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FIB-SEM-based analysis of *Borrelia* intracellular processing by human macrophages

Matthias Klose, Maximilian Scheungrab, Manja Luckner, Gerhard Wanner and Stefan Linder
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MS TITLE: FIB/SEM-based analysis of Borrelia intracellular processing by human macrophages

AUTHORS: Matthias Klose, Maximilian Scheungrab, Manja Luckner, Gerhard Wanner, and Stefan Linder
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

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Reviewer 1

Advance summary and potential significance to field

The authors use FIB SEM and CLEM to investigate Borrelia infecting macrophages and discover a structure they term a tunnel at the tip of invading bacteria. The presence of the tunnel suggests a tug-of-war between the bacterium and the forming phagosome with the bacteria likely moving or being moved outwards while it is pulled into the cell, but the authors show no data supporting or rejecting this claim. The FIB SEM images are of superb quality

Comments for the author

JCS demands functional insight as a criteria for publication, which I am not sure the paper supplies. I do like the paper as a technical advance and would also encourage the editor to think of publishing it as a short report (the figures could readily be combined into three by fusing figures 1 and 2, 3 and 4 and 5 to 7. Especially the paper would fit as such to the upcoming special issue on host pathogen interaction.
Yet prior to publication in JCS or elsewhere, I would ask the authors to quantify in the text and figure legends some key statements, e.g. how many cells were observed per condition (e.g. line 221) also distributions of tunnel length could be shown as panel in the figure (line 186-188) and the diminishing antibody staining could be quantified along the length of the bacterium (line 195-197) Discussion could be much shortened.

minor points:
line 48: please use s instead of sec line 88: please change confirm into test lines 92-96: please add additional references to whole cell cryo electron tomography studies on Borrelia, especially the beautiful work by Jun Liu's group on the motor should be mentioned.
line 137: please add reference after reports 265: what is high resolution, I don't see e.g. microtubules. So resolution is probably worse than 20 nm

Reviewer 2

Advance summary and potential significance to field

This manuscript provides the first 3-dimensional reconstruction of the Borrelia invasion vacuole at the electron-microscope level, obtained using FIB/SEM in combination with CLEM. This is a technically demanding and labour intensive enterprise that, by necessity, reports a comparatively small number of events that are difficult to detect. The authors describe tubules likely representing invasion vacuoles/phagosomes that are partially emptied by the microbe as it tries to extricate itself from the host cells, and others that may be part of the resolution process as Borrelia compacts into a smaller volume. Contacts of the invasion vacuole with the ER are also documented.

Comments for the author

The author should clarify/validate some of their interpretations, specifically:
1) How do they know that the sites where the ER is found to approach the phagosome/invasion vacuole are bona fide contact sites, rather than areas of fortuitous closeness? Considering the extensive distribution of the ER throughout the cytoplasm, how can we know that the putative contacts are functionally significant?
2) How was the ER identified in the FIB-SEM analyses? Was a specific marker used for identification or is the designation based exclusively on morphological appearance? If the latter, what features were used as identifiers and how unambiguous is such a determination?
3) What, if any, is the biological or functional significance of the "tunnels" generated by extrication of Borrelia? The authors state that they were observed in only 2-5% of the instances. How many events were analyzed, were they more than 50?
4) The description of the "sites of contact" between Borrelia and the membrane of the phagosome/invasion vacuole is intriguing. What is their significance are they momentary, unstable

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and inconsequential contacts only? Could they have been artifactually induced by sample processing?
5) How do you distinguish conclusively between a tube formed by extrication from a tube caused by compaction? Were epxeriments performed at different times when only extrication could have happened but compaction had not yet started?
Are compacting phagosomes (but not extricating tubules) positive for Rab5 or Rab7?
6) How does galectin-9 contribute to tubulation? An explicit description of the underlying mechanism with adequate references would be helpful in this context.

Reviewer 3

Advance summary and potential significance to field

This is an excellent manuscript that enormously clarifies the dynamics of the interaction between Borrelia burgdorferi and macrophages during the process of phagocytosis. The manuscript is very well written and clear and provides new insights into the process by using state of the art visual technologies that results in an excellent level of detail.

