

Platelet activation plays a pro-inflammatory role in myasthenia gravis

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript by Wen et al provide insights for a potential role for platelets in Myasthenia Gravis. The study supports increased platelet activation in AChR+ nMG (correlating with severity) leading to interaction with leucocytes (predominantly neutrophils) and dysregulation of CD4+ T cell phenotype, possibly through platelet released RANTES. There is no doubt that platelets interact with and influence leucocytes in inflammatory disease pathology. The key question is why platelets are activated in a predominantly neuromuscular disease like MG and what are the platelet and leukocyte signaling pathways induced. Overall there is a need for in vivo animal studies to demonstrate in vivo relevance and cause-effect.

- Platelets activation needs to be explored in more detail including why and how. Is it cause-effect, a primary causative or secondary phenomenon? This is important for this manuscript as platelet activation is often secondarily associated with inflammation. In vivo mouse studies may be needed for this.
- The authors performed the scRNA Seq of platelets but only from those present in the isolated PBMC fractions where platelets are less than 10% in numbers as compared to PBMCs. The washed platelets should also be studied to determine levels of activation and phenotypes and compared to those interacting with the leucocytes.
- Platelets are known to form aggregates with neutrophils in inflammatory disorders such as ARDS and also shown to contribute to NET formation and NETosis. NET formation should be assessed in the MG patients. The MG patients post treatment (MMS) had reduced platelet-neutrophil aggregates but not monocyte or lymphocyte-platelet aggregates. This should be discussed. The increased myeloid-platelet aggregates in nMG group and its decline in clinical treatment group is an interesting observation. Platelet-neutrophil co-culture assays should also be performed.
- Figure 5 is very important as it provides evidence of platelet surface activation by FC assays, however the platelet cell population in platelet/PBMC pseudocolor plots (CD61 +ve events) does not appear to be a defined platelet population. The non-uniform scatter pattern which is unusual for such FC platelet plots doesn't represent the standard platelet FC plots which uniformly appear when gated by a platelet marker.
- Authors claim increased microparticles in MG group as per Figure 4j which shows the platelet morphology by TEM and the microparticles, however the same figure panel in left HC TEM micrograph shows apparently similar or higher levels of microparticles in HC group also. Quantification of microparticles should be provided.
- Platelets have been reported to affect both CD4 and CD8 T lymphocytes. Are other lymphocytes impacted in myasthenia gravis?
- Result section 3.6 use cytokine neutralization assays where authors select PF4, RANTES and TGFβ1 as targets based on previous literature. The platelet transcriptome results or other results before this did not provide sufficient rationale for using these three as targets for these assays. Were there other upregulated proteins/chemokines in the transcriptomics data which could be the targets? RANTES is a well-studied therapeutic target in autoimmune disorders (Matsui et al 2002, PMID: 12098506; Zang et al, PMID: 11108946). Is the source of RANTES limited to platelets?
- The authors should consider exploring antiplatelet agents such as aspirin in its ability to reduce RANTES and leucocyte

activation.

Minor

- How many events were recorded for FCM assay for platelet and PBMC analysis and what % of events of total were platelets (Figure 5A)?
- The discussion is rather lengthy with unnecessary sub-sections and discussion such as with microparticles.
- A very important reference (Jin et al, Cell Discov, 2021) about immune cell heterogeneity in MG should be discussed.

Reviewer #2

(Remarks to the Author)

Dear editor,

With interest I've read the manuscript regarding myasthenia gravis (MG) and the correlation with altered platelet biology. The authors have explored several technologies and methodologies (incl single cell sequencing, TEM and in vitro immune cell cultures) to understand how altered platelet biology is connected to MG and MG disease severity. Their single cell sequencing experiment identified an increased number of platelets in MG patients suffering from disease, while this returns to normal in patients achieving minimal disease due to treatment. This finding is corroborated in a larger group of patients although the spread among the group is wide. As such it has limited value as a biomarker, but may be interesting for studying a role in the pathophysiology or aetiology of the disease. The rest of the manuscript is focussed on extending this connection between abnormal platelet number and function to MG disease status. To summarize they find: 1. Increased platelet numbers in severe MG patients (3 scRNAseq, larger cohort in serum), 2. altered transcriptional profiles in these cells from patients with disease (n=4), 3. Altered morphology of these platelets (in 1 patient), 4. Enhanced expression of CD62P and sCD40L, 5. an interesting correlation between reduction of PLA formation following IVIg treatment that precedes QMG improvement (n=6), 6. The activated platelets engage more often in interactions with leukocytes (n=many), 7. Medium from MG platelet cultures induce more T cell aggregates (n=>10). The data is convincing although here and there sloppy when it comes to showing the right data. See major comments below. This needs attention. The authors, based on this work, postulate an exciting new hypothesis for this field claiming that this activated platelet phenotype contributes to the autoimmune response observed in these patients. Although the sequence of events is not clear from this manuscript. Meaning we don't know if the platelet dysfunction kickstarts the autoimmune response, is permissive/supportive/ facilitates it or is it a secondary effect of an already derailed immune system? In addition, the authors show some data regarding inhibition of RANTES signaling as a means of limiting the activating nature of platelets in patients (and controls) which may show therapeutic promise in the future. This manuscript presents an interesting new line of research on which very little knowledge exists. I recommend the editor to include a platelet expert for reviewing this manuscript as my expertise is limited to MG and its pathophysiology while the platelets and corresponding methodology are a major component of this manuscript.

The manuscript is well written and does not need much further English editing. Regarding the content of the manuscript I have some specific recommendations, questions and remarks. These have been outlined below in major and minor comments.

Study soundness

The study includes a relatively large number of immunotherapy naïve patients for validation which is important for study power. The single cell data however includes only two. This is understandable given the challenges for these studies however does warrant caution for extending the observations beyond this limited group of individuals. The TEM data was performed on 1 patient sample and although exciting suffers from the same challenge. It would be helpful for the reader to understand which data is generated with different methods from the same donor. That way we can connect the datasets and obtain more confidence in some of the conclusions. The paragraph on the TEM data furthermore refers to several observations with regard to the morphology of these cells however only shows 1 outcome measure in 4i. It would be useful to get the other analysis also in plots to support the observations mentioned in the text. It would be highly recommended to include TEM data from more than just 1 patient if possible.

Noteworthy is that this study contains a single cell dataset that includes untreated AChR MG patients which can be considered an asset and unique to the field. Usually these studies include treated patient samples which suffer from obvious biases.

Major comments

The authors highlight that the focus on platelets in this manuscript comes from the deviation of relative percentage of these cells compared to controls. The data suggest however also major differences on the level of memory B cells. Have the authors looked into this? This is an important observation and one that would deserve more attention in light of the adaptive autoimmune response underlying the disease. Can the authors elaborate on this?

It is unclear to the reviewer why patient nMG4 data is not included in the main figure. If this is because of a batch effect issue, one could still present the data together, but highlight this technical issue. The observation regarding the platelet count is strengthened with this data although the expression profile brings more nuance as to the inter-individual similarities/differences.

The statement line 396-397 "compelling bridge" this statement seems overbearing. It is not clear to the reviewer how this transcriptional data already confirms immunological dysregulation in patients. It suggests skewed numbers of cells that have altered expression profiles, but no dysfunction (on protein level) and certainly not dysfunction causally related to the pathophysiology of MG. It is recommended to rephrase this sentence at this location in the manuscript.

I'm not an expert in SSC analysis, but the flow plots in figure 4 show multiple populations on the y axis with the MMS individuals having 3 (as it seems). Is this what you would expect and which groups of cells are these and can the platelet cell surface markers CD61 and CD62P be compared between groups for each of these cell populations? Furthermore the CD61 value in the MMS condition in the plot is the highest while in the table under b this is not the case. Are these representative flow diagrams? Figure 4F does not seem to reflect the FSC SSC analyses strategy as suggested in the text. In this section it would also be good to mention some thoughts on why the MMS group does not show HC phenotype (as in other outcome measures they do).

Figure 5a would be good to show representative gating plots for the disease/control settings like in figure 4.

Figure s9 would be easier to interpret if the sizes of the aggregates for all conditions were also quantified and presented in a graph. The effect seems rather large so I could imagine this would make sense to present in the main figure.

Figure 6c,d does not show the data referred to in the text. C shows an example of the gating strategy.

The panels from several figures are not chronologically presented in the text. This is sometimes a bit confusing when looking at the figures.

In the discussion the authors mention platelet dysfunction as an upstream factor. What's the evidence for that statement in this manuscript? Please rephrase.

Minor comments

1. Line 111 enrolled enrolment
2. Would be helpful to include data on the use of pyridostigmine or other non-immune treatments
3. Line 114-115 how many patients were now included? 3 or 4?
4. Please clarify how and where healthy donors were recruited.
5. Figure 1 typo in characteristics
6. The supplementary data still contains track changes
7. Supp table 1 thymoma status is called normal. Perhaps make clear to the reader that the thymus is normal or the thymoma status is negative. MGFA classification in the table is at time of withdrawal of blood?? Otherwise this classification and onset symptoms do not match.
8. Figure 1e y axis is not starting at 0
9. Introduction of abbreviation of MK is missing in discussion

Reviewer #3

(Remarks to the Author)

General comments:

In their manuscript, Dr Wen and collaborators analyzed the implication of platelet in Myasthenia Gravis (MG). First, on a small number of patients, they performed a SC-RNAseq of PBMCs to characterize the profile of platelets in controls, MG patients before and after immunosuppressive therapy. Next, on large cohorts of patients, they assessed the activation status of platelets by analyzing CD62P expression, platelet morphology, and sCD40L. They thoroughly investigated the impact of platelet and platelet supernatant on naive CD4 T cells proliferation and cytokine expression profiles in co-culture experiments.

This is a very original and interesting study. However, the important messages do not stand out well because there is too much information that may not be necessary. I am not sure that the SC-RNAseq section contributes much. The paper should focus on the functional studies.

Below are listed comments to further improve the manuscript.

Title:

The title is too long

Abstract:

In the Methods section, indicate that co-culture experiments were also done with platelet supernatant. The Results section does not mention SC-RNAseq conclusions.

