robust statistical power and reliable conclusions in the revised manuscript.

3. the authors claim enhanced plaque stability in the text, but stability isn't clearly tested in the experiments. Either provide additional analysis of plaque stability measurements or remove the comment.

Response: You are correct that our current data do not directly test plaque stability. We have removed the statement regarding enhanced plaque stability from the manuscript.

5. The authors should include citations to the original article for GEO data, GEO database, GDS5083 [Line 120], GDS5083 [line 200] when they are introduced in the results.

Response: Thank you for pointing out the missing citations for the GEO database. Citations for GDS5083 datasets have been added in the text (Lines 120, 204). We also reviewed the manuscript to ensure that other databases were appropriately cited.

6. Figure 1I is too small. Probably move the individual plots to a supplemental figure so they can be read more clearly.

Response: We apologize for the small size of Figure 1I, which makes it difficult to interpret. To address this, Figure 1I has been moved to Supplementary Figure S3 (Figure S3g) and enlarged for clarity.

7. The authors show that a few lipid droplet associated genes are influenced by the deletion of Gpnmb (such as Plin 2, Plin 3). Could the authors also include a heatmap for other foam cell or lipid associated macrophage genes (in addition to the nice heat maps already included)? Foam cell genes were outlined in several prior papers (such as Kim, Circulation Research 2018 and Zernecke Circulation Research 2020).

Response: We appreciate your suggestion to include additional foam cell-associated genes in our heatmap. As suggested, we have included a new heatmap (Supplementary Figure S10f) analyzing additional foam cell-associated genes. The relevant descriptions have also been incorporated into the revised manuscript (Lines 435-437, highlighted in yellow).

We hope these revisions address your concerns and significantly strengthen the manuscript. Thank you again for your time and valuable insights.

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Once again, we would like to express our gratitude to the editor and reviewers for their insightful feedback, which has greatly enhanced the clarity and scientific rigor of our manuscript. We hope that the revised version meets the standards of the journal and is now suitable for publication in ***Nature Communications***. Thank you for your time and consideration.

Sincerely,

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Dear Reviewers,

We sincerely appreciate the opportunity to revise our manuscript titled **"Regulation of Lipoprotein Processing by GPNMB in Foamy Macrophages: Implications for Atherosclerosis" (Manuscript ID: NCOMMS-24-66272-T)**. We extend our heartfelt gratitude for your constructive comments and valuable suggestions, which have significantly improved the quality of our work. We have carefully addressed all the comments and made corresponding revisions to the manuscript. In the revised manuscript and response letter, the newly added data and their corresponding figure legends have been highlighted in yellow for easy reference. Below, we provide a detailed response to each comment.

Reviewer #4

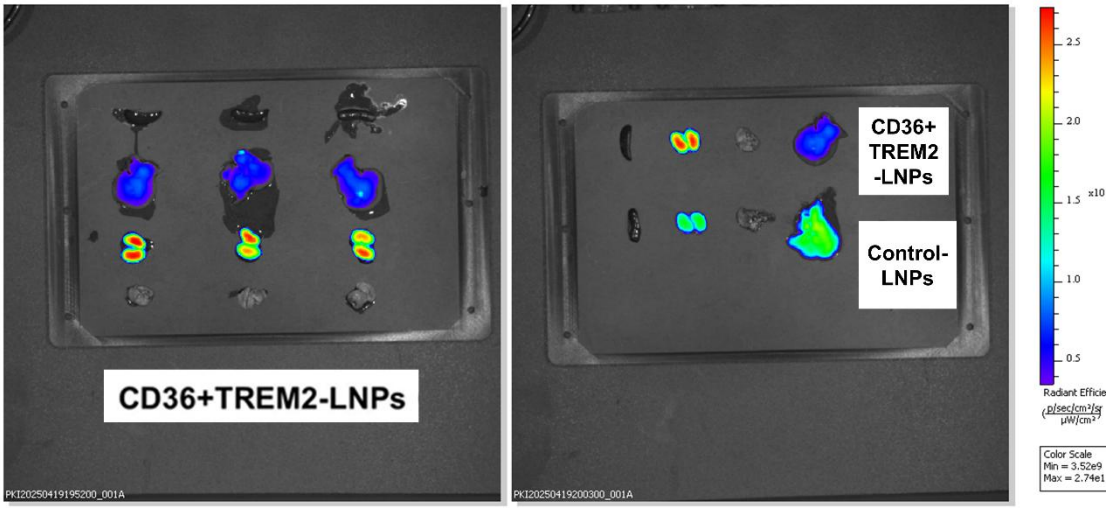
1. Statistical analysis and controls for Western blot data: Several Western blot results lack quantitative statistical analysis, including Figures 2i, 3d, 3e, 3g, S3h, S3j and et al. The authors should provide densitometric quantification with appropriate statistical tests. Additionally, Figure S3j requires a proper negative control (e.g., 0 μg/mL ox-LDL) to validate the specificity of the observed effects.