Comments for the author

I only have a minor critiques/suggestions for improvement:

1. Line 64: The impression is given that TLR2 anchors the spirochete to macrophages, thereby becoming a phagocytic receptor. This needs to be rephrased since several works have demonstrated that this is not the case and the interaction of TLR1/TLR2 with spirochetal lipoproteins only can occur upon bacterial degradation.

2. Lines 373-376. Is there a reference to support this statement? This seems intuitive but lacks formal demonstration.

3. Lines 449-451. The differentiation of human macrophages from monocytes is not sufficiently explained.
Please, confirm that no cytokines (i.e. M-CSF) were used and provide a reference in any case.

First revision

Author response to reviewers' comments

Reviewer 1
The authors use FIB SEM and CLEM to investigate Borrelia infecting macrophages and discover a structure they term a tunnel at the tip of invading bacteria. The presence of the tunnel suggests a tug-of-war between the bacterium and the forming phagosome with the bacteria likely moving or being moved outwards while it is pulled into the cell, but the authors show no data supporting or rejecting this claim. The FIB SEM images are of superb quality.
Thank you for the kind appreciation of our work

major points:
1) JCS demands functional insight as a criteria for publication, which I am not sure the paper supplies. I do like the paper as a technical advance and would also encourage the editor to think of publishing it as a short report (the figures could readily be combined into three by fusing figures 1 and 2, 3 and 4 and 5 to 7. Especially the paper would fit as such to the upcoming special issue on host pathogen interaction.
Fusing the figures as suggested would certainly be possible. However, we think this would lead to a crowding of panels that leads to more complicated reading of the manuscript, especially

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considering the addition of newly generated material (Fig. 4I,J; new Figs. 7 + 8). We leave this point to the editor´s discretion.
Regarding the issue of functional insight, we are now also including novel data on the localization of STIM1-positive ER contact sites at borreliae phagosomes, indicating that store-operated Ca2+ release likely plays an important role in the maturation of these phagosomes (see reviewer 2, point 1).

2) Yet prior to publication in JCS or elsewhere, I would ask the authors to quantify in the text and figure legends some key statements,
2a) how many cells were observed per condition (e.g. line 221)
We have now added the number of cells observed per condition for Fig. 4I, J, Fig. 5A, Fig. 6, Fig. 8.

2b) also distributions of tunnel length could be shown as panel in the figure (line 186-188)
We have now added quantifications of tunnel lengths, together with total lengths of associated borreliae, and of their intra- and extracellular parts in the new Figure 4I, J. A respective paragraph has been added to the Results section (p.7).

2c) and the diminishing antibody staining could be quantified along the length of the bacterium (line 195-197)
We have now quantified the diminishing antibody staining along the length of the internalized Borrelia cell. The respective quantification is now shown in the form of two line graphs in the new Figure 5B panel. A description of the fluorescence-based measurement has been added to the Materials and Methods section.

3) Discussion could be much shortened.
We agree with the reviewer. The previous discussion has now been considerably shortened. Still, based on the referees´ comments, new material has been added.

minor points:
1) line 48: please use s instead of sec
corrected

2) line 88: please change confirm into test
corrected

3) lines 92-96: please add additional references to whole cell cryo electron tomography studies on Borrelia, especially the beautiful work by Jun Liu's group on the motor should be mentioned.
Respective references (Liu et al., 2009; Kudryashev et al., 2011; Chang et al., 2019) have been added.

4) line 137: please add reference after reports
Respective references (Rittig et al., 1992; Linder et al., 2001) have been added

5) line 265: what is high resolution, I don't see e.g. microtubules. So resolution is probably worse than 20 nm
The pixel size of the high-resolution FIB/SEM series is 5 nm laterally (x/y). This results in a resolution of about 12 nm, according to the Nyquist criterion. This is now also mentioned in the legends for Suppl. Video 1. Microtubules can be frequently observed in the FIB/SEM stack. We are including an image of a laterally cut microtubule in the Figure for referee 1.