The abstract concludes "characterized by enhanced adhesion" but this has not been studied.

Materials and Methods:

Participants:

- Line 103: It is mentioned "Patients were excluded based on the... (3) history of malignant tumor and other autoimmune diseases; while in Supplemental tables thymoma patients are clearly indicated.
- All patients are above 35 years old with average ages according to the supplemental tables of 53-55 years. This should be indicated in the text.
- Line 112: 4 patients are mentioned? Checked with lines 330 and 830.
- In table S1, it is not mentioned if patients were thymectomized, while they were all thymectomized in the other cohorts.

SC-RNASeq analysis:

- Platelet analysis by SC-RNAseq has been performed on PBMCs. This is surprising as standard PBMC isolation protocols, such as density gradient centrifugation, are not designed to retain platelets. Platelets are smaller and less dense than PBMCs, and they may be lost during the isolation process. This might also lead to a bias as MG platelet were described as smaller (Fig 4).

- Have the SC-RNAseq (Single-Cell RNA sequencing) data been deposited in public databases?

Flow cytometry and other analyses

- It is not clear if the other analyses (FACS, TEM) have also been performed on PBMCs or on whole blood cells. This should be indicated in the methods sections.

Co-culture experiments

- Lines 245-252: It is not clear if the supernatants were also prepared from different platelet densities and if this was tested. Which option was chosen for the final experiments?

- Line 254, PMA activation is mentioned here but not found in the figure legends. It is not mentioned in the results section either.

- Lines 275-288: There should be a dedicated paragraph on flow cytometry analysis.

- Paragraph 2.7 should be subdivided to make the co-culture experiments easier to understand.

Results

- In the figure legends, all figures should be labeled with a letter and explained in the legends.

For the SC-RNAseq:

- Lines 348-354: why MG4 was not included in the SC-RNAseq analysis?

- Fig 2b left: the aggregation of samples does not seem perfect: for example, memory CD4 T cells (light blue) are dispatched on two different locations, as well as naïve CD8 T cells (light green).

- Fig 2b right: It seems that only MMS1 and MG3 are shown in this graph. Provide the number of platelets analyzed for each patient. Is there a bias related to the use of PBMCs?

- Fig 2c right: explain in the legend what it is.

- Fig 2e and Supplemental Figures 4: HC controls should be added

- Line 371: A fold change (FC) of 1 does not indicate differential expression. The figure shows the Log2 (FC) values. However, no gene appears to be significantly increased, but this is not surprising as only 3 HC and 3 MG samples were compared.

- Fig 3c: The color code in the legend does not match the colors of the clusters in the figure. The names of the clusters should be provided

- Fig 3e is not helpful. According to the x-axis cluster 8 is thus the 7?

- Fig4e: In the legend, explain how the percentage of non-spheroid platelets is obtained.

- Figure 4f and figure 5a correspond to the same CD45 and CD61 FCS analysis but the gated population (CD45-CD61+) is considered as platelet free in Fig 4f and as PLAs in Fig 5a. There might be an error.

- How to relate the data analyzing platelet-myeloid cells aggregates in figure 3 and the fact that there are no changes in figure 4b right)

- Figure 6: This figure is complicated. Maybe the legend should be reviewed to be more helpful. In the legend, it is not explained what is w.P. The experimental design of the supernatant treatment with a plate with cells is confusing. Cells should be above the plate.

- Fig 6a: The act Tn data should be in the figure or at least a line at 1 should be indicated on the graph to show the difference compared to act Tn. And it should be indicated if there is a significant difference with act Tn.

Discussion

- The discussion is too long

- All along the manuscript it is confusing as results from figure 3 mentioned platelet-myeloid cell aggregates, from figure 5 platelet-lymphoid cell aggregates, and from figure 7 platelet-neutrophil aggregates.

- The patients were divided into naïve and MMS groups. Could the duration of symptoms impact the level of platelet activation?

References

- The Reference n°2, is not the more appropriate regarding Treg cell dysregulation in MG.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have adequately addressed all our concerns. An impressive study.

Reviewer #2

(Remarks to the Author)

Thank you for your extensive answers, my concerns have been addressed appropriately

Reviewer #4

(Remarks to the Author)

After careful examination of both the reviewer's comments and the authors' responses, I found that the raised concerns have been adequately addressed.

Specifically:

- To reduce the potential bias arising from platelet isolation from PBMCs, the authors supplemented their analysis with bulk RNA sequencing of washed platelets, which provided additional data. In addition, they ensured the reliability of their findings by processing PBMCs immediately after isolation and they cross-referenced platelet counts and characteristics from PBMC-derived scRNA-seq data with those obtained from whole blood-derived platelets and bulk RNA-seq of washed platelets
- To improve clarity, the authors reported the exact number of platelets analyzed for each patient in the scRNA-seq data set
- Additional clarifications were provided regarding flow cytometry methodology, platelet-leukocyte interactions and the impact of disease duration on platelets activation

Taken together, the authors have made substantial revisions and integrated new data in response to Reviewer 3's comments. I believe that these efforts satisfactorily resolve the major points raised.

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Thank you for the reviewer's valuable comments. Below are our point-by-point responses, highlighted in red for clarity.

Reviewer #1 (Remarks to the Author):

This manuscript by Wen et al provide insights for a potential role for platelets in Myasthenia Gravis. The study supports increased platelet activation in AChR+ nMG (correlating with severity) leading to interaction with leucocytes (predominantly neutrophils) and dysregulation of CD4+ T cell phenotype, possibly through platelet released RANTES. There is no doubt that platelets interact with and influence leucocytes in inflammatory disease pathology. The key question is why platelets are activated in a predominantly neuromuscular disease like MG and what are the platelet and leukocyte signaling pathways induced. Overall there is a need for in vivo animal studies to demonstrate in vivo relevance and cause-effect.

Major comments

- 1.1 Platelets activation needs to be explored in more detail including why and how. Is it cause-effect, a primary causative or secondary phenomenon? This is important for this manuscript as platelet activation is often secondarily associated with inflammation. In vivo mouse studies may be needed for this.

Response: We sincerely appreciate the reviewer's insightful comments regarding the mechanism of platelet activation. We agree that further investigation is needed to determine whether platelet activation in MG represents a causative factor, a consequence, or part of a complex feedback loop. Given its close association with inflammatory processes, understanding the temporal and mechanistic relationship between platelet activation and MG pathogenesis is of particular importance.

To address this, we conducted in vitro co-culture experiments using platelets and neutrophils derived from nMG, MMS, and HC groups. Our findings revealed that platelets co-cultured with neutrophils from nMG patients exhibited significantly higher activation levels compared to those co-cultured with neutrophils from MMS or HC individuals. Notably, platelet activation in the nMG group was comparable to that induced by direct exposure to NETs, suggesting that NETs formation plays a crucial role in platelet activation. Elevated NETs levels in the co-culture supernatants and plasma further supported this hypothesis (see in section 3.5, Figure 5 i-k).

Additionally, we assessed NETs formation in neutrophils co-cultured with platelets from different sources (nMG, MMS, and HC). Our results demonstrated that nMG platelets more potently induced NETs release compared to MMS or HC platelets, highlighting a bidirectional interaction in which activated platelets enhance neutrophil activation and NETs release, which, in turn, further amplifies platelet activation. This positive feedback loop may contribute to the persistence and exacerbation

of systemic inflammation in MG. Interestingly, MMS neutrophils exhibited a partial inhibitory effect on platelet activation, whereas MMS platelets did not exert a reciprocal inhibitory effect on neutrophil NETs formation, suggesting that neutrophil-driven platelet activation may be more prominent than the reverse interaction. These findings are detailed in Sections 2.6, 3.5, and Discussion (paragraph 5) of the manuscript.

In response to the reviewer's suggestion, we further investigated platelet activation *in vivo* using the EAMG rat model, following the protocol established by Zhang, Peng et al. (2021, PMID: 34702288). As illustrated in Figure A, platelet activation (CD62P⁺ platelets) was observed as early as day 7 in both EAMG and control mice, preceding the rise in AChR autoantibody levels (Figure B) and the onset of clinical symptoms (Figure A) around day 35. The early platelet activation in both groups suggests that it was primarily induced by CFA rather than being a direct driver of EAMG pathogenesis. However, following disease onset, platelet activation levels in EAMG mice exceeded those in controls (Figure C), indicating a potential secondary effect of AChR-targeted autoimmunity.

Furthermore, correlation analysis (Figure D) revealed a significant positive association between AChR autoantibody levels and the proportion of activated platelets ($r = 0.4465$, $p = 0.0002$), supporting the hypothesis that autoantibodies may contribute to platelet activation. This is further corroborated by bulk RNA-seq analysis of platelets, which identified an upregulation of Fc receptor-related pathways (Figure 3d in the manuscript), suggesting that immune complexes may be involved in platelet activation.

However, platelet activation levels in EAMG rat did not fully correlate with clinical disease severity. Several factors may account for this discrepancy:

1. Limitations of the EAMG model in replicating human MG pathogenesis: While the EAMG model provides valuable insights into antibody-mediated mechanisms in MG, it does not fully replicate the complex pathogenesis of human MG. In human MG, the thymus plays a pivotal role in initiating the autoimmune response, leading to the production of pathogenic antibodies targeting the neuromuscular junction. This process is accompanied by a cascade of inflammatory mediators that sustain and amplify inflammation (Zhao, Rui et al. 2021, PMID: 33722749), ultimately resulting in clinical muscle weakness. By contrast, the EAMG model involves passive induction of peptide-specific antibodies, bypassing the crucial thymic abnormalities and subsequent inflammatory processes that are central to human MG.
2. Impact of CFA and immunization on platelet activation: During EAMG induction, CFA, which contains mycobacterial antigens, stimulates the innate immune system and promotes platelet activation. Our data indicate that both the EAMG and control groups exhibited a significant increase in CD62P-positive platelets by day 7, suggesting early immune activation. This pattern of platelet activation differs from that observed in human MG patients. Following the second booster immunization, platelet activation levels increased again, with a more pronounced elevation in the EAMG group compared to controls, implying that peptide immunization and the subsequent immune response contributed to this activation.

