Appendix to:

Uptake-independent killing of macrophages by extracellular *Mycobacterium tuberculosis* aggregates

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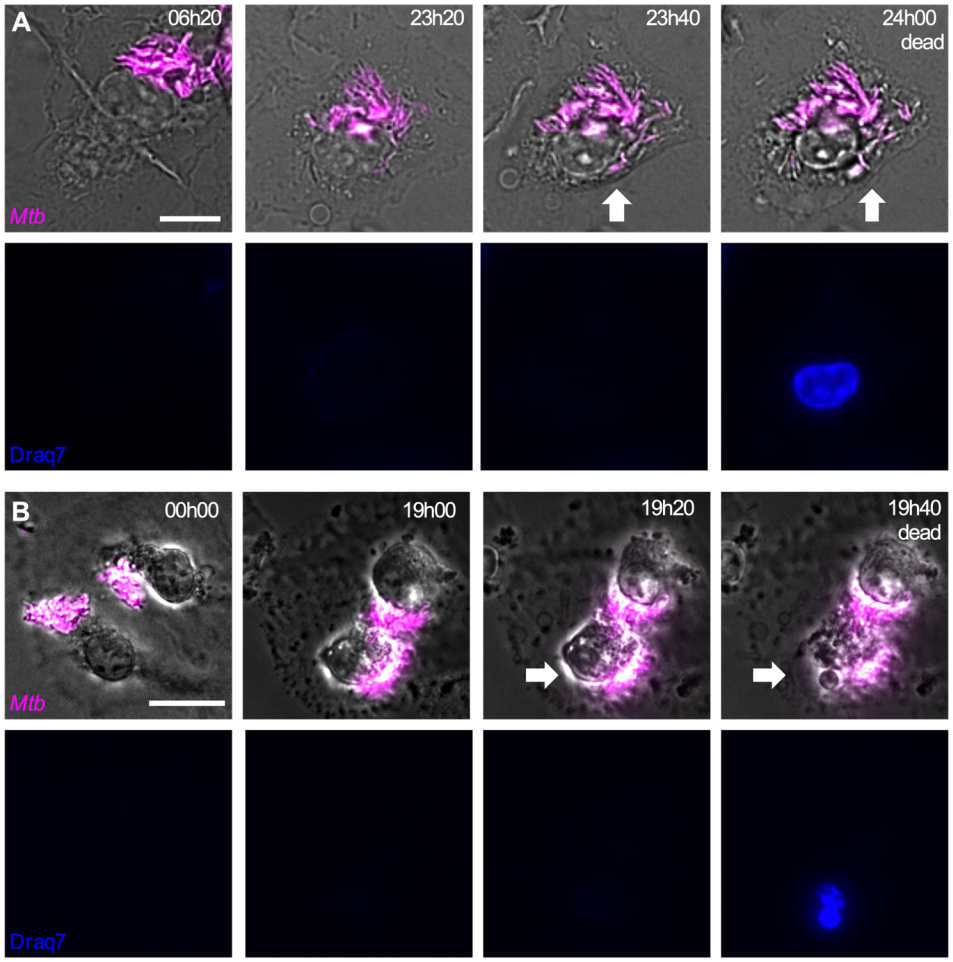
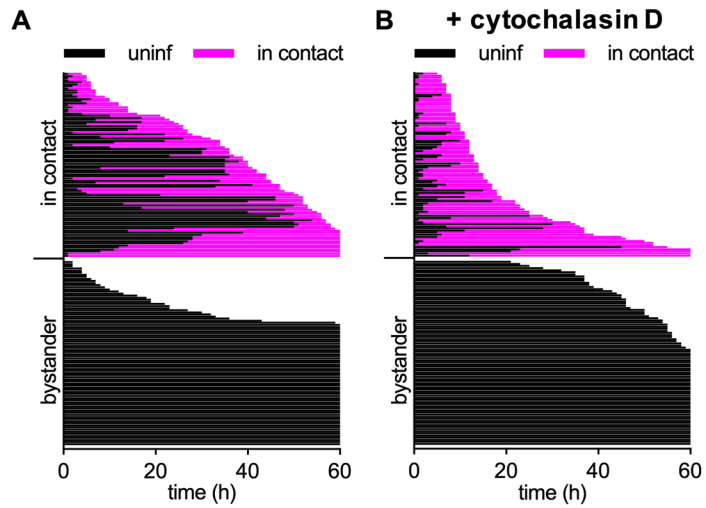
Appendix figures



Appendix figure S1. The macrophage time-of-death identified in brightfield images corresponds to the time-of-death defined by a fluorescent marker of cell death.

