F-actin organization and target constriction during primary macrophage phagocytosis is balanced by competing activity of myosin-I and myosin-II

Sarah Barger, Daan Vorselen, Nils Gauthier, Julie Theriot, and Mira Krendel

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E22-06-0210
TITLE: "F-actin organization and target constriction during primary macrophage phagocytosis is balanced by competing activity of myosin-I and myosin-II"

Dear Dr. Krendel,

Your manuscript E22-06-0210 entitled "F-actin organization and target constriction during primary macrophage phagocytosis is balanced by competing activity of myosin-I and myosin-II" has been evaluated by two experts in the field. You will see that both referees are positive but raise several issues that have to be addressed before the manuscript can be accepted for publication. Especially the reproducibility of the methods regarding comparisons between cell types must be justified.

I will be happy to look at a revised version of your work that specifically addresses this point and all other concerns raised by the reviewers.

I thank you for submitting your work to MBoC and I look forward to receiving a revised version.

Sincerely,

Peter van Haastert

Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Krendel,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The manuscript presents quantitative evidence for the forces generated by murine bone marrow-derived macrophages (BMDM) during the phagocytosis of deformable synthetic particles. The title is supported by the evidence. Specifically, the data support the important conclusion that myosins Ie/f and myosin II contribute in different and counteracting ways to the movements of actin and the forces exerted within phagocytic cups. However, the comparisons with phagocytosis by BMDM and RAW macrophages are not sufficiently supported by the studies. Additionally, the descriptions of the results should include more explanation of the measured parameters and what they mean regarding the forces and dynamics of phagosome formation.

Specific concerns:

1. The introduction or the results section should describe the experimental system sufficiently to justify the quantitative analyses and interpretations. For any given preparation of microspheres, how uniform are the particle dimensions, stiffness, and the density of IgG on their surfaces? How much is the variation observed in experimental measurements due to particle variability and how much is due to cell-to-cell variation? How do these parameters vary between different preparations of particles or cells? This is especially relevant for item #5, below, in which studies of BMDM and RAW were performed at different times.

2. Line 109: "During phagocytosis, this included F-actin structures that do not necessarily follow the particle surface, sometimes containing branches that grow perpendicular to the main pseudopod." Is this extra ruffling a consequence of the continued presence of M-CSF in the medium of BMDM?

3. Line 150: "Whereas RAW cell cups form parallel to the coverslip as well as vertically from the coverslip, BMDM cups were almost exclusively formed growing up", Fig. 2E and line 481. This difference could simply be due to the fact that RAW macrophages are typically more rounded than BMDM, which spread into more flattened (fried egg) shapes on coverslips. A rounded cell would be expected to make some "side-view" phagosomes, parallel to the coverslip plane. 3D reconstructions of RAW and BMDM cell shapes could test this alternative explanation.

4. Line 157: "Instead, this analysis revealed that primary macrophages are pushing on the target at the base of the phagocytic cup," and Caption to Figure 2: "Phagocytosis ... involves local pushing at the cup base" What data is this conclusion based on? I guess it is Figure 2F, right panel, but that was not stated anywhere. Nor was the apparent comparison of deformations in Figure 3G. Moreover, the mechanism of this deformation was not discussed. Did RAW cells exhibit similar deformations with soft particles?

5. Line 179: "Overall, our direct mechanical comparison between RAW macrophages and BMDMs reveals clear differences in internalization behavior between macrophage-like cell lines and primary macrophages." This comparison is not sufficiently justified. As stated in the legend to Figure 2, line 489: "Data on RAW macrophages was taken from previously published work." Figures 2, C, E, and G compare data from BMDM with previously published data using RAW macrophages. These comparisons are valid only if the experiments using the two cell types were performed together. Since they were not, then the x-axes of the plots should separate the data for the different cell types, and the authors must demonstrate that the particles used in both studies were identical with regard to size, stiffness and IgG density.

6. To support the interpretation of competing functions for myosins Ie/f and II, the distributions of those proteins in forming cups should be shown by immunofluorescence.

7. How do rates and extent of phagocytosis compare between wild-type BMDM, dKO and blebbistatin-treated cells? To what extent do differences in these parameters affect the dynamics of contractions, deformations and F-actin?

8. Lines 419, 505: The authors should explain "great circle distances" and how that was used to quantify phagocytic cup progression. Presently, the use of the term is unclear.