Overall, our findings suggest that platelet activation in the EAMG model is primarily linked to CFA exposure and the immune response to the R9-116 peptide, rather than mirroring the thymus-driven

autoimmune mechanisms of human MG. Given the current lack of an animal model that fully recapitulates human MG pathogenesis, we have opted not to include these *in vivo* experiments in the main text, as they do not provide direct mechanistic insights into platelet activation in MG. Future studies should focus on optimizing MG animal models to better reflect disease pathophysiology and further elucidate the role of platelet activation in MG progression.

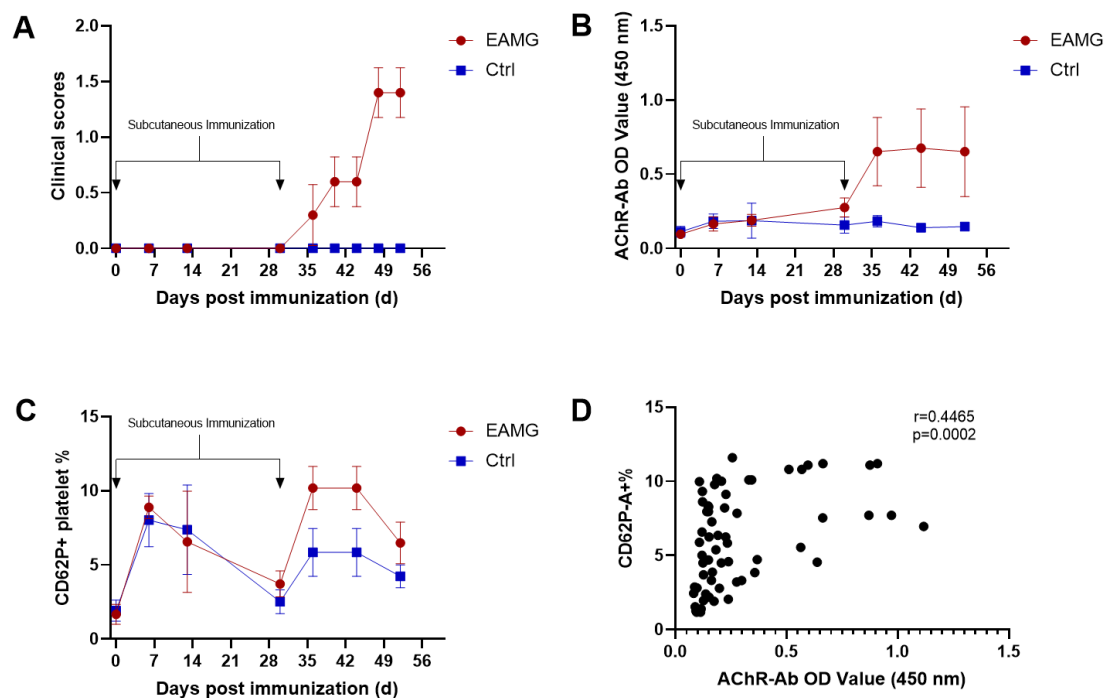


Figure. Longitudinal assessment of clinical scores, AChR antibody levels, and activated platelet percentage in the EAMG rat model.

• 1.2 The authors performed the scRNA Seq of platelets but only from those present in the isolated PBMC fractions where platelets are less than 10% in numbers as compared to PBMCs. The washed platelets should also be studied to determine levels of activation and phenotypes and compared to those interacting with the leucocytes.

Response 1.2: We thank the reviewer for this valuable comment.

In fact, we have already conducted a pilot study on one sample to explore the feasibility of single-cell RNA sequencing (scRNA-seq) of washed platelets. However, the results were suboptimal. We performed the sequencing and obtained 10.62 GB of data, capturing 4,254 cells with a sequencing saturation of 79.39%. This indicates that a substantial portion of the gene information was captured. However, as expected, the median gene count per cell was only 21 genes, suggesting low transcriptional activity in the platelets or insufficient sequencing depth. Additionally, the average read count per cell was 8,056, but the mapping rate was low, with only 23.5% of reads successfully aligning to the reference genome. Taken together, despite a reasonably large amount of data, the overall data quality was suboptimal, with low alignment rates, low gene counts, and high sequencing saturation, which can compromise the accuracy of downstream analyses. Consequently, we decided

not to pursue further single-cell RNA sequencing of additional washed platelets samples.

To date, there are no reports in the literature on single-cell RNA sequencing of purified, washed platelets. We hypothesize that this is due to several key challenges:

1. **Low RNA content and minimal transcriptional activity:** As they lack a nucleus, platelets have a relatively low RNA content, and their transcriptional activity is also minimal. This makes single-cell RNA sequencing less ideal for platelets as the data may not be sufficiently robust to yield meaningful results.
2. **Technical challenges in platelet isolation and capture:** Platelets are small in size, which makes it difficult to isolate and capture individual platelets for single-cell RNA sequencing using current technologies. Whether utilizing "oil-in-water" methods or "microfluidic" systems, both struggle to effectively capture the transcriptomes of individual platelets.
3. **Dynamic nature of platelet activation and function:** Platelets are primarily involved in blood clotting and immune response via receptor-mediated interactions, and their RNA expression is generally low in their resting state. While platelet activation induces changes in RNA expression, this process is dynamic and time-sensitive. Researchers often prefer using other techniques, such as flow cytometry, to analyze platelet function and status, as these methods are less complex and more immediately informative compared to single-cell RNA sequencing.

In contrast, bulk RNA sequencing offers distinct advantages for studying the transcriptomes of such low-abundance cells. This method provides a more comprehensive snapshot of the overall gene expression profile, even from limited amounts of starting material. It can also circumvent some of the challenges of single-cell sequencing by pooling cells, improving sensitivity and allowing for better quantification of gene expression, particularly when dealing with rare populations like platelets.

Hence, we performed bulk RNA sequencing on washed platelets isolated from the peripheral blood of 3 nMG patients and 3 healthy controls. This approach was chosen to complement the PBMC-based single-cell transcriptomic analysis of platelets. The results from this bulk RNA sequencing are now included in the manuscript (Section 3.2, Figure 3).

Regarding the comparison of the activation levels and phenotypes of washed platelets with those interacting with leukocytes, we currently do not have the capability to directly perform such comparisons. It is important to note that the transcriptomes of platelets within aggregates cannot be isolated and analyzed independently from leukocytes, which limits our ability to directly compare the transcriptomic features of free platelets with those in platelet-leukocyte aggregates. Future studies exploring techniques to isolate platelet-specific RNA from these aggregates could provide a more accurate and detailed comparison of platelet activation in the context of leukocyte interactions. This would enhance our understanding of the full phenotypic and transcriptomic changes that occur during platelet-leukocyte interactions, which are central to autoimmune diseases like MG.

- 1.3 Platelets are known to form aggregates with neutrophils in inflammatory disorders such as ARDS and also shown to contribute to NET formation and NETosis. NET formation should be assessed in the MG patients.

Response1.3: We thank the reviewer for this valuable suggestion. In our study, we have observed platelet-neutrophil interactions in MG patients, and we are aware of the potential contribution of these interactions to NETs formation. To address the reviewer's comment, we performed further experiments to compare the formation of NETs in nMG patients with other groups (HC and MMS) in section 3.5 (Figure 5k). Our results indicate that, compared to HC, plasma from nMG patients showed elevated levels of MPO-DNA complexes, suggesting an increased formation of NETs in MG neutrophils.

These findings provide evidence supporting the role of NETs formation in MG and underscore the potential involvement of platelet-neutrophil interactions in amplifying the inflammatory response. We have included these results in the revised manuscript.

1.4 The MG patients post treatment (MMS) had reduced platelet-neutrophil aggregates but not monocyte or lymphocyte-platelet aggregates. This should be discussed.

Response1.4: We thank the reviewer for this important observation. Platelet-neutrophil aggregates play a critical role in inflammatory diseases, and in MG, the interaction between activated platelets and neutrophils may be a central mechanism in the amplification of systemic inflammation. The observed reduction in platelet-neutrophil aggregates after MMS treatment likely reflects the therapeutic effect of immunotherapy, which may modulate neutrophil activation or reduce platelet activation (Schrottmaier, Waltraud C et al. 2015. PMID: 25904241). However, it is important to note that in our study, the lack of significant changes in platelet-monocyte or platelet-lymphocyte aggregates after treatment may suggest that the primary interaction responsible for platelet activation and inflammation in MG is mediated by neutrophils rather than monocytes or lymphocytes.

The differential impact on platelet-neutrophil versus platelet-monocyte/lymphocyte aggregates may be due to the distinct roles of neutrophils in the inflammatory process of MG, where neutrophils are more readily activated and involved in the formation of aggregates with platelets. On the other hand, the interaction between platelets and monocytes or lymphocytes might not be as prominent in this context, or it may require more specific conditions or further activation to show a measurable effect (Zarbock, Alexander et al. 2007. PMID: 16987572).

We have discussed these observations in more detail in the revised manuscript (Discussion, paragraphs 4), acknowledging the importance of neutrophils in the inflammatory process of MG and the potential differential responses of platelet-leukocyte aggregates to immunotherapy.

1.5 The increased myeloid-platelet aggregates in nMG group and its decline in clinical treatment group is an interesting observation. [Platelet-neutrophil co-culture assays](#) should also be performed.

Response1.5: We thank the reviewer for this valuable suggestion. We agree that platelet-neutrophil

co-culture assays could provide important insights into the interaction between platelets and neutrophils, which is crucial for understanding the immune mechanisms underlying MG.

In response to this suggestion, we have conducted additional platelet-neutrophil co-culture assays to investigate how platelet activation influences neutrophil function in MG. The results of these assays have been included in the revised manuscript (section 3.5). These experiments have provided valuable information on the role of platelet-neutrophil interactions in MG and offer further insight into how this interaction contributes to the development of systemic inflammation in MG.

- 1.6 Figure 5 is very important as it provides evidence of platelet surface activation by FC assays, however the platelet cell population in platelet/PBMC pseudocolor plots (CD61 +ve events) does not appear to be a defined platelet population. The non-uniform scatter pattern which is unusual for such FC platelet plots doesn't represent the standard platelet FC plots which uniformly appear when gated by a platelet marker.