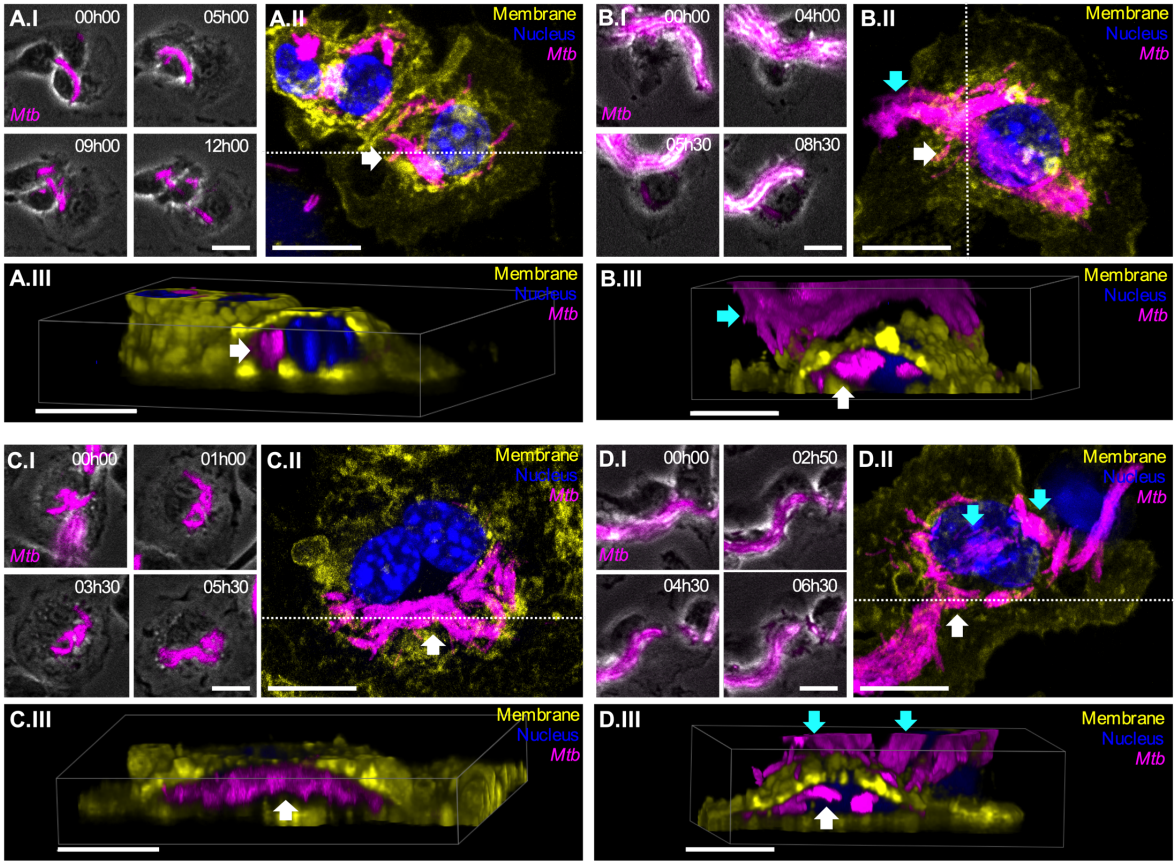
Although it may be difficult to distinguish live and dead cells in individual snapshots, cell-death events are easily identifiable by comparing adjacent frames in image series obtained by time-lapse microscopy (supplementary Movies EV2 and EV7). When macrophages die, they rapidly shrink, lose membrane integrity (white arrows), and stop moving. In brightfield images we define the time-of-death for individual macrophages as the first image frame in which a cell stops moving and loses membrane integrity. At this time, also the intracellular bacteria identified in the fluorescent images and the intracellular structures (vesicles, nucleus) visible in the brightfield images stop moving. The macrophage time-of-death identified in brightfield images overlaps to the time-of-death defined by Draq7 (in blue), a cell-impermeable fluorescent marker commonly used to distinguish dead cells. **(A)**