Reviewer #2 (Remarks to the Author):

This is a very nice study- well executed with excellent statistical analyses. The paper is also very well written. I just had a few minor comments. On line 64 of the Introduction, the authors refer to the "ultrastructural organization of primary macrophage phagocytosis". I would change this as no EM data is presented. Can the authors relate 0.3 kPa to the stiffness of physiological substrates like apoptotic cells? Regarding the authors ideas in the Discussion encapsulated by the following sentence: "It is possible that the balance of these
actin networks (branched vs. anti-parallel bundles) within the phagocytic cup is maintained by myosin-I and myosin-II and that altering this balance results in a shift in their relative contribution to force exertion in phagocytosis", the authors should be careful using LifeAct in future work as it does not label formin generated filaments very well (see Bioarchitecture 2014;4(6):189-202.). Finally, what version of blebbistatin did the authors use (there are now 3- the original, para-nitro BB and para-amino BB)?

We thank the reviewers for their insightful comments and constructive criticisms, and we are glad that all reviewers found this manuscript to be of interest. Following reviewers' suggestions, we have performed additional analyses; we have also revised the text and figures to clarify our observations and hypotheses and to streamline the figure/text organization. Major edits in the text are colored in red in the "red-lined" file. We hope that these changes have addressed the concerns raised by the reviewers and improved the clarity of the manuscript.

Below, we address the individual reviewers' comments:

Reviewer #1 (Remarks to the Author):

The manuscript presents quantitative evidence for the forces generated by murine bone marrow-derived macrophages (BMDM) during the phagocytosis of deformable synthetic particles. The title is supported by the evidence. Specifically, the data support the important conclusion that myosins Ie/f and myosin II contribute in different and counteracting ways to the movements of actin and the forces exerted within phagocytic cups. However, the comparisons with phagocytosis by BMDM and RAW macrophages are not sufficiently supported by the studies. Additionally, the descriptions of the results should include more explanation of the measured parameters and what they mean regarding the forces and dynamics of phagosome formation.

Specific concerns:

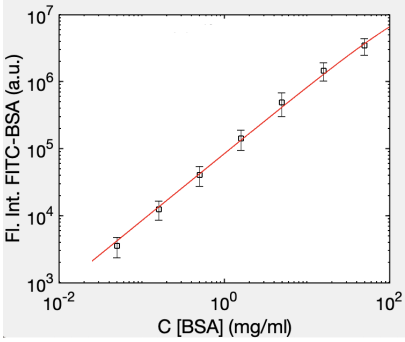
1. The introduction or the results section should describe the experimental system sufficiently to justify the quantitative analyses and interpretations. For any given preparation of microspheres, how uniform are the particle dimensions, stiffness, and the density of IgG on their surfaces? How much is the variation observed in experimental measurements due to particle variability and how much is due to cell-to-cell variation? How do these parameters vary between different preparations of particles or cells? This is especially relevant for item #5, below, in which studies of BMDM and RAW were performed at different times.

We agree that further explanation of the experimental system in relation to the manuscript's goals/findings was necessary, and we have added more descriptions throughout the manuscript (e.g., Line 102-106).

We appreciate the reviewers' concern regarding the comparisons between microspheres of a single preparation and between microsphere preparations. We have previously characterized variability in size and polymer density between particles in microsphere preparations (Vorselen, D., Wang, Y., de Jesus, M.M. *et al.* Microparticle traction force microscopy reveals subcellular force exertion patterns in immune cell–target interactions. *Nat Commun* 11, 20 (2020). https://doi.org/10.1038/s41467-019-13804-z). This revealed that within a single preparation the coefficient of variation (CV) in diameter is ~0.1, which is comparable to commercially available latex beads. We

have previously also assessed the variation in polymer network density through refractive index measurements (which correlate with particle rigidity, and which can be more accurately determined experimentally), which revealed noticeably low variability (CV 0.03 – 0.05, which may still be dominated by the measurement noise and not real variability). A note regarding the variability between DAAMPs has been added on line 418-420.