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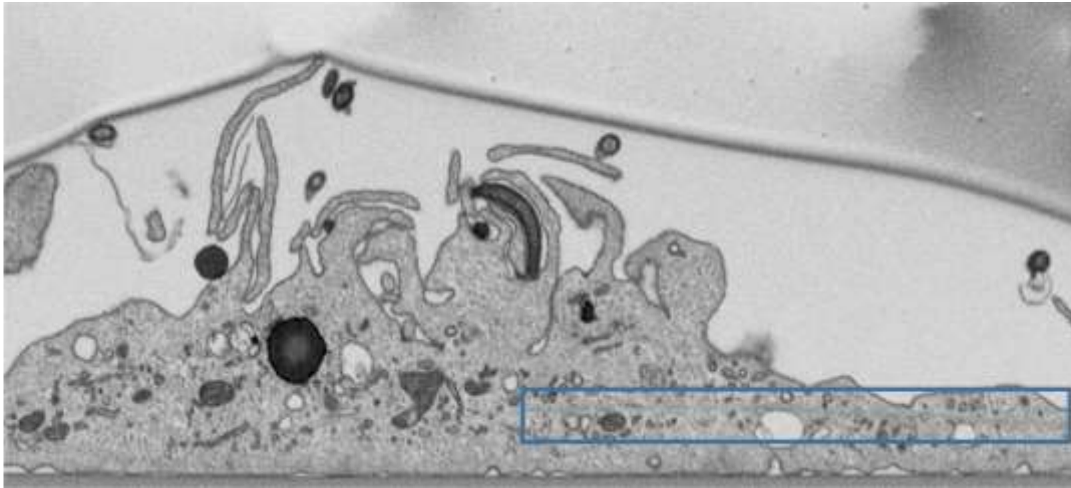

FIB/SEM image taken from stacks shown as animation in Supp. Video 1. Image shows slice of whole macrophage in contact with GFP-expressing borreliae. A laterally cut microtubule is highlighted by the blue box.Resolution of digital image ~12 nm, image width: 18.8 µm.

Reviewer 2
This manuscript provides the first 3-dimensional reconstruction of the Borrelia invasion vacuole at the electron-microscope level, obtained using FIB/SEM in combination with CLEM. This is a technically demanding and labour intensive enterprise that, by necessity, reports a comparatively small number of events that are difficult to detect. The authors describe tubules likely representing invasion vacuoles/phagosomes that are partially emptied by the microbe as it tries to extricate itself from the host cells, and others that may be part of the resolution process as Borrelia compacts into a smaller volume. Contacts of the invasion vacuole with the ER are also documented.

Thank you for the kind appreciation of our work.

major points:

The author should clarify/validate some of their interpretations, specifically:

1) How do they know that the sites where the ER is found to approach the phagosome/invasion vacuole are bona fide contact sites, rather than areas of fortuitous closeness? Considering the extensive distribution of the ER throughout the cytoplasm, how can we know that the putative contacts are functionally significant?

Thank you for pointing this out. We have now tried to clarify this issue by a) rendering ER-phagosome contacts at higher magnification and b) staining endogenous stromal interaction molecule 1 (STIM1) in macrophages with phagocytosed borreliae and expressing either RFP-LactC2 as a phagosome marker or GFP-Sec61 to visualize ER tubules. First, rendering ER-phagosome contacts in higher magnification showed that the ER forms distinct sites of close contact with phagosomes, pointing to a non-random nature of these contacts. Second, as you know, STIM1 is an ER resident transmembrane protein that activates store-operated Ca2+ channels, and has been used as a marker for contacts sites between the ER and mitochondria, the plasma membrane and also phagosomes. Our data show that multiple STIM1 signals localize along borreliae-containing phagosomes, indicating that these observed sites of contact between the ER and phagosomes indeed represent functional ER contact sites. Moreover, Borrelia burgdorferi is only the second bacterium, besides Chlamydia trachomatis, whose phagosomal uptake has been associated with recruitment of STIM1. Our results thus also indicate that STIM1-regulated Ca2+ levels likely play a role in the maturation of Borrelia-containing phagosomes, but also in the intracellular processing of other pathogenic and non-pathogenic bacteria by immune cells. Respective results are now shown in the new Figs. 7 +8, the new Suppl. Video 7, and are mentioned in the Results (pp.8,9) and Discussion sections (p.14)

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2) How was the ER identified in the FIB-SEM analyses? Was a specific marker used for identification or is the designation based exclusively on morphological appearance? If the latter, what features were used as identifiers and how unambiguous is such a determination?

The ER was identified based on its morphological appearance. As in most mammalian cells, the ER in human macrophages shows a characteristic structure and is decorated by 80S ribosomes of a diameter of 20-25 nm. Although ribosomes are not visible on all ER segments of the individual FIB sections, the 3D reconstruction confirms the morphological identification and shows that the ER forms a typcial extensive network.

