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Uptake-independent killing of macrophages by extracellular Mycobacterium tuberculosis aggregates

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Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this manuscript authors show that extracellular Mtb aggregates can cause macrophage killing in a close contact dependent but phagocytosis independent manner. They showed Mtb aggregates can induce plasma membrane perturbations and cytoplasmic Ca2+ influx with live cell microscopy. Next, the authors show that the type of cell death initiated by extracellular aggregates is pyroptosis and they partially supressed cell death with pyroptosis inhibitors. They also identified that PDIM, EsxA/EsxB and EspB all have a role in uptake-independent killing of macrophages even though their impact varies with respect membrane perturbation and Ca2+ influx. Finally, they used a small molecule inhibitor BTP15 to inhibit the effect of ESX-1 during the contact of the extracellular Mtb aggregates with the macrophages and they observed a substantial decrease in membrane perturbation and macrophage killing.

The work describes a very interesting mechanism by which Mtb can kill macrophages that is possibly relevant in the context of infection. In general, there are two main issues with the experiments and the interpretation: the lack of quantitative analysis showing that in a population of macrophages the ones that are in contact with the aggregates die whereas the ones that are not in contact remain alive. This is currently not shown, and it should be added in figure 1. The second is the cell death mode, as the markers used are very different and considering different outcomes (e.g., apoptosis vs. necrosis) are relevant for the infection it is unclear what is being measured here and the impact on bacterial replication.

The authors are showing that infection with Mtb aggregates increase the rate of the macrophage killing but how does this impact infection dissemination and replication of the bacterial aggregates? Is it beneficial for the aggregates? Did the authors check the growth rate of Mtb along with cytochalasin D? How did the authors quantify the interactions of Mtb with macrophages in Figure 1D? Is it enough to conclude with one example of SEM that the mycobacteria with different fragmentation discriminates if the bacteria is intracellular or extracellularly localised? Can authors use an alternative quantitative method to confirm the localization of the bacteria by a quantification by 3D imaging of these two phenotypes with a cytoskeleton marker (or may be even with tdTomato-expressing BMDMs)?

How do we know if the cell is lysed at 30 h in Supplementary Figure 1, did the authors use a marker to detect the cell lysis or is it based on just the observation from the live cell imaging? Movies in supplementary are actually not very informative as there are many ongoing events and it is hard to visualise what the authors claim. A marker of cell death in the movies should be used.

Total macrophage killing after contact in Figure 1L is around 12 hours, whereas it is observed that the macrophage death after contact with cytochalasin D treatment in Figure 1M is even longer than 24 hours. The viability at 12 hours in Figure1M is as fragmented Mtb survival in Figure1L, why there is a difference in timing with respect to macrophage killing?

Did authors perform statistical tests for Figure 1D and Figure 1N? p-values should be added.

In Figure 3, do the observations indicated in the Figure 3 happen in all the macrophages that are in contact with aggregates? This is unclear and critical to support the conclusions. Do all the macrophages that are in contact with Mtb aggregates become Annexin-V positive? In Supplementary Figure 2 there is some information regarding this question, but it will be important to show it as a percentage. Did the authors try to stain Mtb aggregates alone with Annexin-V as a control over the duration of the imaging?

In Figure 4, did the authors continue to image the cells interacting with Mtb aggregates that do not die after Ca2+ accumulation in Supplementary Figure 3D? Do these cells recover from the plasma membrane perturbation? Did the authors consider using another marker for plasma membrane perturbation together with BAPTA?

In Figure 5D-G it will be important if the authors include dots for each macrophage events for the contact conditions as well, as it was done for the bystander condition. How did the authors discriminate between the macrophages that are in contact or not with Mtb aggregates after the staining with Casp-1, pRIP3 and pMLKL? Do the aggregates stay in contact even after the staining procedures? Representative images of the labelling should be included in this figure. The labelling of Figure 5H needs to be corrected both in the text and in the figure legend. Pyroptosis inhibitors did reduce the percentage of cell death, but did it also reduce the number of Annexin-V positive domains? This is important as AnnexinV is a marker of apoptosis and the outcome for Mtb very different.

In Figure 6, The sections for Figure 6 are well described but kept relatively long with too many details, it will be helpful to the reader if the authors can combine the

sections in one header. Figure 6F does not have a statistical test and p-value, it will be important to include the statistical test in the legend and p-values in the figure.

2. Significance:

Significance (Required)

Based on the literature, Mtb infection and replication can trigger different types of cell death and most of the studies have addressed cell death only as an outcome of intracellular replication. This study shows another form of host cell death, associated only to extracellular bacterial aggregates that are in contact with macrophages. Plasma membrane damage initiating pyroptosis has been defined in: "Plasma membrane damage causes NLRP3 activation and pyroptosis during Mycobacterium tuberculosis infection" by K.S. Beckwith et al. (2020). However, the effect of extracellular bacteria on plasma membrane damage was not addressed before and this paper is addressing an important observation with respect to Mtb evasion and dissemination. These observations represent a novel and interesting aspect in the induction of macrophage cell death by Mtb and potentially relevant for the disease. If the authors consider the comments listed above, this manuscript will be a novel and relevant addition to the field of host pathogen interactions in tuberculosis.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

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Yes

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this work, Toniolo an coworkers use single-cell time-lapse fluorescence microscopy to show that extracellular aggregates of Mycobacterium tuberculosis can evade phagocytosis by killing macrophages in a contact-dependent but uptake-independent manner. The authors further show that this process is dependent on the functionality of the ESX-1 type VII secretion system and the presence of mycobacterial phthiocerol dimycocerosate (PDIM). In essence the authors show that M. tuberculosis can induce macrophage death from the outside of the cell, and dissect the different players that are involved in the process.

Major comments:

I was intrigued by all the different findings of this work, which was done by using bone marrow derived murine macrophages, however, my first question to the authors is how they imagine that this process will take under an in vivo situation ? Do they have evidence that these mycobacterial clumps may form during the initial infection process in the lungs ? It would be important to provide more insights and discussion into this question in order to see how relevant the described details are inside the host organism.

Minor comments:

Line 91: here the authors list the different forms of cell death that is induced by MTB infection, and it would be important to add apoptosis as a reported mechanism as well (References: PMID: 23848406, PMID: 28095608)

Line 95: The secretion of EspE was mainly described in M. marinum while in members of the M. tuberculosis complex no virulence phenotype was reported to the best of my knowledge.

Lines 98: In the cited papers it is described that PDIM is required for phagosomal damage/rupture, however, the methods used there do not allow to specifically report about translocation.
The wording should be adapted.