Response 1.6: We thank the reviewer for their insightful comment. We acknowledge that the platelet cell population in the platelet/PBMC pseudocolor plots (CD61+ events) in Figure 5 may appear to lack a defined platelet population. This is due to the fact that the CD61+ population in the plots represents platelet-leukocyte aggregates, rather than a single platelet population.

The non-uniform scatter pattern observed in these plots is likely due to the heterogeneous nature of platelet-leukocyte aggregates. Each leukocyte may have multiple PSGL-1 molecules on its surface, allowing it to bind varying numbers of platelets, leading to a range of CD61 fluorescence intensities (Yang, J et al.1999. PMID: 10348699). This results in a more continuous distribution of CD61+ events, which differs from the uniform distribution typically observed for isolated platelets when gated by a platelet marker.

A similar phenomenon was reported in a previous study by Hottz et al. (Blood, 2020. PMID: 32678428), where CD41 was used as a platelet marker in platelet-leukocyte aggregates. In that study, the CD41+ aggregates also exhibited a non-uniform distribution. This further supports the idea that the observed scatter pattern in our plots is consistent with the formation of platelet-leukocyte aggregates.

- 1.7 Authors claim increased microparticles in MG group as per Figure 4j which shows the platelet morphology by TEM and the microparticles, however the same figure panel in left HC TEM micrograph shows apparently similar or higher levels of microparticles in HC group also. Quantification of microparticles should be provided.

Response 1.7: We thank the reviewer for this insightful comment. We agree that quantification of the microparticles is essential for accurately assessing the differences between the MG and HC groups. To address this, we have increased the sample size for each group and performed a detailed quantification of the microparticles in both groups. The quantification data have been included in the revised manuscript (section 3.3, Figure 4k).

- 1.8 Platelets have been reported to affect both CD4 and CD8 T lymphocytes. Are other lymphocytes impacted in myasthenia gravis?

Response 1.8: We sincerely appreciate the reviewer's insightful comment. We have expanded our single-cell sequencing analysis to further examine platelet interactions with other immune cell populations, as presented in Figure 2f-h. Our results indicate that platelets engage in crosstalk with various immune cells, including other lymphocyte subsets such as B cells and natural killer (NK) cells.

Emerging evidence suggests that platelet-B cell interactions may play a role in autoimmunity. For instance, a descriptive study in systemic lupus erythematosus (SLE) patients reported an increase in platelet-B cell aggregates, which correlated with circulating levels of preswitched memory B cells and immunoglobulins (Zamora et al., 2019. PMID: 30944545). Consistently, our ligand-receptor interaction analysis revealed potential platelet-B cell crosstalk, particularly through the APP-CD74 axis (Figure 2h). However, platelet-B cell interactions appear less frequent than platelet interactions with other immune cells, as B cells lack PSGL-1 expression, a key mediator of platelet adhesion (Scherlinger et al., 2021. PMID: 34193612).

Furthermore, previous studies have demonstrated that platelets actively modulate T cell responses (Chen et al., 2022. PMID: 35589797). Our CellChat analysis also revealed stronger platelet-T cell signaling interactions (Figure 2f). Given the well-established role of CD4⁺ T lymphocytes in MG pathogenesis, our study primarily focused on platelet-T cell interactions. Nevertheless, we acknowledge the potential involvement of platelet-B cell interactions in MG and plan to investigate their functional significance in future studies. This could provide deeper insights into the direct contributions of platelets to MG pathogenesis.

- 1.9.1 Result section 3.6 use cytokine neutralization assays where authors select PF4, RANTES and TGFβ1 as targets based on previous literature. The platelet transcriptome results or other results before this did not provide sufficient rationale for using these three as targets for these assays. Were there other upregulated proteins/chemokines in the transcriptomics data which could be the targets?

Response 1.9.1: We thank the reviewer for this important comment. We acknowledge the concern regarding the selection of PF4, RANTES, and TGFβ1 as targets for the cytokine neutralization assays, as these choices were primarily based on previous literature. Indeed, the platelet transcriptome results in our study did not provide sufficient direct evidence to specifically support these three targets. Partially because of the limited RNA content in platelets and their weak association with the proteome pose significant challenges to the application of platelet transcriptome research.

However, it is important to note that PF4, RANTES, and TGFβ1 are well-established in the literature for their roles in platelet function and immune modulation. These factors have been implicated in various immune responses and platelet-leukocyte interactions, making them reasonable candidates for our neutralization assays.

Further analysis using bulk-seq, we identified several differentially expressed transcription factors in MG platelets, such as STAT, ETS, and THR-like TF families, which could be considered as potential targets for future studies (see Figure 3e). These findings open new avenues for investigation, and we will explore these candidates in subsequent research to better understand their role in platelet function and immune modulation in MG.

1.9.2 RANTES is a well-studied therapeutic target in autoimmune disorders (Matsui et al 2002, PMID: 12098506; Zang et al, PMID: 11108946). Is the source of RANTES limited to platelets?

Response 1.9.2: We thank the reviewer for this insightful comment. Indeed, RANTES is not exclusively derived from platelets; it is also secreted by various other cell types, including T cells. To ensure that our study focused specifically on platelet-derived RANTES, we designed our neutralization antibody experiments using a platelet-only culture system. In this system, RANTES was neutralized to confirm that the experimental results were solely related to platelet-derived RANTES.

Additionally, we conducted further experiments to evaluate RANTES levels released by platelets from different samples after a period of incubation under identical conditions. This allowed us to further characterize and confirm the differences in platelet-derived RANTES secretion between groups.

- The authors should consider exploring antiplatelet agents such as aspirin in its ability to reduce RANTES and leucocyte activation.

Response: We thank the reviewer for this insightful suggestion. We have previously conducted a preliminary experiment in which platelets were treated with aspirin (100 μ M for 1 hour), and the supernatant was co-cultured with CD4 T cells. The results showed a slight reduction in RANTES levels, but this was not statistically significant, and aspirin treatment did not have a clear impact on platelet-induced CD4 T cell activation (proliferation and differentiation). This finding is consistent with our clinical observations (Figure 4e), where the proportion of activated platelets in patients on antiplatelet therapy did not show a significant decrease.

The treatment of platelets with aspirin did not reduce their activation capacity. We speculate because the platelets in MG may already be in a "pre-activated" state in vivo. As a result, aspirin treatment may not prevent the release of RANTES from these pre-activated platelets. Additionally, MG patients may exhibit a systemic inflammatory state or platelet dysfunction that keeps platelets in a highly activated state. Even after aspirin treatment, platelets may continue to secrete RANTES through non-COX-1-dependent pathways.

Furthermore, a study (Tan, Shuai et al. 2020. PMID: 32671959) reported that aspirin treatment, specifically inhibition of thromboxane A2 (TxA2) synthesis, did not influence platelet-enhanced CD4 T cell responses, supporting our observation that aspirin may not be effective in modulating platelet-mediated immune activation in the context of MG.

Platelet activation is a complex process mediated by multiple receptors, and there are various

antiplatelet agents available. In future studies, we aim to further clarify the key pathways involved in platelet activation in MG and identify potential targets and drugs that could suppress platelet activation and mitigate immune dysregulation in this context.

Minor

- How many events were recorded for FCM assay for platelet and PBMC analysis and what % of events of total were platelets (Figure 5A)?

Response: In the FCM assay for platelet and PBMC analysis, approximately 50,000 events were recorded for PBMCs, and platelets accounted for about 10% of the total recorded events.

- The discussion is rather lengthy with unnecessary sub-sections and discussion such as with microparticles.

Response: Thank you for your valuable feedback. We appreciate your observation regarding the length and structure of the discussion section. We have carefully revised this section to streamline the content, remove unnecessary sub-sections, and condense discussions, to ensure that the focus remains clear and concise.

- A very important reference (Jin et al, Cell Discov, 2021) about immune cell heterogeneity in MG should be discussed.

Response: We thank the reviewer for pointing out the important reference (Jin et al, 2021. PMID: 34521820) on immune cell heterogeneity in MG. In the revised manuscript, we have cited this reference in the third paragraph of discussion to enhance the context of our study and further support our findings.

Reviewer #2 (Remarks to the Author):

Dear editor,

With interest I've read the manuscript regarding myasthenia gravis (MG) and the correlation with altered platelet biology. The authors have explored several technologies and methodologies (incl single cell sequencing, TEM and in vitro immune cell cultures) to understand how altered platelet biology is connected to MG and MG disease severity. Their single cell sequencing experiment identified an increased number of platelets in MG patients suffering from disease, while this returns to normal in patients achieving minimal disease due to treatment. This finding is corroborated in a larger group of patients although the spread among the group is wide. As such it has limited value as a biomarker, but may be interesting for studying a role in the pathophysiology or aetiology of the disease. The rest of the manuscript is focussed on extending this connection between abnormal platelet number and function to MG disease status.

To summarize they find: 1. Increased platelet numbers in severe MG patients (3 scRNAseq, larger cohort in serum), 2. altered transcriptional profiles in these cells from patients with disease (n=4), 3. Altered morphology of these platelets (in 1 patient), 4. Enhanced expression of CD62P and sCD40L, 5. an interesting correlation between reduction of PLA formation following IVIg treatment that precedes QMG improvement (n=6), 6. The activated platelets engage more often in interactions with leukocytes (n=many), 7. Medium from MG platelet cultures induce more T cell aggregates (n=>10).

The data is convincing although here and there sloppy when it comes to showing the right data. See major comments below. This needs attention. The authors, based on this work, postulate an exciting new hypothesis for this field claiming that this activated platelet phenotype contributes to the autoimmune response observed in these patients. Although the sequence of events is not clear from this manuscript. Meaning we don't know if the platelet dysfunction kickstarts the autoimmune response, is permissive/supportive/ facilitates it or is it a secondary effect of an already derailed immune system?

In addition, the authors show some data regarding inhibition of RANTES signaling as a means of limiting the activating nature of platelets in patients (and controls) which may show therapeutic promise in the future. This manuscript presents an interesting new line of research on which very little knowledge exists. I recommend the editor to include a platelet expert for reviewing this manuscript as my expertise is limited to MG and its pathophysiology while the platelets and corresponding methodology are a major component of this manuscript.