Representative example of a dying *Mtb*-infected macrophage. Snapshots from Movie EV4. **(B)** Representative example of cytochalasin D-treated macrophage that dies upon contact with an extracellular *Mtb* aggregate. Snapshots from Movie EV7. Scale bars, 10 μm.



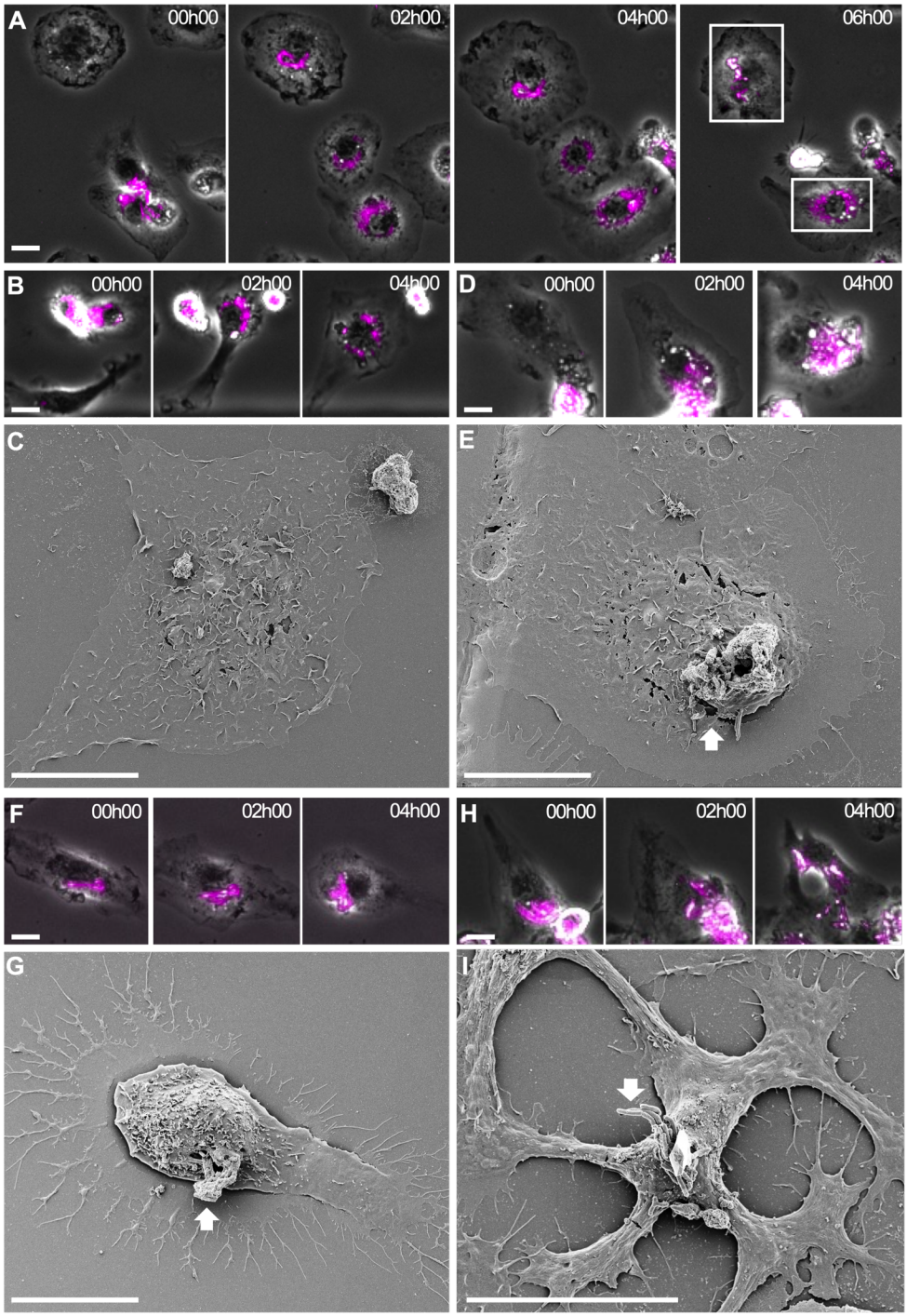
Appendix figure S2. Physical interaction between bacterial aggregates and macrophages is required to induce death of the infected cells.