Line 206: Here it is described that in Figure 3A the BMDMs were expressing tdTomato fluorescence and the bacteria GFP, and the same is also repeated in the Figure legend of Fig3A. However, on the images, BMDMs are shown green and bacterial clumps purple (as also indicated in the description directly on the images) Please check and explain/correct this discrepancy.

Line 304: Here the authors could mention that this finding is similar to results found previously in reference PMID: 28095608 and opposite to the results reported previously in PMID: 28505176.

Line 321: It should be mentioned that CFP10 (EsxB) can also be secreted without its EsxA partner (under certain circumstances , i.e. when the EspACD operon is not expressed due to a phoP regulatory mutation (PMID: 28706226)). However, in Figure S7 an EspAdeletion mutant shows loss of EsxB secretion. This should be checked and discussed how the data here compare with data and strains published previously.
The finding that EspB can substitute the loss of virulence due to loss of EsxA/ESAT-6 secretion is astonishing and also is different to previous observations that strain H37Ra and MTBVAC (two attenuated strains that have no or very little EsxA secretion due to a regulation defect of the espACD operon PMID: 18282096; PMID: 28706226). How does the hypothesis put forward by the authors match with these previously published data ?
In the same context, it is to notice that the authors report in the paragraph between lines 310-330 about EsxA/EsxB secretion, however, looking at the Western blots of figure S7, there is no blot showing results using an antibody against EsxA. Given the previously published results that EsxA/EsxB secretion may also be disconnected (PMID: 28706226), the wording of the text in this paragraph should be adapted or the results from Western Blots using EsxA antibodies be added.

Line 395: Here the authors write that BTP15, a small molecule that in a previous study was shown to inhibit EsxA secretion at higher concentrations (starting from 1.5 uM and higher). However, no effect on the expression of EsxA was described for that compound in reference PMID: 25299337. Thus the corresponding sentence in line 395 needs to adapted to that situation.
Moreover, most concentrations of the compounds used are reported in uM, except for BTP15. It would be easier for the reader if the concentration used for BTP15 could

Also be reported in uM.

Line 475 The comment on the pore forming activity has to be made with caution, as recombinant EsxA produced from E. coli cultures has been shown to often retain detergent PMID: 28119503 that may be responsible for pore forming activity of recombinant EsxA observed in quite some studies, whereas EsxA purified from M. tuberculosis cultures did not show the detergent, but still retained membranolytic activity. This point should be clarified and discussed, and the wording adapted, as EsxA is not a classical poreforming toxin, but excerts the membrane-lysing activity together with other partners (PDIM) in a yet unknown way upon cell contact.

2. Significance:

Significance (Required)

The findings in this work extend the current knowledge on cell infection by M. tuberculosis in a significant way and put extracellular M. tuberculosis clumps in a new context. These data obtained by single-cell time-lapse fluorescence microscopy also need to be discussed for predicting the relevance for an in vivo situation inside the host organism.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

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No

Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

This is an excellent study distinguished by the volume of observations, rigor of analysis and clarity of presentation. The results are novel, biologically interesting and pathophysiologically important. The ability of aggregated M. tuberculosis to kill macrophages has been reported, but the understanding was that proliferation of Mtb within macrophages killed them. Here, the authors observe that macrophages are susceptible to pyroptotic death triggered by contact with extracellular Mtb aggregates, and that this is not recapitulated by contact with a comparable number of Mtb as single bacilli. The authors go some way to tracing the mechanism and uncover a complex inter-dependence on PDIM and on components of the mycobacterial ESX-1 secretory system.

The following comments will helpfully improve the study further.

Major points

The chief measurement in this study is death of individual macrophages as judged by the observer in videomicroscopy. However, the criteria for calling a macrophage "dead" are not defined with any morphological detail, beyond noting that the cell stops moving and lyses. Of course a cell will stop moving if it has lysed, but do not some if not most cells stop moving before they lyse? If so, lysis alone would seem to be the time-point marker for cell death. Yet from the images in Fig 1E and F, I cannot tell that the cells called "dead" have lysed. Watching the videos, the time of lysis is not clear to me. Eventually, shrunken cell bodies are obvious but it is not clear if these are residua of cells that had been said to "lyse" at an earlier time.

The use of BTP15 as a specific inhibitor of ESX-1 is problematic. The source of the compound is not stated. The concentration used, 20 mg/mL, is well above the reported IC50 (1.2 uM) for its presumed target, a mycobacterial histidine kinase, and above the concentrations (0.3-0.6 uM) reported to inhibit Mtb's secretion of EsxA almost completely. It is concerning that the concentrations that were reported to work so well

on the whole cell are lower than the IC50 for the presumed target, because uptake into Mtb and intrabacterial metabolism will typically lead to a lower potency for an inhibitor against the whole bacterium than against the isolated enzyme; and because 50% inhibition of an enzyme rarely gives a functional effect as complete as what is shown in the cited reference. In other words, it is not clear that the histidine kinase is the functionally relevant target of BTP15 in Mtb. The original report did not consider BTP15's possible effect on mammalian cells and the present authors likewise do not take that into consideration with respect to possible effects on the macrophages. No concentration-response or time course experiments with BTP15 are presented. Most important, unless I missed it, there is apparently no demonstration that the compound inhibited ESX-1-dependent secretion in the present authors' hands, no matter by what mechanism. Without this, I am reluctant to accept that the results with BTP15 demonstrate a dependence of extracellular-aggregate-induced macrophage death on ESX-1-mediated secretion from Mtb. I would recommend that the authors either provide a direct demonstration of BTP15's effect on ESX-1 dependent secretion at concentrations near those that worked on whole cells in the original report, or drop the BTP15 studies from the paper. That said, the genetic experiments remain unequivocal, so the paper's conclusions would not be affected.

The experiments, or at least the discussion, could consider what may distinguish single Mtb cells from aggregated Mtb in some way relevant to the present observations. The authors seem to assume that all the Mtb cells in their preparations are biochemically equivalent and that their distribution into single-cell or aggregate subpopulations is stochastic. What if it is deterministic instead? For example, what if these two subpopulations are defined by differential expression of PDIM, so that the greater macrophage-killing effect of aggregates than single cells in equivalent numbers reflects a greater amount of PDIM in the aggregates, rather than some sort of valency-of-contact effect? The authors could compare the PDIM-to-DNA ratio in the single cell and aggregated subpopulations, or at least discuss this possibility.

Minor points

Some of the experiments compare "low", "medium" and "high" numbers of Mtb, but I could not find a definition of these numbers.

There seem to be no positive or negative controls for any of the antibodies used for cell staining (anti-cleaved caspase 1, antiphospho RIP3, anti-phospho MLKKL).