Regarding the density of IgG on the particle surface: Each dataset was collected over multiple days, and IgG functionalization was performed separately on each day that experiments were performed. We haven't quantified the IgG density on the particle surface, because this is non-trivial in case of porous beads. We have, however, previously quantified the amount of covalently bound BSA throughout the particles, which is the first step in our two-step functionalization protocol (the second is the functionalization with the anti-BSA IgG). The BSA functionalization shows little variation between samples and high reproducibility between different batches (see a typical binding curve below). Furthermore, we have not observed a clear day-to-day variation within each experimental condition, even though the IgG functionalization was typically performed independently every day. This indicates that the variability caused by the IgG conjugation is small compared to the differences observed between the experimental groups (BMDMs vs RAWs).



Concentration of FITC-BSA in solution vs fluorescent signal on beads. Error bar indicate standard errors of the mean.

We haven't thoroughly quantified variation between microsphere preparations. However, all comparisons in this manuscript were made between microparticles from a single batch of each particle type. Furthermore, the same microsphere preparation was used for the 1.4 kPa DAAMPs used to obtain the BMDM data described in this manuscript and the previously published RAW cell data. The batch-to-batch variation between distinct particle preparations is therefore irrelevant for the interpretation of the presented results.

We note that we had also used the same imaging equipment, and the data was collected within a 2-months span for both cell types, even though the data analysis was performed at two separate times due to the large volume of data collected. We have added this information to the manuscript at lines 373-378. Overall, the observations from the previously published manuscript and the current study can indeed be directly compared.

2. Line 109: "During phagocytosis, this included F-actin structures that do not necessarily follow the particle surface, sometimes containing branches that grow perpendicular to the main pseudopod." Is this extra ruffling a consequence of the continued presence of M-CSF in the medium of BMDM?

We agree that this is one possible interpretation, although the concentration of M-CSF used and the duration of M-CSF treatment make it somewhat less likely. In our imaging experiments, M-CSF was continuously present in the culture medium at the concentration of 20 ng/ml, which corresponds to ~3.2-10 U/ml, while previous experiments examining stimulation of macropinocytosis ruffling by M-CSF used a higher concentration and a short treatment duration since the M-CSF-induced pinocytosis rate declines after 30 minutes of treatment. We have added comments to that effect on line 121-125.

3. Line 150: "Whereas RAW cell cups form parallel to the coverslip as well as vertically from the coverslip, BMDM cups were almost exclusively formed growing up", Fig. 2E and line 481. This difference could simply be due to the fact that RAW macrophages are typically more rounded than BMDM, which spread into more flattened (fried egg) shapes on coverslips. A rounded cell would be expected to make some "side-view" phagosomes, parallel to the coverslip plane. 3D reconstructions of RAW and BMDM cell shapes could test this alternative explanation.

We thank the reviewer for bringing up this excellent point. 3D projections of RAW cells and BMDMs indeed indicate that RAWs stay in a more rounded shape. Whether this effect is sufficient to explain the observed differences in cup angles would however require quantitatively taking into account cell shape as well as cell density (which could limit phagocytosis horizontal to the coverslip). Unfortunately, our data is not suitable for the systematic segmentation of cells and the subsequent quantification of cell shape so we cannot make a conclusive statement on this. We now mention the alternative explanation by the reviewer on lines 162-164.

4. Line 157: "Instead, this analysis revealed that primary macrophages are pushing on the target at the base of the phagocytic cup," and Caption to Figure 2: "Phagocytosis ... involves local pushing at the cup base" What data is this conclusion based on? I guess it is Figure 2F, right panel, but that was not stated anywhere. Nor was the apparent comparison of deformations in Figure 3G. Moreover, the mechanism of this deformation was not discussed. Did RAW cells exhibit similar deformations with soft particles?

We agree with the reviewer that it wasn't clear what these conclusions were drawn from. We now have updated the text to reference specific figures and more fully explain these quantifications. In addition, we have added an additional figure panel (2G) to show the relative elongation of targets in phagocytic cups. For both 0.3 kPa and 1.4 kPa DAAMPs we see compression along the phagocytic axis, which is consistent with pushing at the base of the phagocytic cup. For RAW macrophage and 1.4 kPa targets we see clear elongation along the phagocytic axis. We did not challenge the RAW

macrophages with softer (0.3 kPa DAAMPs), but we believe this comparison between BMDMs and RAW macrophages using 1.4 kPa targets is an equally valid comparison. Furthermore, we now suggest a potential mechanism (Line 182–184). We note that Figure 2F contains the same WT data as figure 3G.