Untreated **(A)** or cytochalasin D-treated **(B)** BMDMs infected with aggregates of *Mtb* Erd-tdTomato and imaged by time-lapse microscopy at 1-hour intervals for 60 hours. Each line represents the life span of an individual cell; the fraction of the line in black represents the time spent as uninfected, whereas the fraction of the line in magenta represents the time spent interacting with an *Mtb* aggregate. n=88 for each subclass (bystander, in contact).



Appendix figure S3. Correlative time-lapse microscopy and immunofluorescence on BMDMs infected with *Mtb* aggregates.

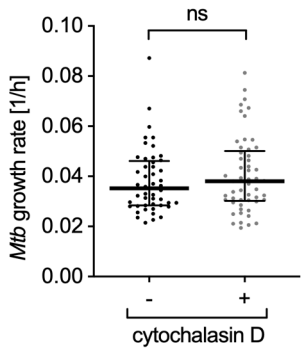
(A-D) BMDMs infected with aggregates of *Mtb* were imaged by time-lapse microscopy (every 30 min for up to 13.5h) followed by fixation, immunostaining and imaging by confocal microscopy. *Mtb* in magenta, nuclei stained with Hoecsth (blue), membrane staining with anti-CD45 antibody in yellow. White arrows indicate intracellular bacteria, cyan arrows indicate extracellular bacteria. All scale bars, 10 μM. **(A.I,B.I,C.I,D.I)** Time-lapse microscopy image-series of macrophages that interacts with *Mtb* aggregates and fragment (A-C) or do not fragment (B-D) them. **(A.II,B.II,C.II,D.II)** Max intensity projection of confocal microscopy images of the same macrophages shown in panels I. **(A.III,B.III,C.III,D.III)** 3-D reconstruction of the cells imaged in panels II, images are cropped in x or y in the position indicated by the white dotted lines in panels II to show the inside of the cell.



Appendix figure S4. Correlative time-lapse microscopy and scanning electron microscopy (SEM) on BMDMs infected with *Mtb* aggregates.

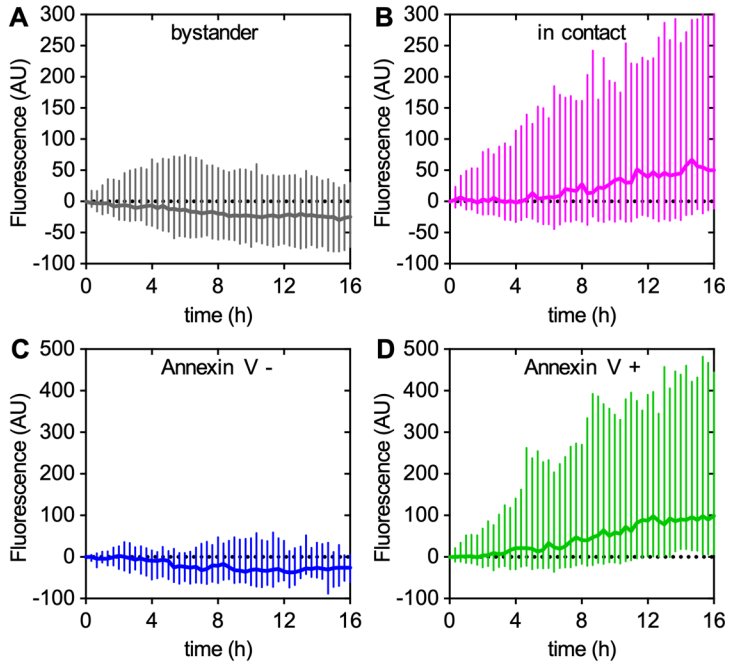
(A-H) BMDMs infected with aggregates of *Mtb* were imaged by time-lapse microscopy at 2-hour intervals for 12 hours, followed by SEM. **(A)** Time-lapse microscopy image-series of the macrophages shown in figure 1I,J (bottom square) and in figure 1K-M (top square). **(B)** Time-lapse microscopy image-series of a macrophage that shows the typical “bullseye” pattern of bacterial redistribution upon