2. Significance:

Significance (Required)

The results are novel, biologically interesting and pathophysiologically important.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

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Full Revision

Manuscript number: RC-2022-01654
Corresponding author(s): Chiara, Toniolo

1. General Statements (optional)

We thank the reviewers for their thorough and insightful evaluations of our manuscript and for their constructive feedback, which have significantly improved the quality of our manuscript. We were pleased to read that all three reviewers found our work novel, interesting, and relevant. In this revised manuscript, we have done our best to address all of the points raised by the reviewers by performing new experiments and revising sections of the text, as requested.

2. Point-by-point description of the revisions

This section is mandatory. Please insert a point-by-point reply describing the revisions that were already carried out and included in the transferred manuscript.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In this manuscript authors show that extracellular Mtb aggregates can cause macrophage killing in a close contact dependent but phagocytosis independent manner. They showed Mtb aggregates can induce plasma membrane perturbations and cytoplasmic Ca2+ influx with live cell microscopy. Next, the authors show that the type of cell death initiated by extracellular aggregates is pyroptosis and they partially supressed cell death with pyroptosis inhibitors. They also identified that PDIM, EsxA/EsxB and EspB all have a role in uptake-independent killing of macrophages even though their impact varies with respect membrane perturbation and Ca2+ influx. Finally, they used a small molecule inhibitor BTP15 to inhibit the effect of ESX-1 during the contact of the extracellular Mtb aggregates with the macrophages and they observed a substantial decrease in membrane perturbation and macrophage killing.
The work describes a very interesting mechanism by which Mtb can kill macrophages that is possibly relevant in the context of infection.

1. In general, there are two main issues with the experiments and the interpretation: the lack of quantitative analysis showing that in a population of macrophages the ones that are in contact with the aggregates die whereas the ones that are not in contact remain alive. This is currently not shown, and it should be added in figure 1.

All our data are based on the visual inspection and annotation of time-lapse microscopy image series, from which it is conclusive that death happens more often among cells in contact with *Mtb* aggregates (see movies S3 and S6 for representative examples). However, we acknowledge the reviewer's suggestion that quantitative data supporting this observation might

help to convey this conclusion more effectively. Therefore, we have quantified the percentage of dead cells in: I) macrophages in uninfected controls; II) macrophages that establish contact with an *Mtb* aggregate; III) bystander macrophages that never contact an *Mtb* aggregate despite being in the same sample as the infected cells, in experiments with (figure 1D) or without (figure 1Q) cytochalasin D treatment. These data have been incorporated as two additional plots in figure 1 in the revised manuscript. We find that uninfected and bystander cells have similar survival probabilities over the time-course of an experiment, whereas most of the cells that physically interact with *Mtb* aggregates die by the end of the experiment. To further validate these observations, we have also plotted the lifespans of infected cells vs. bystander cells without (figure S3A) and with (figure S3B) cytochalasin D treatment. In these plots, the lifespan of an individual cell is represented by a line; the fraction of the line coloured in black corresponds to the time spent as bystander and the fraction of the line in magenta corresponds to the time spent in contact with an *Mtb* aggregate. We hope that these new data convincingly show that bystander cells (black lines) survive longer compared to cells that interact with *Mtb* aggregates (black-magenta lines).

2. The second is the cell death mode, as the markers used are very different and considering different outcomes (e.g., apoptosis vs. necrosis) are relevant for the infection it is unclear what is being measured here and the impact on bacterial replication.

As the reviewer points out, it has previously been shown that different cell death pathways can affect viability and propagation of intracellular bacteria (1, 2). Since in our experiments we are specifically analyzing extracellular bacteria, we cannot directly comment on how cell death affects intracellular bacterial replication. However, to address the reviewer's comment, we have included additional data in figure S13A of the revised manuscript showing that specific inhibitors of cell death do not affect the growth or replication of extracellular *Mtb*. These results suggest that while these molecules do not affect *Mtb* growth per se, the suppression of these specific death pathways also does not significantly affect the microenvironment to alter *Mtb* growth (i.e., access to nutrients or molecules released by dead cells). In addition, we have included new data in figure S12 demonstrating the responsiveness of our isolated macrophages to the various cocktails of molecules typically used to induce apoptosis, pyroptosis, or necroptosis.

The authors are showing that infection with Mtb aggregates increase the rate of the macrophage killing but how does this impact infection dissemination and replication of the bacterial aggregates? Is it beneficial for the aggregates? Did the authors check the growth rate of Mtb along with cytochalasin D?

A previous study has shown that phagocytosis of *Mtb* aggregates leads to macrophage death more efficiently than phagocytosis of a similar number of individual bacteria (3). It has also been shown that *Mtb* growing on the debris of dead host macrophages forms cytotoxic aggregates that kill the newly interacting macrophages (3). These observations suggest a model in which host cell death induced by *Mtb* aggregates supports faster extracellular growth and propagation of infection (3). This study was cited in the Introduction section of our

manuscript, and our data support these observations. In the revised manuscript, we show that single *Mtb* bacilli or *Mtb* aggregates induce macrophage death in a dose-dependent manner (figure S7A,B); however, bacterial aggregates kill more efficiently when compared to similar numbers of non-aggregated bacilli (figure S7A,B). We also show that infection with *Mtb* aggregates leads to faster bacterial propagation compared to infection with similar numbers of individual bacteria (figure S7C,D). These observations, combined with our data showing that *Mtb* aggregation also enhances uptake-**independent** killing of macrophages (figure 2), suggest that *Mtb* aggregates induce rapid host cell death, allowing the bacteria to escape intracellular stresses, grow faster outside host cells (figure S1B), and propagate to other cells. To address the reviewer's concern whether cytochalasin D affects *Mtb* growth, the revised manuscript includes additional data confirming that cytochalasin D does not affect the growth of *Mtb* aggregates (figure S6).

3. How did the authors quantify the interactions of Mtb with macrophages in Figure 1D?

The interactions of *Mtb* with macrophages were quantified through manual annotation of the time-lapse microscopy image series. If the *Mtb* aggregates disaggregated upon interaction with the macrophage, resulting in redistribution of smaller aggregates of bacteria, we categorized them as "fragmented". On the other hand, if the aggregates remained clustered, we categorized them as "not fragmented". Representative snapshots of these two patterns are presented in figure 1E and 1F and we have included additional representative examples in movies S4 and S5 of the revised manuscript. These interactions are quantified and plotted in figure 1N of the revised manuscript (figure 1D in the original version).