Response: We acknowledge your concern. To address this, we have added densitometric quantification with appropriate statistical analyses (unpaired *t*-tests or one-way ANOVA with Tukey's post hoc test) for all the relevant figures. The results were included in the revised panels. Specifically, this includes the following statistical data panels: Figure 2I-Figure 2J, Figure 3D, Figure 3E, Figure 3G-Figure 3H, and Figure S3j (now S3i). The revised description of these results has also been highlighted in the manuscript (Lines 185-187 for Figure S3i, 221-230 for Figure 3H). Additionally, a 0 μg/mL ox-LDL negative control has been incorporated into Fig. S3j (now S3i) to confirm the effect specificity.

2. Unexpected biodistribution of CD36+TREM2-LNPs in Figure 8h: The increased renal accumulation of CD36+TREM2-LNPs when compared to control LNPs is unexpected and warrants further discussion. The authors should clarify whether this reflects active targeting or nonspecific clearance. Moreover, the near absence of hepatic fluorescence is surprising, given that liver accumulation remains a major challenge for most LNPs, even those engineered for extrahepatic delivery. The author should explain this observation. This does not look reasonable thus far. Repeated experiments might be necessary to ensure accuracy and reliability.

Response: We appreciate your astute observation regarding the correlation strength in Figure 8H. We sincerely apologize for the misinterpretation caused by inappropriate data processing (specifically, excessive background subtraction in Figure 8H). We have reprocessed the original data and replaced the corresponding panel. As revealed in the updated Figure 8H, in conjunction with Figure 8G, considerable LNP signal presence is also observable in the liver of the targeted group, albeit significantly lower than that in the non-targeted group. Specifically, we present *ex vivo* fluorescence images of the remaining mice in the CD36+TREM2-LNPs group across various organs (particularly the liver and kidneys), which demonstrate the discernible presence of the fluorescent signal in the liver and reveal limited inter-individual variation within the group, as corroborated by the statistical data in Figure 8G.

Moreover, in response, we have supplemented the Discussion section (Line 622-647) with a comprehensive analysis of the observed biodistribution profile in Figure 8H.



3. Quantification of *in vivo* imaging data: The fluorescence intensity data in Figures 8h and 8i should be supplemented with statistical analysis. Regions of interest (ROIs) and normalization methods must be clearly described to ensure reproducibility.

Response: Thanks for your constructive suggestion. We have now supplemented Figures 8H and 8I with quantitative statistical analyses, and the revised description of these results has been highlighted in the manuscript (Lines 537-539 for Figure 8H, 550-553 for Figure 8I (now Figure 8J)). Additionally, we provide explicit methodological details in the revised Materials and Methods (subsection "***In vivo fluorescence imaging, Photoacoustic imaging, and In vivo Microscopy***", highlighted with yellow). These updates ensure rigor, reproducibility, and statistical validity of the biodistribution data.

We hope these revisions address your concerns and significantly strengthen the manuscript. Thank you again for your time and valuable insights.

Once again, we would like to express our gratitude to the editor and reviewers for their insightful feedback, which has greatly enhanced the clarity and scientific rigor of our manuscript. We hope that the revised version meets the standards of the journal and is now suitable for publication in ***Nature Communications***. Thank you for your time and consideration.

Sincerely,

Li Tang, Ph.D.
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