5. Line 179: "Overall, our direct mechanical comparison between RAW macrophages and BMDMs reveals clear differences in internalization behavior between macrophage-like cell lines and primary macrophages." This comparison is not sufficiently justified. As stated in the legend to Figure 2, line 489: "Data on RAW macrophages was taken from previously published work." Figures 2, C, E, and G compare data from BMDM with previously published data using RAW macrophages. These comparisons are valid only if the experiments using the two cell types were performed together. Since they were not, then the x-axes of the plots should separate the data for the different cell types, and the authors must demonstrate that the particles used in both studies were identical with regard to size, stiffness and IgG density.

As discussed in our response to comment 1, the experiments with the BMDM and RAW cells were performed in parallel, using the same preparations of microparticles. This is noted in the revised text at lines 373-378.

6. To support the interpretation of competing functions for myosins Ie/f and II, the distributions of those proteins in forming cups should be shown by immunofluorescence.

We agree that this would be very informative. Unfortunately, immunofluorescence staining of cells interacting with the antibody-coated deformable microparticles was problematic in these experiments, because the microparticles produced very high background in our experiments and made immunofluorescence localization nearly impossible. An alternative approach, transfection with fluorescently tagged myosin constructs, is very challenging to perform in BMDM. We have added a discussion of our previous observations of myosin-I and -II distribution in RAW cells (Vorselen et al., 2021) to the line 283-287 to help in interpretation of the findings reported in the current manuscript.

7. How do rates and extent of phagocytosis compare between wild-type BMDM, dKO and blebbistatin-treated cells? To what extent do differences in these parameters affect the dynamics of contractions, deformations and F-actin?

These experiments certainly represent logical next steps in examining the contributions of the various cytoskeletal components to macrophage phagocytosis. Addressing these questions would require a significant amount of new experimentation, and partly because both first authors are no longer working on this project, this work is beyond the scope of the current manuscript and could be best addressed in a new paper.

8. Lines 419, 505: The authors should explain "great circle distances" and how that was used to quantify phagocytic cup progression. Presently, the use of the term is unclear.

Great circle distance is the shortest distance between two features on a sphere, measured along the surface of that sphere. We have added this clarification in the legend of Figure 3.

Reviewer #2 (Remarks to the Author):

This is a very nice study- well executed with excellent statistical analyses. The paper is also very well written. I just had a few minor comments.

On line 64 of the Introduction, the authors refer to the "ultrastructural organization of primary macrophage phagocytosis". I would change this as no EM data is presented.

We agree and have changed the text to “the detailed structural organization of phagocytic cups”.

Can the authors relate 0.3 kPa to the stiffness of physiological substrates like apoptotic cells?

Thank you for this suggestion. Necrotic cells and cancerous cells have been measured to be in the 0.3-0,5 kPa range. We now cite this study in the Results section (Lines 102-106). Using DAAMPs allowed us to make and functionalize targets with characteristics (size, stiffness) that are physiologically relevant to what a primary cell might encounter, such as apoptotic cells.

Regarding the authors ideas in the Discussion encapsulated by the following sentence: "It is possible that the balance of these actin networks (branched vs. anti-parallel bundles) within the phagocytic cup is maintained by myosin-I and myosin-II and that altering this balance results in a shift in their relative contribution to force exertion in phagocytosis", the authors should be careful using LifeAct in future work as it does not label formin generated filaments very well (see Bioarchitecture 2014;4(6):189-202.).

We appreciate the reviewer’s comments regarding the interpretation of LifeAct labeling in future work. In the case of the discussion referenced by the reviewer, we were referring to the existing literature on the myosin II vs. myosin I interactions with the linear vs. branched actin networks, which does not rely on the LifeAct labeling.

Finally, what version of blebbistatin did the authors use (there are now 3- the original, para-nitro BB and para-amino BB)?

The original blebbistatin was used and this has now been noted in the methods.

RE: Manuscript #E22-06-0210R
TITLE: "F-actin organization and target constriction during primary macrophage phagocytosis is balanced by competing activity of myosin-I and myosin-II"

Dear Dr. Krendel,

Your manuscript E22-06-0210 entitled "F-actin organization and target constriction during primary macrophage phagocytosis is balanced by competing activity of myosin-I and myosin-II" has been evaluated by two experts in the field.

All points have been addressed well in the revised manuscript E22-06-0210R and the paper is acceptable for publication.

I thank you for submitting your work to MBoC.

Sincerely,
Peter Van Haastert
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Krendel:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

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