3) What, if any, is the biological or functional significance of the "tunnels" generated by extrication of Borrelia? The authors state that they were observed in only 2-5% of the instances. How many events were analyzed, were they more than 50?

Based on our analyses, we theorize that tunnels are the result of partial extrication of the highly motile borreliae. They are thus more indicators of previous internalization of spirochetes, and likely have no funtional significance of their own. We are now including quantifications of tunnel length, and also of respective extra- and intracellular parts of associated borreliae (Fig. 4I,J) for 20 bona fide observations of tunnels. We would like to point out that, although RFP-LactC2 has proven to be a good marker for tunnels, the presence of RFP-LactC2 signals also at plasma membrane complicates the identification of true tunnels as opposed to borreliae extending along plasma membrane ruffles. This necessitates the capturing of confocal image stacks and 3D analysis, making this a time-consuming endeavour (see also referee 1, points 2a, 2b).

4) The description of the "sites of contact" between Borrelia and the membrane of the phagosome/invasion vacuole is intriguing. What is their significance, are they momentary, unstable and inconsequential contacts only? Could they have been artifactually induced by sample processing?

The sites of contact between Borrelia and the phagosome membrane are most probably not artefacts. Indeed, the cytoplasm shrinks slightly when fixed with glutaraldehyde (and other aldehydes), as in FIB/SEM sample preparations, and phagosomal invaginations are thus slightly dilated. We therefore assume that the contact sites between Borrelia and phagosomal membrane are more numerous or more extensive than we detect. As observed in live cell imaging, initial phagosomes might not follow the outline of highly bent spirochetes completely. This should allow room for slight intraphagosomal movements of spirochetes, indicating a transient nature of the observed contacts. Moreover, tunnel formation and thus partial extrication of borreliae also indicates that contacts with the phagosomal membrane are not permanent. However, once phagosomes are completely sealed and compaction begins, relative movement of borreliae stops (illustrated in Figure for referee 2) and spirochete-phagosome contacts likely become more extensive and also more stable. So far, we have no indication that these contacts are significant during the phagosomal process. They seem to be more a consequence of size restrictions within and compaction of phagosomes. These points are now also mentioned in the Discussion section (p.12).

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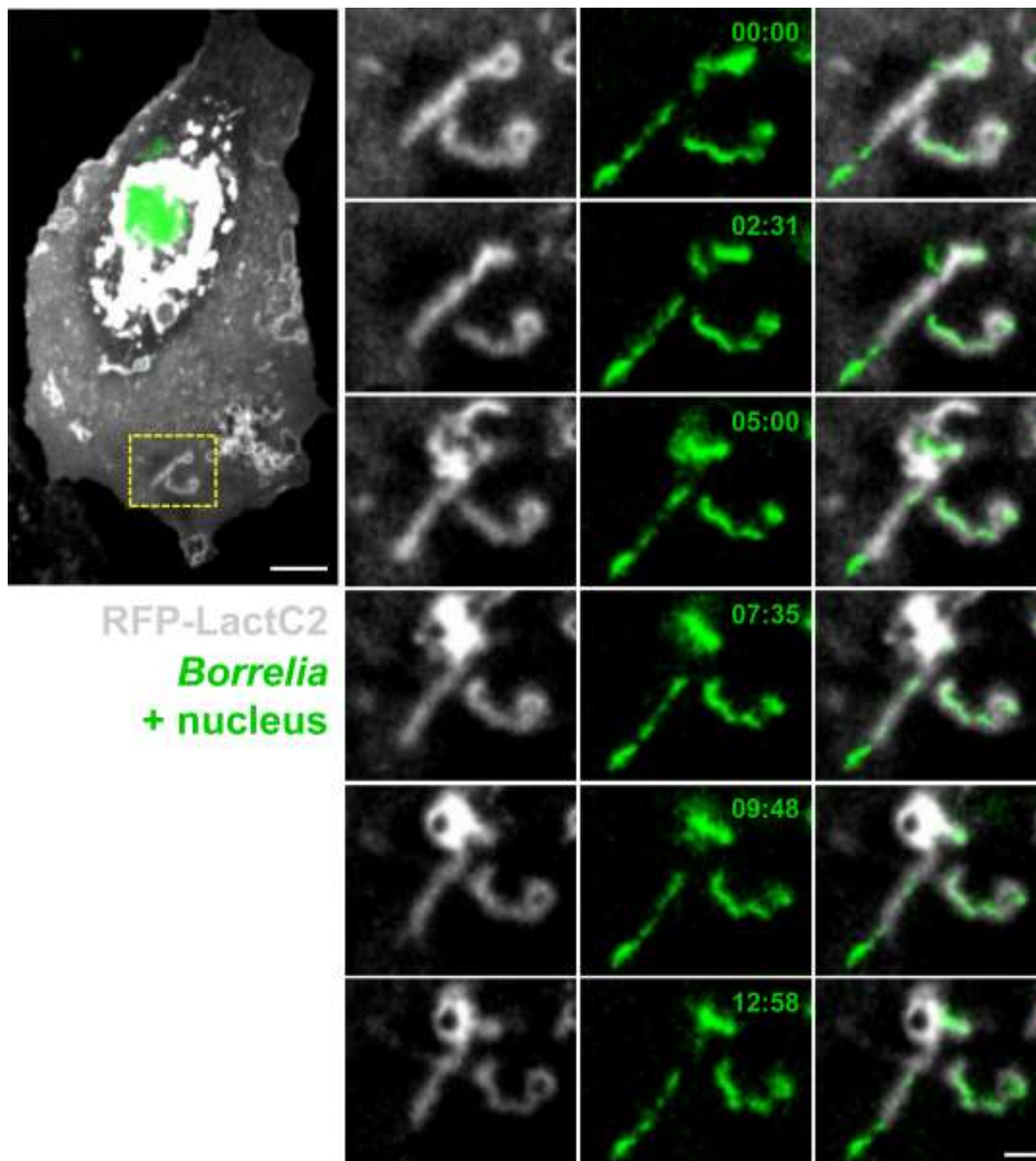