The manuscript is well written and does not need much further English editing. Regarding the content of the manuscript I have some specific recommendations, questions and remarks. These have been outlined below in major and minor comments.

Study soundness

The study includes a relatively large number of immunotherapy naïve patients for validation which is important for study power. The single cell data however includes only two. This is understandable given the challenges for these studies however does warrant caution for extending the observations beyond this limited group of individuals.

The TEM data was performed on 1 patient sample and although exciting suffers from the same challenge. It would be helpful for the reader to understand which data is generated with different methods from the same donor. That way we can connect the datasets and obtain more confidence in some of the conclusions. The paragraph on the TEM data furthermore refers to several observations with regard to the morphology of these cells however only shows 1 outcome measure in 4i. It

would be useful to get the other analysis also in plots to support the observations mentioned in the text. It would be highly recommended to include TEM data from more than just 1 patient if possible.

Response 2.2: We thank the reviewer for this valuable suggestion. We agree that including TEM data from multiple patients would strengthen the findings and provide a more comprehensive understanding of platelet morphology in MG. Currently, Figure 4i includes one key outcome measure (the proportion of non-quiescent platelets), but we acknowledge that other aspects of the morphological analysis were not fully represented in the figures. In response to this, we have supplemented the TEM data with additional samples and performed quantitative analysis of PEVs. The updated results are presented in section 3.4 of the revised manuscript.

Noteworthy is that this study contains a single cell dataset that includes untreated AChR MG patients which can be considered an asset and unique to the field. Usually these studies include treated patient samples which suffer from obvious biases.

Major comments

The authors highlight that the focus on platelets in this manuscript comes from the deviation of relative percentage of these cells compared to controls. The data suggest however also major differences on the level of memory B cells. Have the authors looked into this? This is an important observation and one that would deserve more attention in light of the adaptive autoimmune response underlying the disease. Can the authors elaborate on this?

Response 2.4: We thank the reviewer for bringing up the important role of memory B cells in MG. Memory B cells circulate through peripheral blood, secondary lymphoid tissues, and bone marrow, and differentiate into plasmablasts and plasma cells more rapidly than naïve B cells when an antigen triggers an immune response. They also regulate the number of plasma cells in tissue and peripheral blood. In autoimmune diseases, a phenomenon called “homing” occurs, where memory B cells migrate to and embed in target tissues, activating and promoting antibody production. In MG, activated circulating memory B cells likely migrate to secondary lymphoid organs, reducing the number of memory B cells in the blood, where they differentiate into plasmablasts and plasma cells, producing autoantibodies such as anti-AChR antibodies.

In fact, studies have shown a reduction in circulating memory B cells in MG patients, similar to what is observed in other autoimmune diseases like Sjögren’s syndrome (Hansen, Arne et al. 2002. PMID: 12209521). Interestingly, treatment with immunosuppressants in MG patients has been shown to reduce the percentage of naïve B cells and increase the percentage of memory B cells in peripheral blood (Kohler, Siegfried et al. 2013. PMID: 24099983), which might be relevant for therapeutic strategies aimed at modulating the immune response.

In our single-cell sequencing data, we observed an increase in the proportion of myeloid cells and platelets in MG patients, alongside a decrease in T and B cells, including memory B cells. This decrease in memory B cells in the peripheral blood of MG patients is consistent with findings in

other autoimmune diseases. In Section 3.1 of the results, we have included a description of the changes in the proportions of various B cell subpopulations. However, since Platelet-B cell interactions are less common than interactions of platelets with other immune cells, mainly because B cells do not express PSGL1 (Scherlinger, Marc et al. 2021. PMID: 34193612), and this study primarily focuses on the interaction between platelets and T cells, we did not discuss memory B cells in great detail. In future studies, we plan to further investigate the role of memory B cells in MG and explore their involvement in the pathophysiology of the disease.

Thank you again for this valuable suggestion.

It is unclear to the reviewer why patient nMG4 data is not included in the main figure. If this is because of a batch effect issue, one could still present the data together, but highlight this technical issue. The observation regarding the platelet count is strengthened with this data although the expression profile brings more nuance as to the inter-individual similarities/differences.

Response 2.5: We thank the reviewer for this thoughtful comment. The data for patient nMG4, a male MG patient, was included in the supplementary materials because this patient was recruited to investigate whether the observed increase in platelet count also occurs in male MG patients, as the first three MG cases were female. However, there were no corresponding matched male healthy controls (HC) or post-MMS samples available for nMG4, which would have been necessary for a robust comparison. To avoid potential bias in the single-cell data comparison, we decided to present the nMG4 data in the supplementary materials instead of the main figure.

While these data are not part of the primary analysis, they do support the overall observation of increased platelet count in MG patients. We have added a note in the main text in section 3.1 to clarify this decision and highlight the inclusion of nMG4 data in the supplementary materials. We appreciate the reviewer's suggestion and agree that transparency in presenting such data is critical to strengthening the findings.

The statement line 396-397 "compelling bridge" this statement seems overbearing. It is not clear to the reviewer how this transcriptional data already confirms immunological dysregulation in patients. It suggests skewed numbers of cells that have altered expression profiles, but no dysfunction (on protein level) and certainly not dysfunction causally related to the pathophysiology of MG. It is recommended to rephrase this sentence at this location in the manuscript.

Response 2.6: We appreciate your insightful comments and have revised the manuscript according to your suggestion regarding the statement in lines 396-397. The wording has been adjusted to better reflect the scope of our findings, emphasizing the observed transcriptional changes without overstating their implications.

2.7.1 I'm not an expert in SSC analysis, but the flow plots in figure 4 show multiple populations on the y axis with the MMS individuals having 3 (as it seems). Is this what you would expect and which groups of cells are these and can the platelet cell surface markers CD61 and CD62P be compared between groups

for each of these cell populations? Furthermore the CD61 value in the MMS condition in the plot is the highest while in the table under b this is not the case. Are these representative flow diagrams?

Response: We would like to thank the reviewer for the valuable question. Regarding the multiple populations shown on the y-axis in the SSC analysis in Figure 4a, we believe these results are consistent with our expectations and reflect the diverse cell populations present in peripheral blood. Specifically, the multiple populations observed in the MMS group primarily consist of platelet-leukocyte aggregates (including neutrophils, monocytes, and lymphocytes) as well as free platelets. The proportions of these cell populations may vary between different samples, but this does not affect the gating of CD61+CD62P+ platelets.

Furthermore, to more precisely distinguish the different cell populations, we refined the gating strategy using CD45 and CD15 markers to identify neutrophils, monocytes, and lymphocytes. We then compared the expression of CD61 within these populations across different groups, specifically referring to neutrophil-platelet aggregates, monocyte-platelet aggregates, and lymphocyte-platelet aggregates (as shown in Figure 5b-d).

Regarding the comment on the CD61 value in the MMS condition in the plot being the highest, we believe this refers to the higher number of CD61+ events observed in the MMS group in Figure 4a. However, when focusing on CD62P+ activated platelets, the proportion of these cells within the CD61+ population in MMS is not as high. Therefore, there is no conflict between the flow plot (Figure 4a) and the table under part b, which reflects the proportion of activated CD62P+ platelets. To avoid any confusion, we have replaced the nMG and HC plots with more typical examples.

We hope this clarification addresses your concerns.

2.7.3 Figure 4F does not seem to reflect the FSC SSC analyses strategy as suggested in the text. In this section it would also be good to mention some thoughts on why the MMS group does not show HC phenotype (as in other outcome measures they do).

Response: Thank you for bringing this to our attention. In Figure 4f, we indeed utilized a gating strategy to isolate the platelet population, and the FSC/SSC parameters were then calculated automatically in FlowJO software for this specific population. We realize that the description in the text may not have been sufficiently clear, and we have revised the text to better explain this strategy (see in figure legend).

While the MMS group demonstrated improvements in other measures (e.g., platelet count, formation of platelet-leukocyte aggregates), it is possible that platelet activation states do not completely revert to the HC phenotype due to residual disease activity or incomplete resolution of inflammation. Additionally, the treatments used in the MMS group may directly or indirectly modulate platelet activation, leading to persistent differences. In fact, we observed other indications suggesting that MMS platelets have not fully returned to the normal phenotype. For example, electron microscopy showed that MMS platelets had a higher number of microparticles (PMPs) per platelet (Fig. 4k), while the NETs formed by neutrophils co-cultured with MMS platelets were still

slightly elevated compared to HC (Fig. 5h). Furthermore, CD4⁺ T cell proliferation in the presence of MMS platelet supernatant was higher than in the HC group (Fig. 6k). Together, these findings suggest that platelets in the MMS group may remain mildly activated or show a tendency for activation compared to healthy controls.

We have incorporated this discussion into the revised manuscript (see Discussion, paragraph 7).

2.8 Figure 5a would be good to show representative gating plots for the disease/control settings like in figure 4.

Response 2.8: Thank you for your valuable suggestion and we have now included representative gating plots in Figure 5a to more clearly illustrate the analysis results for the disease and control groups.

Figure s9 would be easier to interpret if the sizes of the aggregates for all conditions were also quantified and presented in a graph. The effect seems rather large so I could imagine this would make sense to present in the main figure.

Response: We thank the reviewer for this insightful suggestion. We have conducted a quantitative analysis of the aggregate sizes and presented the results in a graph. Additionally, we have included this graph in the main figure (Fig. 6) to enhance clarity and the overall impact of the data. Thank you again for your valuable input.

Figure 6c,d does not show the data referred to in the text. C shows an example of the gating strategy.

Response: Thank you for your observation. We have revised Figure 6 and its corresponding legend to clarify the content and ensure consistency with the text. We hope this modification addresses your concern.

The panels from several figures are not chronologically presented in the text. This is sometimes a bit confusing when looking at the figures.

Response: We thank the reviewer for pointing out the issue with the chronological presentation of panels in several figures. We have revised the manuscript by adjusting the panel order in the figures to ensure consistency with the descriptions in the text.

In the discussion the authors mention platelet dysfunction as an upstream factor. What's the evidence for that statement in this manuscript? Please rephrase.