interaction with a bacterial aggregate. **(C)** Correlative SEM image of the macrophage shown in (B). **(D,F)** Time-lapse microscopy image-series of macrophages interacting with extracellular *Mtb* aggregates without fragmentation and redistribution of bacteria. **(E,G)** Correlative SEM images of (D) and (F) respectively. **(H)** Time-lapse microscopy image-series of a macrophage that interacts with an extracellular *Mtb* aggregates and fragments it. **(I)** Correlative SEM image of (H). This example shows that even some aggregates that get fragmented can be partially retained on the surface of the macrophage. Scale bars, 20 μm.



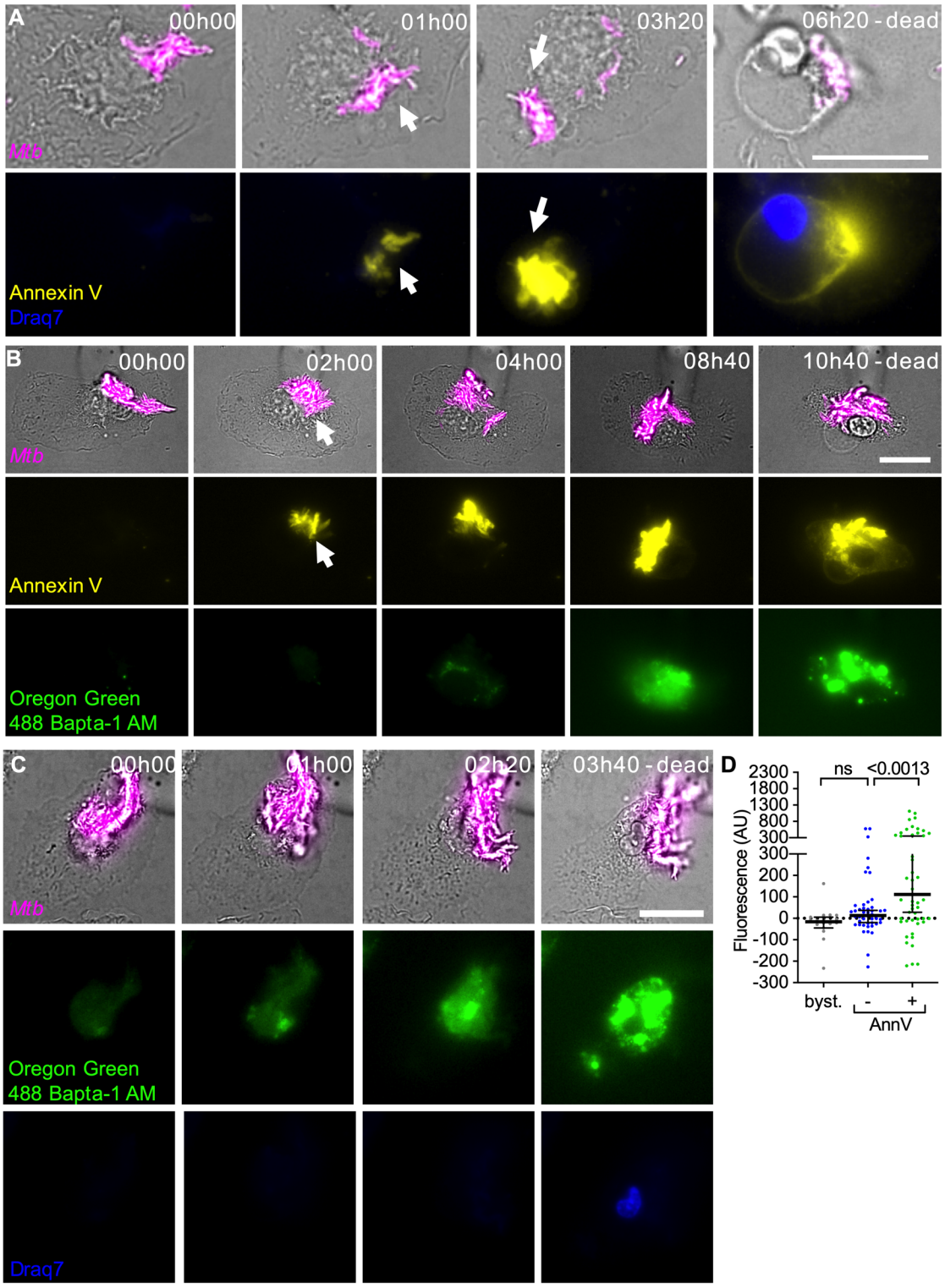
Appendix figure S5. Cytochalasin D does not affect the growth of *Mtb* aggregates.