Still images from confocal time lapse imaged by live-cell spinning disk microscopy with macrophage overexpressing RFP-LactC2 (grey) and infected with wildtype *B. burgdorferi* visualized by Hoechst 33342 staining of their DNA (green). Yellow dashed box indicates detail region shown enlarged in gallery panels. Time since start of experiment is indicated in minutes:seconds. Scale bars: 10 µm for overview image and 2 µm for insets.

5a) How do you distinguish conclusively between a tube formed by extrication from a tube caused by compaction?
Distinction between tunnels, probably caused by extrication of borreliae, and membrane tubules, caused by active extrusion of material from the phagosome surface is based on both diameter and relative location of structures. As former sites of borreliae phagosomes, tunnels have a diameter that roughly corresponds to the diameter of borreliae (~0.5 µm), even considering partial shrinkage. Moreover, tunnels are by definition only formed at the distal end of partially phagocytosed borreliae. By contrast, membrane tubules at phagosomes show smaller diameters (~0.2 µm) and can be formed all over the surface of borreliae phagosomes (see Fig. 6). Both criteria are now also mentioned in the Discussion section (pp.13,14).

5b) Were epxeriments performed at different times, when only extrication could have happened but compaction had not yet started?

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Over the years, we have tried repeatedly to synchronize phagocytosis of borreliae by macrophages, including centrifugation and keeping cell cultures at 4°C. However, all such attempts were unsuccessful, which is likely due to the high motility of spirochetes and their varying length. Borreliae are thus phagocytosed within a time window of 45 min, with widely varying time points for individual uptake steps of different phagocytic events within this time frame.

5c) Are compacting phagosomes (but not extricating tubules) positive for Rab5 or Rab7?
Borreliae containing phagosomes show membrane tubulation at the sites of contact between the phagosomal coat, which is enriched with Rab22a, and vesicles that are positive for Rab5a (Naj and Linder, Cell Rep, 2015). Contact with these vesicles is dynamic, while the vesicles remain anchored to the ER. Neither the phagosome nor the extruded tubules are positive for Rab5a. Rab7 is acquired by borreliae containing phagosomes only at later time points, when phagosomes are already mostly compacted. (see also reviewer 3, point 2)