Response: Thank you for pointing out this important aspect. Upon review, we acknowledge that the current evidence presented in our manuscript does not definitively establish platelet dysfunction as an upstream factor in the pathogenesis of MG. We have revised the discussion to adopt a more cautious and scientifically accurate interpretation. Specifically, we now suggest that platelet activation may contribute to sustaining the inflammatory state associated with MG, rather than implying a direct upstream role. We believe this modification better reflects the evidence and aligns with your concerns.

Minor comments

1. Line 111 enrolled enrolment

Response: We appreciate your attention to detail and have corrected the wording in Line 111.

2. Would be helpful to include data on the use of pyridostigmine or other non-immune treatments

Response: Thank you for your valuable suggestion and we have supplemented the patient information table to include data on the use of non-immune treatments like pyridostigmine.

3. Line 114–115 how many patients were now included? 3 or 4?

Response: Thank you for pointing out this discrepancy. In the single-cell data analysis section, we included a total of 3 MG patients. We sincerely apologize for the writing error in the original text and have corrected it. We appreciate your careful review and attention to detail again.

4. Please clarify how and where healthy donors were recruited.

Response: Thank you for your comment. The healthy controls were selected from the hospital medical staff, nurses, and their family members. All participants underwent thorough medical examinations to rule out any neurological or autoimmune diseases. We have now clarified this information in Section 2.1 of the manuscript.

5. Figure 1 typo in characteristics

Response: Thank you for pointing out the typographical error in Figure 1. We have corrected the spelling of "characteristics" and carefully reviewed the figure to ensure there are no additional typographical or formatting issues.

6. The supplementary data still contains track changes

Response: We sincerely apologize for the oversight in the supplementary data. The track changes in the document were unintended and have now been fully removed. We have carefully reviewed the supplementary files to ensure that no additional formatting issues remain. A clean version of the supplementary data has been prepared and is included in this revised submission. Thank you for bringing this to our attention.

7. Supp table 1 thymoma status is called normal. Perhaps make clear to the reader that the thymus is normal or the thymoma status is negative. MGFA classification in the table is at time of withdrawal of blood?? Otherwise this classification and onset symptoms do not match.

Response: Thank you for your insightful comment. We acknowledge that describing the "thymoma" status as "normal" was imprecise, and we apologize for any confusion it may have caused. To enhance clarity, we have revised the description in Supplementary Table 1 to ensure it accurately reflects the thymic status and avoids potential misinterpretation

We confirm that the MGFA classification in the table corresponds to the time of blood withdrawal, during which several patients were in their most severe state. Your observation has made us realize

that presenting only the onset symptoms in the table may lead to misunderstanding. To address this, we have revised the table to include the symptoms at the time of enrollment, ensuring greater clarity and consistency in the data presentation.

8. Figure 1e y axis is not starting at 0

Response: Thank you for pointing out this issue. We believe you may be referring to the platelet count comparison between groups in Figure 2, rather than Figure 1e. We have revised Figure 2e so that the y-axis now starts at 0, ensuring an accurate representation of the data. The updated figure has been included in the revised manuscript. Thank you again for your valuable feedback.

9. Introduction of abbreviation of MK is missing in discussion

Response: Thank you for your comment. We have revised the discussion section to include the full introduction of the abbreviation "MK" (megakaryocyte) upon its first use.

Reviewer #3 (Remarks to the Author):

General comments:

In their manuscript, Dr Wen and collaborators analyzed the implication of platelet in Myasthenia Gravis (MG). First, on a small number of patients, they performed a SC-RNAseq of PBMCs to characterize the profile of platelets in controls, MG patients before and after immunosuppressive therapy. Next, on large cohorts of patients, they assessed the activation status of platelets by analyzing CD62P expression, platelet morphology, and sCD40L. They thoroughly investigated the impact of platelet and platelet supernatant on naive CD4 T cells proliferation and cytokine expression profiles in co-culture experiments.

This is a very original and interesting study. However, the important messages do not stand out well because there is too much information that may not be necessary. I am not sure that the SC-RNAseq section contributes much. The paper should focus on the functional studies.

Below are listed comments to further improve the manuscript.

Title:

3.1 The title is too long

Response: Thank you for your comment. We have revised the title to make it more concise while retaining the key elements of the study. The updated title is "Platelet Activation and its Interaction with Neutrophils and CD4+ T Cells in Myasthenia Gravis". This revised title maintains the focus on the core findings and avoids unnecessary detail.

Abstract:

3.2 In the Methods section, indicate that co-culture experiments were also done with platelet supernatant.

Response: Thank you for pointing this out. We have revised the Methods section to explicitly mention that in vitro co-culture experiments were performed using both intact platelets and platelet supernatant.

3.3 The Results section does not mention SC-RNAseq conclusions.

Response: We appreciate your observation. In the revised manuscript, we have included the main conclusions from the single-cell RNA sequencing (scRNA-seq) analysis in the Results section.

3.4 The abstract concludes “characterized by enhanced adhesion” but this has not been studied.

Response: Thank you for bringing this to our attention. We agree that the wording in the original abstract could be misleading. We have revised the abstract to clarify that the findings related to platelet-leukocyte aggregates are based on experimental evidence (flow cytometry and in vitro analysis). The phrase “enhanced adhesion” has been removed to avoid overinterpretation, and the abstract now accurately reflects the observed results, including platelet-leukocyte interactions and aggregate formation.

Materials and Methods :

3.5 Participants:

– Line 103: It is mentioned “Patients were excluded based on the... (3) history of malignant tumor and other autoimmune diseases; while in Supplemental tables thymoma patients are clearly indicated.

Response: We appreciate the reviewer’s insightful comment. We acknowledge the apparent inconsistency between the exclusion criteria in Line 103 and the inclusion of thymoma patients in the supplemental tables. To clarify, our study specifically focuses on myasthenia gravis (MG), in which thymoma is a well-recognized associated condition rather than a general malignancy or secondary autoimmune disorder. Therefore, while patients with other malignant tumors or autoimmune diseases were excluded, those with thymoma were included as a distinct clinical subgroup for analysis. To avoid any ambiguity, we have revised the exclusion criteria in Section 2.1 to explicitly state that patients with malignancies or autoimmune diseases other than thymoma were excluded.

3.6 – All patients are above 35 years old with average ages according to the supplemental tables of 53–55 years. This should be indicated in the text.

Response: We thank the reviewer for highlighting this point. We agree that the age distribution of the patients is an important characteristic that should be explicitly stated in the text. Based on the supplemental tables, all patients included in this study were above 35 years old, with an average age range of 53–55 years. This information has now been added to the “Study participants and sample collection” section of the manuscript to provide a clearer description of the study population.

3.7 – Line 112: 4 patients are mentioned? Checked with lines 330 and 830.

Response: Thank you for pointing out this discrepancy. In the single-cell data analysis section, we included a total of 3 MG patients. We sincerely apologize for the writing error in the original text and have corrected it. We appreciate your careful review and attention to detail again.

3.8 – In table S1, it is not mentioned if patients were thymectomized, while they were all thymectomized in the other cohorts.

Response: We thank the reviewer for pointing out this omission. As clarified in the study, the patients included in Table S1 did not undergo thymectomy at the time of sequencing. However, following treatment and disease stabilization, all patients eventually underwent thymectomy in accordance with international guidelines for managing myasthenia gravis. We have updated Table S1 to include a column specifying whether each patient had undergone thymectomy at the time of sequencing. Additionally, thymectomy status has been incorporated into other supplemental tables containing patient information to ensure consistency across all data presentations.

3.9 SC-RNASeq analysis:

– Platelet analysis by SC-RNAseq has been performed on PBMCs. This is surprising as standard PBMC isolation protocols, such as density gradient centrifugation, are not designed to retain platelets. Platelets are smaller and less dense than PBMCs, and they may be lost during the isolation process. This might also lead to a bias as MG platelet were described as smaller (Fig 4).

Response: Thank you for your valuable comments. We indeed used the standard PBMC isolation method (density gradient centrifugation) in our experiments and are aware that platelets are smaller and less dense, which may result in partial loss of platelets during the isolation process. We also acknowledge the potential bias pointed out by the reviewer, particularly regarding the difference in platelet size between MG patients and healthy controls (as shown in Fig. 4), which may affect the behavior of platelets during the isolation process and their presence in the final sample.

To further validate and reduce this potential bias, we supplemented the analysis with bulk RNA sequencing of washed platelets (instead of relying solely on platelet data from PBMCs) to ensure a more comprehensive examination of platelets. Through this additional experiment, we were able to explore the characteristics of platelets from MG patients more thoroughly while compensating for the potential loss of platelets during PBMC isolation.

These additional measures have helped us better control for potential biases and have strengthened the reliability of our results. We have updated the relevant data and discussion sections of the manuscript (section 2.3, 3.2 and paragraph 2 in discussion) to further address the possible impact of these factors on the outcomes.

3.10 – Have the SC-RNAseq (Single-Cell RNA sequencing) data been deposited in public databases?

Response: Thank you for your question regarding the availability of the single-cell RNA sequencing (scRNA-seq) data. The sequencing data have been deposited in the GSA-human database and are

accessible under the accession code subHRA011221. To facilitate access during the peer-review process, reviewer tokens can be provided. The data can be accessed using the DAC number HDAC004274.

3.11 Flow cytometry and other analyses

– It is not clear if the other analyses (FACS, TEM) have also been performed on PBMCs or on whole blood cells. This should be indicated in the methods sections.

Response: We thank the reviewer for raising this important point. To address this concern, we have clarified the sample sources used for both FACS and TEM analyses in the revised manuscript. Specifically, we have now explicitly stated in the methods section that the FACS analysis was conducted on whole blood samples that underwent red blood cell lysis, as described in section 2.3. Similarly, the TEM analysis utilized platelets directly isolated from whole blood, as detailed in sections 2.4.1 and 2.5.

3.12 Co-culture experiments

– Lines 245–252: It is not clear if the supernatants were also prepared from different platelet densities and if this was tested. Which option was chosen for the final experiments?