4. Is it enough to conclude with one example of SEM that the mycobacteria with different fragmentation discriminates if the bacteria is intracellular or extracellularly localised? Can authors use an alternative quantitative method to confirm the localization of the bacteria by a quantification by 3D imaging of these two phenotypes with a cytoskeleton marker (or may be even with tdTomato-expressing BMDMs)?

In the revised manuscript, we provide additional examples of correlative time-lapse microscopy and SEM images (supplementary figure S5). As suggested by the reviewer, in the revised manuscript we further validate these conclusions using an alternative approach based on correlative time-lapse microscopy followed by confocal 3D imaging. After time-lapse imaging, we fixed the samples and labelled the plasma membrane of the macrophages with a fluorescent anti-CD45 antibody to define the cell boundaries and identify bacteria that are intracellular vs. extracellular. Representative images obtained using this approach have been added to figure 1 and additional examples are shown in supplementary figure S4 of the revised manuscript. The acquisition, processing, and analysis of these 3D images are time-consuming and prevent us from performing an exhaustive quantitative analysis. However, we are confident in our conclusions, since in all of the cells that we analyzed we found that aggregates that are not fragmented within 6 hours of stable interaction with macrophages are visible on the outer side of the plasma membrane.

5. How do we know if the cell is lysed at 30 h in Supplementary Figure 1, did the authors use a marker to detect the cell lysis or is it based on just the observation from the live cell imaging? Movies in supplementary are actually not very informative as there are many ongoing events and it is hard to visualise what the authors claim. A marker of cell death in the movies should be used.

In this study, we used brightfield time-lapse microscopy images to identify cell death. Dying macrophages rapidly change shape, lose membrane integrity, and stop moving. Moreover, the intracellular structures and bacteria also stop moving at the time of death of the host cell. While these events can be difficult to distinguish by examining individual snapshots, they are readily identifiable by careful frame-by-frame examination of time-lapse microscopy image series. To exemplify this process, in the revised manuscript we show in supplementary figure S2A how we identify macrophage cell death events. We also include Draq7 (a live cell-impermeable dye commonly used to identify dead cells by flow cytometry and microscopy) in the growth medium during time-lapse imaging in order to label dead macrophages. The timing of staining validates and confirms our strategy of using brightfield time-lapse images to define the time-of-death of individual cells. To further assist readers, in the revised manuscript we provide the time-lapse microscopy movie used to generate this figure (movie S4). Similar images and movies have also been added for cells treated with cytochalasin D (figure S2B; movie S7). As suggested by the reviewer, we also replaced figure S1A with a new figure that shows a representative example of an *Mtb* intracellular microcolony that, upon death of the host macrophage, grows and forms a large extracellular aggregate on the debris of the dead cell (Draq7-positive). Movie S2 was used to generate this figure. Finally, we replaced figures 1E,F with new figures incorporating the Draq7 staining to label macrophage cell death and we include the time-lapse microscopy movies used to generate these figures (movies S4, S5).

6. Total macrophage killing after contact in Figure 1L is around 12 hours, whereas it is observed that the macrophage death after contact with cytochalasin D treatment in Figure 1M is even longer than 24 hours. The viability at 12 hours in Figure1M is as fragmented Mtb survival in Figure1L, why there is a difference in timing with respect to macrophage killing?

We thank the reviewer for this interesting observation. Indeed, we find that macrophages treated with cytochalasin D do take longer to die upon establishing stable interaction with *Mtb* aggregates in comparison to untreated cells. Although we do not have a clear explanation for this difference in timing, we speculate that by inhibiting actin polymerization and consequently cell motility, cytochalasin D might slow the expansion of the macrophage plasma membrane and the establishment of a larger interface of contact between the cell and the bacterial aggregate, which could influence the timing of cell death.

7. Did authors perform statistical tests for Figure 1D and Figure 1N? p-values should be added.

Figure 1D (figure 1N in the revised manuscript) shows the percentage of interactions between macrophages and *Mtb* aggregates that do or do not lead to fragmentation of the aggregate. Each dot represents the percentage of these events in one experimental replicate. We included this plot to show that reproducibly in all our replicates approximately 20% of the interactions do not lead to fragmentation of the aggregate. Since the purpose of this plot is not to compare the "fragmented" and "non-fragmented" populations but rather to highlight the reproducibility of the phenomenon, we do not think it would be appropriate to add a p-value. However, figure 1N (figure 1Q in the revised manuscript) has been updated and modified to include statistical analysis and a p-value.

8. In Figure 3, do the observations indicated in the Figure 3 happen in all the macrophages that are in contact with aggregates? This is unclear and critical to support the conclusions. Do all the macrophages that are in contact with Mtb aggregates become Annexin-V positive? In Supplementary Figure 2 there is some information regarding this question, but it will be important to show it as a percentage.

In response to the reviewer's suggestion, we have modified the figure to include quantitation of Annexin-V staining. Approximately 75% of the macrophages that interact with an *Mtb* aggregate show detectable local Annexin V-positive membrane domains at the site of contact with the aggregate during a typical 60 hour-long experiment. Since most of the macrophages show local Annexin V-positive membrane domains within the first 12 hours upon contact with an *Mtb* aggregate (figure 3C), we used this criterion for comparison of different conditions or strains (for example, those shown in figure 6F). In addition, we added figure 3D, which shows the behaviour of 105 macrophages upon contact with *Mtb* aggregates in a typical experiment. In this plot, each line represents the lifespan of an individual cell; the fraction of the line in black represents the time spent as bystander, the fraction of the line in magenta represents the time spent interacting with an *Mtb* aggregate, and the fraction in green represents the time upon formation of local Annexin V-positive membrane domains at the site of contact with the *Mtb* aggregate. We believe that this additional information further supports our conclusions that most of the cells in contact with an *Mtb* aggregate show local Annexin V-positive membrane domains and that cells that show this pattern die faster than cells that do not develop local Annexin V-positive membrane domains.

9. Did the authors try to stain Mtb aggregates alone with Annexin-V as a control over the duration of the imaging?

We thank the reviewer for suggesting this control. In supplementary figure S8C of the revised manuscript, we include a representative example of a time-lapse microscopy image series showing *Mtb* aggregates that never interact with a live macrophage althought they are adjacent to a dead cell. As observed in the Annexin V fluorescence images (yellow), these *Mtb* aggregates never become Annexin-V positive during the course of the experiment (60 hours).

10. In Figure 4, did the authors continue to image the cells interacting with Mtb aggregates that do not die after Ca2+ accumulation in Supplementary Figure 3D? Do these cells recover from the plasma membrane perturbation? Did the authors consider using another marker for plasma membrane perturbation together with BAPTA?