BMDMs infected with aggregates of fluorescent *Mtb* and incubated without (-) or with (+) cytochalasin D were imaged by time-lapse microscopy at 1-hour intervals for 72 hours. The bacterial growth rate was calculated from microscopy time-series by measuring the fluorescent area over time of individual *Mtb* microcolonies. Each symbol represents an *Mtb* microcolony (n > 45 bacterial aggregates per condition). Black lines indicate median values and interquartile ranges. *P*-value calculated using an unpaired Mann-Whitney test; ns, *P* value > 0.05.



Appendix figure S6. Extracellular *Mtb* aggregates induce calcium accumulation over time in cytochalasin D-treated macrophages.

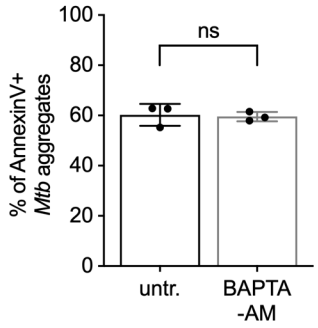
BMDMs stained with Oregon Green 488 Bapta-1 AM and treated with cytochalasin D were infected with aggregates of *Mtb* Erd-tdTomato and imaged by time-lapse microscopy at 20-minute intervals for 24 hours. Oregon Green 488 Bapta-1 AM fluorescence values at each time point were normalized to the time of first contact for infected cells or to time 0 for uninfected bystander cells. Lines represent median fluorescence values for all cells, error bars represent interquartile ranges. **(A,B)** Cells that survived until the end of the experiment and that are bystander (**A**, n=122) or in stable contact with *Mtb* aggregates (**B**, n=88). The distributions of the fluorescence values at 16 h in A and B are significantly different, *p* value = 0.0009, calculated using a Welch's t test. **(C,D)** Cells in stable contact with *Mtb* aggregates that do not (**C**, n=32) or do (**D**, n=91) develop Annexin V-positive membrane domains. Both the cells that stay alive and that die during the course of the experiment are included in the plots. The distributions of the fluorescence values at 16 h in C and D are significantly different, *p* value < 0.0001, calculated using a Welch's t test.



Appendix figure S7. Extracellular *Mtb* aggregates induce formation of Annexin V-positive plasma membrane domains, intracellular calcium accumulation, and death in macrophages not treated with cytochalasin D.