Response: We thank the reviewer for highlighting this important point. To clarify, in our study, we tested various Tn cells to platelets ratios, including 1:10, 1:100, 1:500, and 1:1000, with the total co-culture volume fixed at 200 μ L. Each ratio corresponded to a different platelet concentration, with 1:100 resulting in a platelet concentration of 1×10^8 /mL. Based on the results of the direct co-culture experiments, we identified 1:100 as the optimal ratio for Tn cell activation and platelet interaction.

For the final experiments, we used the 1:100 ratio to compare the effects of direct (physical contact) and indirect (supernatant-dependent) co-culture. These details have been added to the revised manuscript in section 2.7 for greater clarity.

3.13 – Line 254, PMA activation is mentioned here but not found in the figure legends. It is not mentioned in the results section either.

Response: We thank the reviewer for highlighting this important oversight. PMA and ionomycin were used to activate CD4⁺ T cells on the fourth day of co-culture to assess cytokine production. This information has been added to the relevant figure legends and results section to ensure consistency between the methods, results, and figure descriptions.

3.14 – Lines 275–288: There should be a dedicated paragraph on flow cytometry analysis.

Response: We appreciate the reviewer's suggestion to provide a dedicated paragraph on flow cytometry analysis. In the revised manuscript, we have reorganized this section to include a standalone paragraph (2.7.4) specifically focused on the flow cytometry analysis.

3.15 – Paragraph 2.7 should be subdivided to make the co-culture experiments easier to understand.

Response: We appreciate the reviewer's valuable suggestion. In the revised manuscript, section 2.7 has been subdivided into distinct subsections to enhance readability and improve the clarity of the co-culture experiments. The revised section now includes the following subsections: *Preparation of washed platelets*, *Co-culture experimental setup*, *Stimulation and cell staining*, and *Flow cytometry analysis*. This restructuring provides a more logical flow, making the experimental design and procedures easier to understand for readers.

Results

3.16 – In the figure legends, all figures should be labeled with a letter and explained in the legends.

Response: We thank the reviewer for this important suggestion. In the revised manuscript, we have ensured that all figures are properly labeled with letters (e.g., Fig. 1a, Fig. 1b) and that these labels are consistently referenced throughout the text. Additionally, we have updated the figure legends to include detailed explanations for each labeled subfigure, clearly describing the experimental setup, data representation, and key findings.

3.17 For the SC-RNAseq:

–Lines 348–354: why MG4 was not included in the SC-RNAseq analysis?

Response: We appreciate the reviewer's question. The data for MG4, an elderly male MG patient, were excluded from the primary scRNA-seq analysis to avoid potential bias. This decision was made due to the lack of corresponding matched male healthy control (HC) samples and post-MMS samples for MG4, which are critical for robust comparative analysis. Including MG4 without appropriate controls might have introduced confounding variables, particularly given the potential influence of age and sex on scRNA-seq profiles.

However, to assess whether the observed increase in platelet count and associated gene expression changes are influenced by age or sex, we performed a separate scRNA-seq analysis of PBMCs from MG4. The results, presented in Fig. S3, demonstrate that MG4 exhibited both increased platelet counts and elevated expression of platelet degranulation genes, aligning with the findings from the primary analysis. These supplementary data provide additional evidence supporting the robustness of our observations while highlighting the independence of these phenomena from age and sex.

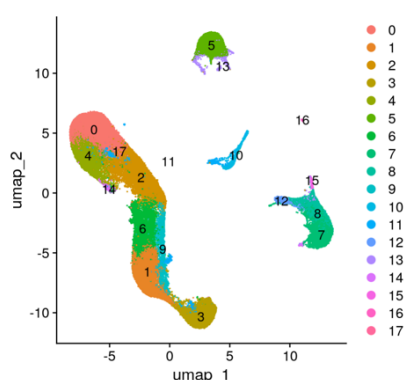
We have added further clarification to the manuscript in section 3.1 to address this point and thank the reviewer for the opportunity to improve the clarity of our study.

3.18 – Fig 2b left: the aggregation of samples does not seem perfect: for example, memory CD4 T cells (light blue) are dispatched on two different locations, as well as naïve CD8 T cells (light green).

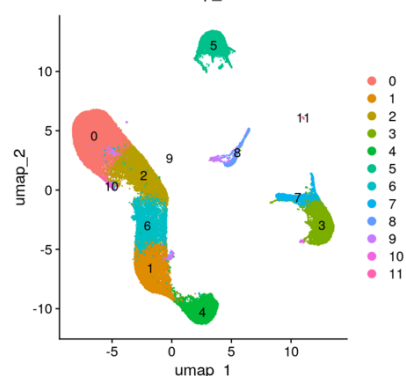
Response: We appreciate the reviewer's insightful comment. To address this concern, we have systematically adjusted the clustering parameters and performed additional dimensionality reduction and re-clustering analyses. Despite these efforts, we were unable to achieve a perfectly cohesive clustering pattern where each cluster forms a completely distinct and contiguous group.

Below, we present representative results from 3 independent parameter adjustments, illustrating that the issue persists across different clustering settings. After thorough evaluation, we believe this phenomenon is inherently associated with the limitations of dimensionality reduction techniques and high-dimensional single-cell data representation. Similar observations have been reported in previous studies, such as Jin et al. (2021, PMID: 34521820, Fig. 1b) and Xie et al. (2020, PMID: 34691592, Fig. 2a), where clusters of the same cell type appear in spatially distinct locations. This may be attributed to biological heterogeneity within the same cell type, technical variability in feature selection, or intrinsic limitations of the algorithm used for visualization.

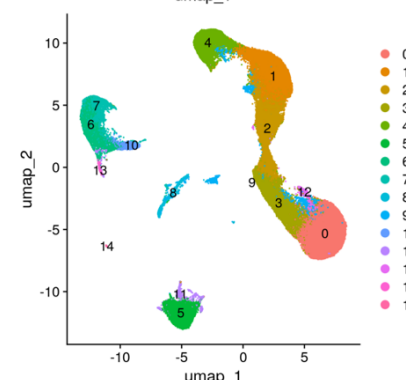
Given these considerations, we have decided to retain our original clustering results. We hope this explanation sufficiently addresses the reviewer's concern. Thank you for your valuable feedback.



```
FindNeighbors(merged, reduction = "pca", dims = 1:10)
FindClusters(merged, resolution = 0.5)
RunUMAP(merged, reduction = "pca", dims = 1:10)
```



```
FindNeighbors(merged, reduction = "pca", dims = 1:10)
FindClusters(merged, resolution = 0.3)
RunUMAP(merged, reduction = "pca", dims = 1:10)
```



```
FindNeighbors(merged, reduction = "pca", dims = 1:8)
FindClusters(merged, resolution = 0.5)
RunUMAP(merged, reduction = "pca", dims = 1:8)
```

3.19 – Fig 2b right: It seems that only MMS1 and MG3 are shown in this graph. Provide the number of platelets analyzed for each patient. Is there a bias

related to the use of PBMCs?

Response: We thank the reviewer for raising this question. The graph in Fig. 2b (right) represents the integrated data from all samples. However, due to the higher number of cells captured in the scRNA-seq data for MMS1 and MG3, these samples appear more prominent in the visualization. To improve clarity, we have now provided the exact number of platelets analyzed for each patient in the scRNA-seq dataset, which is now included in Supplementary Table S2. Additionally, we have adjusted the figure so that the platelets from each sample group are represented in a uniform color scheme, with a blended representation to better illustrate platelet enrichment in the MG group (see in Figure 2b right).

We also appreciate the reviewer's question regarding potential bias in analyzing platelets from PBMC-derived scRNA-seq data. To minimize such biases and ensure the reliability of our findings, we implemented the following measures:

1. Reduction of handling bias: PBMCs were processed immediately after isolation to prevent platelet activation or degradation, thereby preserving their native state.
2. Validation through multiple datasets: We cross-referenced platelet counts and characteristics from PBMC-derived scRNA-seq data with those obtained from whole blood-derived platelet data (section 3.1) and washed platelet bulk RNA-seq (section 3.2). The consistency of trends across these datasets supports the representativeness of PBMC-derived platelets.

3.20 – Fig 2c right: explain in the legend what it is.

Response: We thank the reviewer for their comment. In the revised manuscript, we have updated the legend for Fig. 2c to include a clear explanation of the right panel.

3.21 – Fig 2e and Supplemental Figures 4: HC controls should be added

Response: We thank the reviewer for this valuable suggestion. We agree that the inclusion of HC controls in Fig. 2e and Supplemental Fig. 4 is important for proper comparison and to strengthen the validity of our findings. We have now included the data from the HC group in these figures to provide a more comprehensive comparison.

3.22 – Line 371: A fold change (FC) of 1 does not indicate differential expression. The figure shows the Log2 (FC) values. However, no gene appears to be significantly increased, but this is not surprising as only 3 HC and 3 MG samples were compared.

Response: We appreciate the reviewer's comment and agree that a fold change (FC) of 1 indicates no change in gene expression. Furthermore, we acknowledge that no genes meet strict thresholds for significant differential expression, likely due to the small sample size (3 HC and 3 MG), which limits the statistical power of the analysis.

To address this limitation and enhance the robustness of our findings, we have removed this portion of the results to prevent potential misinterpretation. Furthermore, we performed bulk RNA sequencing on washed platelets to increase sequencing depth and conducted a differential expression analysis (DEG), with the results now presented in Figure 3. Thank you for bringing this to our attention and allowing us to improve the clarity and rigor of our manuscript.

3.23 – Fig 3c: The color code in the legend does not match the colors of the clusters in the figure. The names of the clusters should be provided

Response: We thank the reviewer for pointing out these important issues. To enhance clarity and avoid potential confusion, we have revised the manuscript and removed the scRNA-seq platelet-myeloid aggregate analysis from the results (previously presented in Figure 3c-e).

3.24 – Fig 3e is not helpful. According to the x-axis cluster 8 is thus the 7?

Response: We appreciate the reviewer's feedback. As mentioned in section 3.2 of the manuscript, we identified 10 myeloid cell subpopulations, designated as clusters 0-9, ordered by cell count from highest to lowest, as shown in Fig. 3c (upper). Therefore, the x-axis label "8" corresponds to cluster 8.