Unfortunately, we are not able to image macrophages stained with Oregon Green 488 Bapta-1 AM for more than 36 hours because they lose fluorescence over time, possibly due to partial dye degradation or secretion. Another issue is that macrophages do not establish synchronous interaction with *Mtb* aggregates (figure 3D; figure S3B). In order to pool together results from many cells, we analyze all the cells that interact with *Mtb* within the first 20 hours and we define as timepoint 0 the time at which each individual cell establishes interaction with the bacteria. To compare similar time windows for each cell, we use fluorescence values measured at 16 hours post-interaction with bacteria as a readout. This time window is sufficient to observe formation of local Annexin V plasma membrane domains and death in a relevant number of macrophages (figure 1P; figure 3D). Not all of the contacted cells die within the timeframe of our experiments; however, we believe that if we imaged cells that accumulate Ca^{2+} for longer durations, we would find that all such cells eventually die. This assumption is consistent with the observation that calcium chelation reduces inflammasome activation and death in macrophages in contact with *Mtb* aggregates (figure 5D; figure 4E).

With respect to the reviewer's query whether cells recover from plasma membrane perturbation, in our time-lapse microscopy experiments, we observe that when macrophages form local Annexin V-positive plasma membrane domains at the site of contact with *Mtb* aggregates, they never revert to an Annexin V-negative status afterwards (figure 3D; movie S7; movie S8). Our SEM data show that *Mtb* aggregates colocalizing with Annexin V-positive domains are not partially covered by intact membrane, in contrast to those associated with Annexin V-negative macrophages, although they do present vesicles and membrane debris on their surface (figure 3F,G). In the revised manuscript, we include additional fluorescence microscopy images showing that Annexin V-positive foci colocalize with markers for the macrophages' plasma membrane (figure S8A,B) as well as with more distal areas of the bacterial aggregates, where we do not observe any positive plasma membrane staining (figure S8B). Similarly, although *Mtb* aggregates that are never in contact with macrophages never become Annexin V-positive (figure S8C), we see that upon macrophage death, aggregates in contact with dead cells retain some Annexin V-positive material on their surface (figure S8C; movie S8). Vesicle budding and shedding is a common ESCRT III-mediated membrane repair strategy that allows removal of damaged portions of the plasma membrane and wound resealing (4). Thus, we think that in our experiments the Annexin V-positive foci might represent both damaged membrane areas and released macrophage plasma membrane vesicles that stick to the hydrophobic surface of the bacterial aggregates. This means that the time of appearance of Annexin V-positive domains marks the time when the macrophage membrane experiences a damaging event. Interestingly, we do not observe a gradual increase in fluorescence intensity of the Annexin V-positive domains, but rather multiple single intensity peaks over time (movie S8). This might suggest that multiple discrete damaging events occur over time.

11. In Figure 5D-G it will be important if the authors include dots for each macrophage events for the contact conditions as well, as it was done for the bystander condition.

We apologize for using a too-pale shade of magenta in the earlier version of the manuscript, which apparently made the dots in these figures hard to visualize. In the revised manuscript, we use a darker shade of magenta to show the dots corresponding to the fluorescence values of the macrophages in contact with *Mtb* aggregates.

12. How did the authors discriminate between the macrophages that are in contact or not with Mtb aggregates after the staining with Casp-1, pRIP3 and pMLKL? Do the aggregates stay in contact even after the staining procedures? Representative images of the labelling should be included in this figure.

Before fixation, we make sure to remove the medium gently to avoid disrupting the interactions between cells and bacteria. This step most likely removes the floating bacterial aggregates that are not in stable contact with cells but apparently does not detach aggregates that stably interact with cells. Our correlative time-lapse microscopy and immunofluorescence images (figure 1; figure S4), as well as our correlative time-lapse microscopy and SEM images (figure S5; figure 3F,G), confirm that *Mtb* aggregates that interact with cells during time-lapse imaging are retained on the surface of those cells upon fixation and processing for immunofluorescence or electron microscopy. As we can observe in figure 5B (cell indicated by the white arrow), *Mtb* aggregates are retained on the debris of dead cells. In figure 5 we distinguish between “in contact” macrophages and “bystander” macrophages by inspecting brightfield images showing the cells and the respective fluorescence images corresponding to the bacteria. If the body of a macrophage identified in the brightfield image overlaps with a bacterial aggregate identified in the fluorescence channel, we define the macrophage as “in contact”; otherwise, it is annotated as “bystander”. We provide representative images in figure S12 and we clarify the definition of “in contact” and “bystander” in the figure legend of figure 5.

13. The labelling of Figure 5H needs to be corrected both in the text and in the figure legend.

We thank the reviewer for bringing our attention to this error, which has been corrected in the revised manuscript.

14. Pyroptosis inhibitors did reduce the percentage of cell death, but did it also reduce the number of Annexin-V positive domains? This is important as AnnexinV is a marker of apoptosis and the outcome for Mtb very different.

As pointed out by the reviewer, Annexin V staining is often used as a marker for apoptosis. Typically, apoptotic cells stain positive for Annexin V but negative for other membrane-impermeable markers such as propidium iodide, because they expose phosphatidylserine (bound by Annexin V) on the outer leaflet of the plasma membrane without losing plasma

membrane integrity (5). Apoptotic cells often look round and their plasma membrane is stained homogeneously by fluorescently labelled Annexin V (5). In our experiments, we observe that macrophages in contact with *Mtb* aggregates become Annexin V-positive; however, this happens only at the site of contact with the bacteria (figure 3A; movie S7). Only when cells die and get stained by membrane-impermeable dies such as Draq7 do they also get stained with Annexin V over the entire membrane debris. We thus use Annexin V staining as a marker for membrane perturbation rather than for cell death. If we were using the Annexin V as a marker for cell death, we would expect to see a reduction in Annexin V-positive cells in samples treated with pyroptosis inhibitors. In these samples, we do observe a reduced percentage of cell death in comparison to untreated controls; however, we still observe a comparable percentage of macrophages that stain positive for Annexin V locally, i.e., at the site of contact with bacterial aggregates (supplementary figure S13B). In line with this observation, treated vs. untreated macrophages in contact with *Mtb* aggregates accumulate similar levels of intracellular calcium. These observations are consistent with our model suggesting that contact with *Mtb* aggregates induces membrane perturbation, calcium accumulation, inflammasome activation, and pyroptosis in contacted macrophages. Since the death inhibitors used in our study specifically target pyroptosis effectors, we do not expect them to affect upstream events such as membrane perturbation and calcium accumulation.

15. In Figure 6, The sections for Figure 6 are well described but kept relatively long with too many details, it will be helpful to the reader if the authors can combine the sections in one header.