6) How does galectin-9 contribute to tubulation? An explicit description of the underlying mechanism with adequate references would be helpful in this context.
We agree that the role of galectin-9 in phagosomal membrane tubulation is intriguing. We recently identified galectin-9 as an interaction partner of the 11 amino acid residue C-terminus of SNX3 and as a novel regulator of borreliae phagosome compaction (Klose et al., JCB, 2019). Using single and combinatorial knockdowns, we could show that galectin-9 acts in the same pathway as SNX3. Moreover, we could show that both carbohydrate binding domains (CRDs) of galectin-9 are required for this effect. However, use of point mutations showed that none of the currently known residues involved in binding specific carbohydrates, such Ala 46 for binding of Forssman pentasaccharides, or Arg221 for sialylated oligosaccharides, are apparently important for phagosomal tubulation. Still, identification of flotillin-2 as a second marker protein for galectin-9 positive vesicles should be helpful to further clarify the underlying mechanism. These investigations are a current research focus in the lab. We have now added more information on the respective role of galectin-9, as currently known, to the Discussion (p.13).

Reviewer 3
This is an excellent manuscript that enormously clarifies the dynamics of the interaction between Borrelia burgdorferi and macrophages during the process of phagocytosis. The manuscript is very well written and clear and provides new insights into the process by using state of the art visual technologies that results in an excellent level of detail.
I only have a minor critiques/suggestions for improvement:
Thank you for the very kind appreciation of our work.

Minor points:
1) Line 64: The impression is given that TLR2 anchors the spirochete to macrophages, thereby becoming a phagocytic receptor. This needs to be rephrased since several works have demonstrated that this is not the case and the interaction of TLR1/TLR2 with spirochetal lipoproteins only can occur upon bacterial degradation.
Thank you for pointing this out. To avoid misunderstandings, we are no longer mentioning TLR2 in the context of phagocyte-Borrelia recognition and have also removed the respective citation.

2) Lines 373-376. Is there a reference to support this statement? This seems intuitive but lacks formal demonstration.
We showed previously that membrane tubules are extruded and detached from the phagosomal surface at sites of contact between the Rab22a-positive phagosomal coat and Rab5a-positive vesicles (Naj and Linder, Cell Rep, 2015). This coincides with the progressive shrinkage of phagosomes and the resulting compaction of internalized borreliae. While this is not a final proof of a causal relationship between the phenomena, it still strongly suggests that membrane tubulation is at least one of the mechanisms that supports phagosome compaction. We have now added the respective citation to the text. (see also reviewer 2, point 5)

3) Lines 449-451. The differentiation of human macrophages from monocytes is not sufficiently explained. Please, confirm that no cytokines (i.e. M-CSF) were used and provide a reference in any case.
Macrophages are routinely differentiated in the lab from peripheral blood monocytes by the addition of 20% autologous human serum, without further addition of cytokines and cultivation for

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at least 6 days under these conditions. This procedure has been standardized in the lab during the last 20 years. Differentiation into macrophages is checked by expression of marker proteins such as CD68, high phagocytic capacity, cell size (>20 µm diameter) and and typical radially symmetric morphology under non-stimulating conditions as well as restructuring of the actin cytoskeleton, including the formation of >100 podosomes per cell. We have now added more details on the procedure and added a respective reference (Cervero et al., 2013).

Second decision letter

MS ID#: JOCES/2020/252320

MS TITLE: FIB/SEM-based analysis of Borrelia intracellular processing by human macrophages

AUTHORS: Matthias Klose, Maximilian Scheungrab, Manja Luckner, Gerhard Wanner, and Stefan Linder
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors addressed the questions both by quantifying their findings and by adding a new experiment.

Comments for the author

Thanks for a nice and precise revision, which in my view did improve the paper.
The authors addressed the points I raised. The paper still doesn't strictly provide functional insight but I naturally leave the importance of that to the editor.

Reviewer 2

Advance summary and potential significance to field

The authors have adequately addressed my queries and suggestions.

Comments for the author

The authors have adequately addressed my queries and suggestions.

Reviewer 3

Advance summary and potential significance to field

This is an excellent manuscript that enormously clarifies the dynamics of the interaction between Borrelia burgdorferi and macrophages during the process of phagocytosis. The manuscript is very well written and clear and provides new insights into the process by using state of the art visual technologies that results in an excellent level of detail.

Comments for the author

The authors have responded satisfactorily to the questions and I have no more queries.

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