The purpose of this figure was to highlight the high expression of characteristic myeloid cell genes in cluster 8. However, we acknowledge that this information may be unnecessary, as cluster 8 is inherently a subpopulation of myeloid cells. To avoid redundancy, we have decided to remove this figure from the manuscript. Thank you for your helpful suggestion, which has led to a more streamlined presentation of the data.

3.25 – Fig4e: In the legend, explain how the percentage of non-spheroid platelets is obtained.

Response: We thank the reviewer for the suggestion. We have updated this result in Fig. 4j and revised the corresponding figure legend to explicitly explain how the percentage of non-spheroid platelets is calculated.

3.26 – Figure 4f and figure 5a correspond to the same CD45 and CD61 FCS analysis but the gated population (CD45-CD61+) is considered as platelet free in Fig 4f and as PLAs in Fig 5a. There might be an error.

Response: We appreciate the reviewer's careful observation. Upon reviewing the figures, we would like to clarify the distinction between the populations shown in Fig. 4f and Fig. 5a:

In Fig. 4f, the gated population CD45-CD61+ corresponds to free platelets, where CD45 fluorescence intensity is $< 10^3$. These are identified as platelets that are CD45-negative and CD61-positive, indicating that they are circulating platelets not associated with other cells.

In Fig. 5a, the gated population CD45+CD61+ represents platelet-leukocyte aggregates (PLAs), where CD45 fluorescence intensity is $> 10^3$. These aggregates consist of platelets (CD61+) bound to leukocytes (CD45+), which are clearly distinguished by the higher CD45 expression compared to free platelets.

We have updated the figure legends to clarify these differences in gating and the definition of each population. This revision should help avoid any confusion regarding the two populations and improve the clarity of the data presentation.

3.27 – How to relate the data analyzing platelet-myeloid cells aggregates in figure 3 and the fact that there are no changes in figure 4b right)

Response: We thank the reviewer for these insightful questions. The apparent discrepancy between the increase in myeloid-platelet aggregates (C8) observed in the scRNA-seq analysis (Fig. 3) and the lack of significant change in monocyte-platelet aggregates observed in the peripheral blood FCM analysis ('Fig. 4b right', or Fig. 5b, if that is what you intended to reference) may be due to several factors related to the different methodologies and the nature of the analyzed populations.

1. Differences between scRNA-seq and FCM analysis: The scRNA-seq analysis in Fig. 3 focused on PBMCs, which provides a more detailed and granular view of cell interactions at the single-cell level, including rare cell populations such as myeloid-platelet aggregates (C8). These aggregates may be present in low numbers but show distinct gene expression profiles that are not easily detectable by FCM, which analyzes bulk populations in peripheral blood. This could explain why we observe an increase in C8 in the scRNA-seq data from nMG patients, but a lack of significant change in monocyte-platelet aggregates in the FCM analysis in Fig. 4b right.
2. Specificity of the FCM analysis: In the FCM analysis (Fig. 4b), we focused on monocyte-platelet aggregates, which may not fully capture the broader category of platelet-myeloid cell aggregates that are detected in the scRNA-seq data. The scRNA-seq analysis (Fig. 3) identifies multiple types of myeloid cells interacting with platelets, including monocytes, dendritic cells, and others, which may not all be reflected in the FCM analysis of monocyte-platelet aggregates. Therefore, the absence of significant changes in monocyte-platelet aggregates in FCM does not necessarily contradict the findings of increased platelet-myeloid aggregates observed in the scRNA-seq data.
3. Platelet-myeloid aggregate formation in the scRNA-seq data: The scRNA-seq data identifies an increase in platelet-myeloid aggregates in nMG patients, as illustrated by the clustering of myeloid cells and platelet markers in C8 (Fig. 3). This suggests that in MG, activated platelets may interact more frequently with myeloid cells, forming aggregates that are detected by single-cell RNA sequencing. However, the FCM analysis of monocyte-platelet aggregates may not capture all types of platelet-myeloid interactions, especially if the aggregates formed are transient or occur in lower numbers.

Overall, the scRNA-seq provides a more comprehensive view of rare and transient interactions, while FCM analysis captures more prominent and stable aggregates in the peripheral blood. To avoid potential confusion and to improve clarity, we have decided to revise the manuscript and remove the scRNA-seq platelet-myeloid aggregate analysis from the results. We believe this will streamline the interpretation of our findings and reduce ambiguity related to potential methodological differences between the two datasets.

3.28 – Figure 6: This figure is complicated. Maybe the legend should be reviewed to be more helpful. In the legend, it is not explained what is w.P. The experimental design of the supernatant treatment with a plate with cells is confusing. Cells should be above the plate.

Response: We thank the reviewer for the valuable feedback. We understand that Figure 6 may appear complicated, and we appreciate your suggestion to improve the clarity of the legend and the experimental design illustration.

In response to your comments, we have adjusted the figure numbering and sequence to align with the corresponding references in the manuscript. The legend has been revised to provide a clearer explanation of "w.P." to avoid any confusion regarding its meaning. Furthermore, we have updated the experimental design illustration to position the cells above the plate during the supernatant treatment, which should help clarify the setup.

These revisions have been made in the updated figure to make the experimental design more intuitive and the legend more informative. Thank you again for your helpful suggestions.

3.29 – Fig 6a: The act Tn data should be in the figure or at least a line at 1 should be indicated on the graph to show the difference compared to act Tn. And it should be indicated if there is a significant difference with act Tn.

Response: We appreciate the reviewer's comment and agree with the suggestion to improve clarity in Fig. 6a. In response, we have added the act Tn data to the figure and included a horizontal line at a value of 1 to represent the baseline level standardized using the act Tn data. Since there were no significant differences between act Tn and the other conditions, non-significant results have not been depicted in the figure. Thank you again for your helpful suggestions to help clarify the data presentation.

Discussion

3.30 – The discussion is too long

Response: We appreciate the reviewer's feedback regarding the length of the discussion. In response, we have carefully revised the discussion to make it more concise while preserving the key points and necessary context.

3.31 – All along the manuscript it is confusing as results from figure 3 mentioned platelet-myeloid cell aggregates, from figure 5 platelet-lymphoid cell aggregates, and from figure 7 platelet-neutrophil aggregates.

Response: We thank the reviewer for pointing out the confusion regarding the terminology of different platelet-cell aggregates in the manuscript. To address this concern, we have made the following revisions:

1. Since the concept of "platelet-myeloid cell aggregates" only appeared in the single-cell sequencing data section and overlapped with the three types of aggregates identified in the flow cytometry analysis, we have removed the single-cell sequencing results related to platelet-myeloid cell aggregates. Given its relatively minor contribution to the overall study, this revision enhances clarity and ensures better alignment with the subsequent terminology.
2. We have carefully revised the figure legends to explicitly specify the cell types involved in

each platelet-cell aggregate analyzed, ensuring consistency and clarity.

3. We have standardized the terminology and abbreviations throughout the manuscript to maintain coherence and avoid confusion. This allows readers to clearly follow the distinctions and relationships between the different platelet-cell aggregates across the figures.

We hope these revisions could resolve the reviewer's concerns and significantly improve the clarity and readability of the manuscript.

3.32 – The patients were divided into naïve and MMS groups. Could the duration of symptoms impact the level of platelet activation?

Response: We thank the reviewer for this important question. We agree that the duration of symptoms could potentially impact the level of platelet activation, and we appreciate the reviewer's suggestion to consider this factor. In our study, we have not explicitly accounted for symptom duration as a confounding variable, which could indeed influence the platelet activation status.

In response to this, we further stratified the patients into different disease duration groups within both the nMG and MMS groups. We then compared the level of platelet activation across these subgroups to determine if there were any differences associated with the duration of symptoms (S5a-b). Our results indicated no significant differences in platelet activation levels across the disease duration subgroups, suggesting that platelet activation is not substantially influenced by the duration of symptoms in this cohort.

We have included these additional analyses in the manuscript to provide a more comprehensive understanding of the potential impact of disease duration on platelet activation.

References

3.33 – The Reference n° 2, is not the more appropriate regarding Treg cell dysregulation in MG.

Response: We thank the reviewer for this insightful comment. We agree that Reference 2 may not be the most appropriate source to support the description of Treg cell dysregulation in MG in the introduction. Upon reviewing the manuscript, we have added a new reference (Ann N Y Acad Sci. 2018 Feb;1413(1):154-162.) that includes more relevant studies, which better support the concept of Treg cell dysfunction in MG. We appreciate the reviewer's suggestion, which has helped us improve the accuracy and relevance of our references.

References

1. Zhang, P. et al. Toll-like receptor 9 antagonist suppresses humoral immunity in experimental autoimmune myasthenia gravis. *Mol. Immunol.* **94**, 200-208 (2018).

Point-by-point response to the reviewers' comments

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed all our concerns. An impressive study.

Response: Thank you for your positive feedback. We appreciate your thoughtful review and are glad that our responses addressed your concerns.

Reviewer #2 (Remarks to the Author):

Thank you for your extensive answers, my concerns have been addressed appropriately

Response: Thank you for your feedback. We are glad that our responses have addressed your concerns appropriately.

Reviewer #4 (Remarks to the Author):

After careful examination of both the reviewer's comments and the authors' responses, I found that the raised concerns have been adequately addressed.

Specifically:

- To reduce the potential bias arising from platelet isolation from PBMCs, the authors supplemented their analysis with bulk RNA sequencing of washed platelets, which provided additional data. In addition, they ensured the reliability of their findings by processing PBMCs immediately after isolation and they cross-referenced platelet counts and characteristics from PBMC-derived scRNA-seq data with those obtained from whole blood-derived platelets and bulk RNA-seq of washed platelets
- To improve clarity, the authors reported the exact number of platelets analyzed for each patient in the scRNA-seq data set
- Additional clarifications were provided regarding flow cytometry methodology, platelet-leukocyte interactions and the impact of disease duration on platelets activation

Taken together, the authors have made substantial revisions and integrated new data in response to Reviewer 3's comments. I believe that these efforts satisfactorily resolve the major points raised.

Response: Thank you for your thoughtful review. We appreciate your recognition of the revisions and additional data provided. We are glad that our efforts have addressed the concerns raised and improved the clarity and reliability of the study.