We agree that the text linked to figure 6 is long. We tried to make these sections as concise as possible; however, we are concerned that combining all of the sections under a single header might be at the expense of clarity. Thus, unless the reviewer objects, we would prefer to maintain the use of multiple headers.

16. Figure 6F does not have a statistical test and p-value, it will be important to include the statistical test in the legend and p-values in the figure.

As recommended by the reviewer, we have analyzed the results in figure 6F by using a one-way ANOVA test and we have added the calculated p-values to the figure.

Reviewer #1 (Significance (Required)):

Based on the literature, Mtb infection and replication can trigger different types of cell death and most of the studies have addressed cell death only as an outcome of intracellular replication. This study shows another form of host cell death, associated only to extracellular bacterial aggregates that are in contact with macrophages. Plasma membrane damage initiating pyroptosis has been defined in: "Plasma membrane damage causes NLRP3 activation and pyroptosis during Mycobacterium tuberculosis infection" by K.S. Beckwith et al. (2020). However, the effect of extracellular bacteria on plasma membrane damage was not addressed

before and this paper is addressing an important observation with respect to Mtb evasion and dissemination. These observations represent a novel and interesting aspect in the induction of macrophage cell death by Mtb and potentially relevant for the disease. If the authors consider the comments listed above, this manuscript will be a novel and relevant addition to the field of host pathogen interactions in tuberculosis.

We thank the reviewer for their perspective and their positive comments about our work.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

In this work, Toniolo and coworkers use single-cell time-lapse fluorescence microscopy to show that extracellular aggregates of Mycobacterium tuberculosis can evade phagocytosis by killing macrophages in a contact-dependent but uptake-independent manner. The authors further show that this process is dependent on the functionality of the ESX-1 type VII secretion system and the presence of mycobacterial phthiocerol dimycocerosate (PDIM). In essence the authors show that M. tuberculosis can induce macrophage death from the outside of the cell, and dissect the different players that are involved in the process.

Major comments:

1. I was intrigued by all the different findings of this work, which was done by using bone marrow derived murine macrophages, however, my first question to the authors is how they imagine that this process will take under an in vivo situation? Do they have evidence that these mycobacterial clumps may form during the initial infection process in the lungs? It would be important to provide more insights and discussion into this question in order to see how relevant the described details are inside the host organism.

Formation of *Mtb* aggregates in tuberculosis lesions have been documented in several animal models (6, 7) and in humans (8–11). While it is unclear whether mycobacterial aggregates form during the earliest stages of infection, extracellular bacterial aggregates have been observed in animal models of infection within the first month post-infection, and they are often associated with necrotic foci. Moreover, masses of *Mtb* growing as pellicle-like aggregates are often observed on the surface of cavities in human tuberculosis patients. These observations confirm that *Mtb* aggregates can form during a tuberculosis infection and that a significant fraction of bacteria are extracellular during different stages of infection. As we observe that macrophages undergo contact-dependent uptake-independent death also in the absence of cytochalasin D *in vitro*, we assume that this may also happen *in vivo* when *Mtb* aggregates are formed or released outside host cells. This process may promote bacterial propagation at early stages of infection as well as at later stages when necrotic granulomas and cavities are formed.

In the revised manuscript we present and discuss our observations in the context of the *in vivo* phenotypes reported in the scientific literature and we include additional references showing

that extracellular *Mtb* aggregates are often observed *in vivo*. We also propose this concept already in the Introduction section to better link our observations to possible *in vivo* scenarios.

Minor comments:

Line 91: here the authors list the different forms of cell death that is induced by MTB infection, and it would be important to add apoptosis as a reported mechanism as well (References: PMID: 23848406, PMID: 28095608)

As suggested by the reviewer, in the revised manuscript we have modified the Introduction section to include apoptosis as a *Mtb*-induced mechanism of macrophage death and we have cited the two publications recommended by the reviewer.

2. Line 95: The secretion of EspE was mainly described in M. marinum while in members of the M. tuberculosis complex no virulence phenotype was reported to the best of my knowledge.

In agreement with the reviewer's comment, we have modified the sentence and removed EspE from the list of virulence factors.

3. Lines 98: In the cited papers it is described that PDIM is required for phagosomal damage/rupture, however, the methods used there do not allow to specifically report about translocation. The wording should be adapted.

We thank the reviewer for this insightful comment and we have modified the text accordingly.

4. Line 206: Here it is described that in Figure 3A the BMDMs were expressing tdTomato fluorescence and the bacteria GFP, and the same is also repeated in the Figure legend of Fig3A. However, on the images, BMDMs are shown green and bacterial clumps purple (as also indicated in the description directly on the images) Please check and explain/correct this discrepancy.

We apologize that the color scheme in figure 3A is confusing. In this figure we used tdTomato-expressing BMDMs and GFP-expressing bacteria; however, we have pseudo-colored the fluorescence images for the sake of consistency with the other figures in the manuscript, which always show bacteria in magenta. We have clarified this point in the figure legend of the revised manuscript.

5. Line 304: Here the authors could mention that this finding is similar to results found previously in reference PMID: 28095608 and opposite to the results reported previously in PMID: 28505176.

Full Revision

As recommended by the reviewer, we have added a sentence comparing our results with previous studies and we have cited the two references suggested by the reviewer.

6. Line 321: It should be mentioned that CFP10 (EsxB) can also be secreted without its EsxA partner (under certain circumstances, i.e. when the EspACD operon is not expressed due to a phoP regulatory mutation (PMID: 28706226)). However, in Figure S7 an EspAdeletion mutant shows loss of EsxB secretion. This should be checked and discussed how the data here compare with data and strains published previously.

We thank the reviewer for pointing out this interesting point. Our proteomics data revealed that both our *esxA* mutant and our *espA* mutants abolish secretion of both EsxA and EsxB, in line with previously published data (12–14). We do not know why the *espA* mutant behaves differently from the MTBVAC strain concerning secretion of EsxA and EsxB (although we note that regulatory mutations may have complex pleiotropic effects). In the revised manuscript, we have modified this section to include references highlighting that secretion of these proteins may be uncoupled in some circumstances.

7. The finding that EspB can substitute the loss of virulence due to loss of EsxA/ESAT-6 secretion is astonishing and also is different to previous observations that strain H37Ra and MTBVAC (two attenuated strains that have no or very little EsxA secretion due to a regulation defect of the espACD operon PMID: 18282096; PMID: 28706226). How does the hypothesis put forward by the authors match with these previously published data ?

We thank the reviewer for this interesting comment. We would like to clarify that we are not claiming that EspB and EsxA are in general redundant and that EspB can substitute EsxA as a virulence factor. In our experiments we show that EspB can induce contact-dependent uptake-independent death in macrophages in contact with *Mtb* aggregates *in vitro* even in the absence of EsxA; however, the precise role of EspB during infection in mice or humans remains to be elucidated and is outside the scope of this manuscript. A previous study comparing *Mtb* ESX-1 mutants with different secretion patterns linked EspB secretion to *Mtb* virulence *in vivo* (14); however, the behavior of an isogenic *espB* deletion strain *in vivo* was not reported. A *M. marinum espB* mutant was shown to have reduced virulence; however, in contrast to *Mtb*, deletion of *espB* also affects secretion of EsxA in this organism (15). As the reviewer points out, the *Mtb* strains H37Ra and MTBVAC do not secrete EsxA due to a mutated *phoP* gene. Previous literature has shown that *espB* expression is also dependent on PhoP (16). We thus speculate that these strains might behave similarly to our *espA espB* mutant strain in the context of contact-dependent uptake-independent induction of macrophage death, although we think that this point is outside the scope of our manuscript.

8. In the same context, it is to notice that the authors report in the paragraph between lines 310-330 about EsxA/EsxB secretion, however, looking at the Western blots of figure S7, there is no blot showing results using an antibody against EsxA. Given the previously published results that EsxA/EsxB secretion may also be disconnected (PMID: 28706226),

the wording of the text in this paragraph should be adapted or the results from Western Blots using EsxA antibodies be added.

We agree with the reviewer's comment. Unfortunately, we currently do not have access to a good antibody for EsxA. A commercial monoclonal antibody that was previously available for immunoblotting has been discontinued. We tried several other antibodies that were previously shown to work in *M. marinum*, but none of these antibodies were effective in *M. tuberculosis*. We agree that analysing secretion of EsxB alone might not be sufficient to support claims about EsxA secretion. For this reason, we performed quantitative proteome analysis of the secretome in all of the relevant mutant strains. In our revised manuscript, we are careful to make sure that whenever we refer to EsxA/EsxB secretion we always provide proteomics data to support our conclusions.

9. Line 395: Here the authors write that BTP15, a small molecule that in a previous study was shown to inhibit EsxA secretion at higher concentrations (starting from 1.5 uM and higher). However, no effect on the expression of EsxA was described for that compound in reference PMID: 25299337. Thus the corresponding sentence in line 395 needs to adapted to that situation.

We thank the reviewer for noticing this error, which we have corrected in the revised manuscript.

10. Moreover, most concentrations of the compounds used are reported in uM, except for BTP15. It would be easier for the reader if the concentration used for BTP15 could also be reported in uM.

As suggested by the reviewer, in the revised manuscript we report the concentration of BTP15 in µM.

11. Line 475 The comment on the pore forming activity has to be made with caution, as recombinant EsxA produced from E. coli cultures has been shown to often retain detergent PMID: 28119503 that may be responsible for pore forming activity of recombinant EsxA observed in quite some studies, whereas EsxA purified from M. tuberculosis cultures did not show the detergent, but still retained membranolytic activity. This point should be clarified and discussed, and the wording adapted, as EsxA is not a classical poreforming toxin, but excerts the membrane-lysing activity together with other partners (PDIM) in a yet unknown way upon cell contact.

We thank the reviewer for this comment. In the revised manuscript, we have modified the text accordingly and included the sugggested reference.

Reviewer #2 (Significance (Required)):

Full Revision

The findings in this work extend the current knowledge on cell infection by M. tuberculosis in a significant way and put extracellular M. tuberculosis clumps in a new context. These data obtained by single-cell time-lapse fluorescence microscopy also need to be discussed for predicting the relevance for an in vivo situation inside the host organism.

As suggested by the reviewer, in the revised manuscript we discuss additional examples from the literature showing that *Mtb* aggregates can form during infection and that many bacteria are extracellular and associated with necrotic foci during different stages of the disease in animal models of infection and in human patients. We believe that these previously published observations support the *in vivo* relevance of the process we observe *in vitro*.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

This is an excellent study distinguished by the volume of observations, rigor of analysis and clarity of presentation. The results are novel, biologically interesting and pathophysiologically important. The ability of aggregated M. tuberculosis to kill macrophages has been reported, but the understanding was that proliferation of Mtb within macrophages killed them. Here, the authors observe that macrophages are susceptible to pyroptotic death triggered by contact with extracellular Mtb aggregates, and that this is not recapitulated by contact with a comparable number of Mtb as single bacilli. The authors go some way to tracing the mechanism and uncover a complex inter-dependence on PDIM and on components of the mycobacterial ESX-1 secretory system.

The following comments will helpfully improve the study further.

Major points

1. The chief measurement in this study is death of individual macrophages as judged by the observer in videomicroscopy. However, the criteria for calling a macrophage "dead" are not defined with any morphological detail, beyond noting that the cell stops moving and lyses. Of course a cell will stop moving if it has lysed, but do not some if not most cells stop moving before they lyse? If so, lysis alone would seem to be the time-point marker for cell death. Yet from the images in Fig 1E and F, I cannot tell that the cells called "dead" have lysed. Watching the videos, the time of lysis is not clear to me. Eventually, shrunken cell bodies are obvious but it is not clear if these are residua of cells that had been said to "lyse" at an earlier time.

In this study, we used brightfield time-lapse microscopy images to identify cell death. Dying macrophages rapidly change shape, lose membrane integrity, and stop moving. Moreover, the intracellular structures and bacteria also stop moving at the time of death of the host cell. While these events can be difficult to distinguish by examining individual snapshots, they are readily identifiable by careful frame-by-frame examination of time-lapse microscopy image series. To exemplify this process, in the revised manuscript we show in supplementary figure S2A how we

identify macrophage cell death events. We also include Draq7 (a live cell-impermeable dye commonly used to identify dead cells by flow cytometry and microscopy) in the growth medium during time-lapse imaging in order to label dead macrophages. The timing of staining validates and confirms our strategy of using brightfield time-lapse images to define the time-of-death of individual cells. To further assist readers, in the revised manuscript we provide the time-lapse microscopy movie used to generate this figure (movie S4). Similar images and movies have also been added for cells treated with cytochalasin D (figure S2B; movie S7). As suggested by the reviewer, we also replaced figures 1E,F with new figures incorporating the Draq7 staining to label macrophage cell death and we include the time-lapse microscopy movies used to generate these figures (movies S4, S5).

2. The use of BTP15 as a specific inhibitor of ESX-1 is problematic. The source of the compound is not stated.

The BTP15 molecule was kindly provided by Prof. Stewart Cole, the corresponding author of the article describing the identification of this compound and its effect on Esx-1 secretion (17). We have included this information in the Materials and Methods section.

3. The concentration used, 20 ug/mL, is well above the reported IC50 (1.2 uM) for its presumed target, a mycobacterial histidine kinase, and above the concentrations (0.3-0.6 uM) reported to inhibit Mtb's secretion of EsxA almost completely. It is concerning that the concentrations that were reported to work so well on the whole cell are lower than the IC50 for the presumed target, because uptake into Mtb and intrabacterial metabolism will typically lead to a lower potency for an inhibitor against the whole bacterium than against the isolated enzyme; and because 50% inhibition of an enzyme rarely gives a functional effect as complete as what is shown in the cited reference. In other words, it is not clear that the histidine kinase is the functionally relevant target of BTP15 in Mtb. The original report did not consider BTP15's possible effect on mammalian cells and the present authors likewise do not take that into consideration with respect to possible effects on the macrophages. No concentration-response or time course experiments with BTP15 are presented. Most important, unless I missed it, there is apparently no demonstration that the compound inhibited ESX-1-dependent secretion in the present authors' hands, no matter by what mechanism. Without this, I am reluctant to accept that the results with BTP15 demonstrate a dependence of extracellular-aggregate-induced macrophage death on ESX-1-mediated secretion from Mtb. I would recommend that the authors either provide a direct demonstration of BTP15's effect on ESX-1 dependent secretion at concentrations near those that worked on whole cells in the original report, or drop the BTP15 studies from the paper. That said, the genetic experiments remain unequivocal, so the paper's conclusions would not be affected.

We agree with the reviewer that in the original version of our manuscript we did not provide direct evidence demonstrating that BTP15 inhibits ESX-1 secretion and that it does not affect the host cells. We addressed the first issue by quantifying (by Western blot) the secretion of

EsxB and EspB in *Mtb* cultures treated with different concentrations of BTP15. We show that BTP15 reduces secretion of these two proteins in a dose-dependent manner. These data have been included in figures S21A-B of the revised manuscript. In line with this observation, we also show that BTP15 reduces uptake-independent killing of macrophages by *Mtb* aggregates in a dose-dependent manner (figure 6H). To show that the dose-dependent effect observed in macrophages does not depend on a direct effect of BTP15 on the host cells, we treated *Mtb* with different concentrations of BTP15 for 48 hours and removed the compound by washing the bacteria prior to infection. We observe that *Mtb* aggregates that have been treated with BTP15 show reduced uptake-independent killing of macrophages, even when bacteria have been pre-treated and the small molecule is not present during the incubation with the cells (figure S21C). We hope that these additional results provide clear evidence that BTP15 reduces *Mtb*-mediated contact-dependent uptake-independent killing of macrophages by inhibiting ESX-1 secretion, consistent with our genetic data. We think these results are important because they provide a chemical validation of our genetic data. To the best of our knowledge, BTP15 is the only available compound known to inhibit ESX-1 secretion, and in the revised manuscript we confirm that this compound has the previously described effect on *Mtb* also in our hands. Unfortunately, we had to use concentrations higher than those previously reported to inhibit ESX-1 secretion in order to achieve the observed effects. As we had access only to prediluted aliquots that had been stored for a long time, we cannot rule out the posibility that the compound might have undergone partial degradation during storage.

4. The experiments, or at least the discussion, could consider what may distinguish single Mtb cells from aggregated Mtb in some way relevant to the present observations. The authors seem to assume that all the Mtb cells in their preparations are biochemically equivalent and that their distribution into single-cell or aggregate subpopulations is stochastic. What if it is deterministic instead? For example, what if these two subpopulations are defined by differential expression of PDIM, so that the greater macrophage-killing effect of aggregates than single cells in equivalent numbers reflects a greater amount of PDIM in the aggregates, rather than some sort of valency-of-contact effect? The authors could compare the PDIM-to-DNA ratio in the single cell and aggregated subpopulations, or at least discuss this possibility.

We thank the reviewer for proposing this extremely interesting idea. In the revised manuscript, we have added a discussion of this point (lines 487-489) and we have floated various possible explanations. However, we believe that experimental dissection of the underlying mechanism could be a very lengthy undertaking and we hope that the reviewer will agree that this is outside the scope of the current manuscript.

Minor points

5. Some of the experiments compare "low", "medium" and "high" numbers of Mtb, but I could not find a definition of these numbers.

We apologize for this oversight. In the revised manuscript, we have clarified the definition of these gates in the figure 2 legend.

6. There seem to be no positive or negative controls for any of the antibodies used for cell staining (anti-cleaved caspase 1, antiphospho RIP3, anti-phospho MLKKL).

As recommended by the reviewer, the revised manuscript includes controls for all of the antibodies used for immunostaining. In figure S12 we provide representative immunostaining images and fluorescence quantification of uninfected untreated macrophages (negative controls) and of uninfected macrophages treated with cocktails of molecules typically used to induce apoptosis, pyroptosis, or necroptosis (positive controls).

Reviewer #3 (Significance (Required)):

The results are novel, biologically interesting and pathophysiologically important.

We thank the reviewer for their appreciation of our findings.

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1st Editorial Decision **26th Jan 2023**

Thank you for submitting your revised Review Commons manuscript to The EMBO Journal. Your manuscript has now been seen by all original reviewers, who find that their main concerns have been addressed and now recommend publication of the manuscript. I will therefore be happy to accept the manuscript for publication in The EMBO Journal after its reformatting along the guidelines included in the attached document.

Please feel free to contact me if you have any further questions regarding this final editorial revision. Please use the link below to upload the revised files.

Thank you for the opportunity to consider your work for publication, and I look forward to receiving your revised manuscript.

Referee #1:

The authors have responded adequately to my previous comments and have improved the manuscript

Referee #2:

The revision is highly responsive to the review. I have no further suggestions for improvement.

Referee #3:

Many thanks to the authors for taking on board my comments and address my concerns in such a detailed manner. The study has significantly improved with all the additional data and I am happy to recommend for publication.

The authors addressed the remaining editorial issues.

Thank you for addressing the final editorial issues. I am now pleased to inform you that your manuscript has been accepted for publication.

EMBO Press Author Checklist

Corresponding Author Name: Chiara Toniolo
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2023-113490

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Abridged guidelines for figures

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The data shown in figures should satisfy the following conditions:

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- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	all bacterial strains generated in this study are available (Material and Methods), proteomics data generated in this study have been deposited to PRIDE (Data Availability)

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For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Material and Methods

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Material and Methods and table 2

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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Material and Methods

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Material and Methods and Acknoledgement sections

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Material and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	