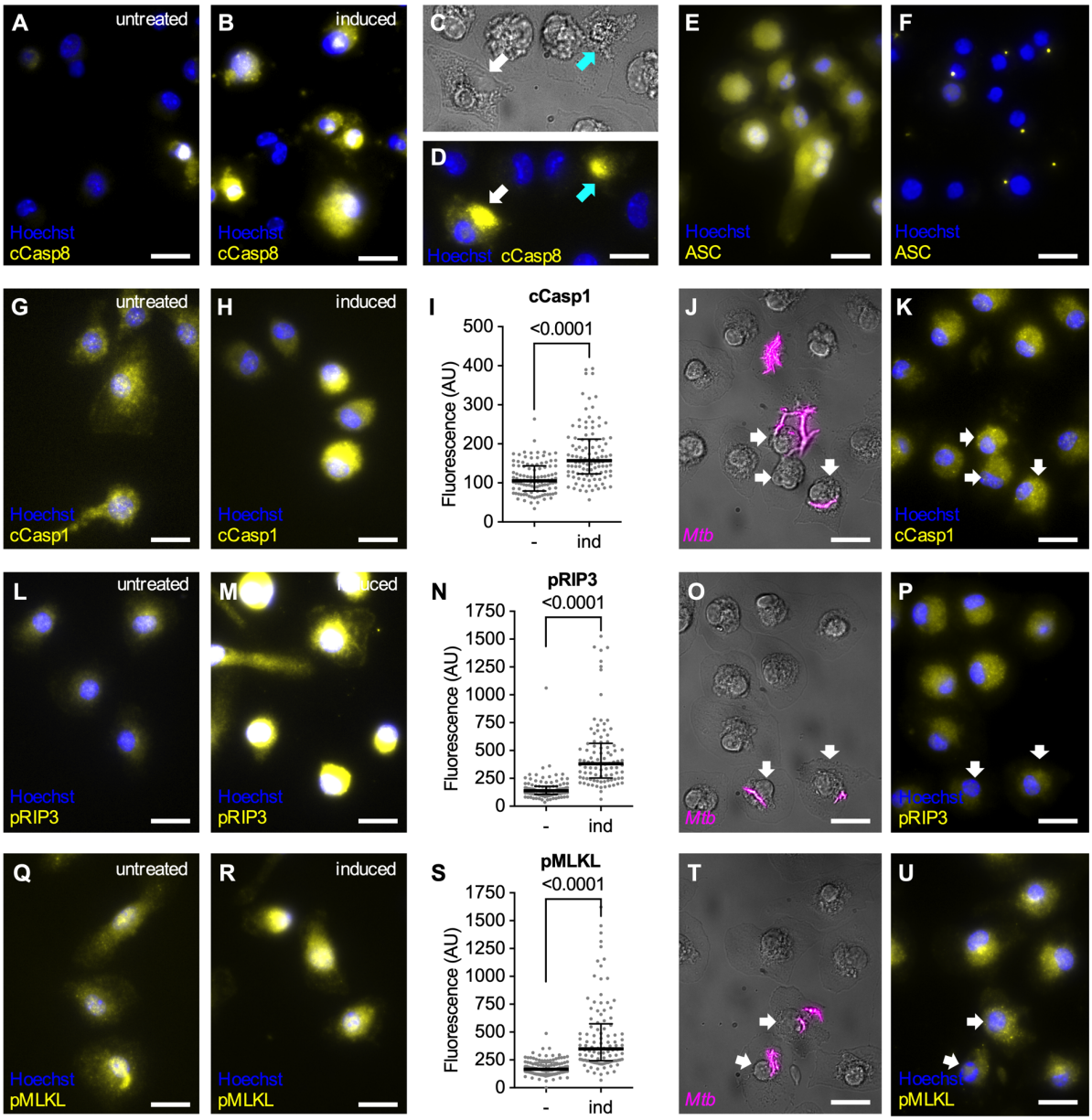
BMDMs were infected with aggregates of *Mtb* Erd-tdTomato and imaged by time-lapse microscopy at 20-minute intervals for 24 hours. Scale bars, 20 μm. **(A)**

Example of a BMDM (brightfield) interacting with an extracellular *Mtb* aggregate (magenta). Annexin V-positive plasma membrane domains (yellow). Nucleus of dead cell stained with Draq7 (blue). White arrows indicate co-localization between bacteria and a local Annexin V-positive plasma membrane domain. **(B)** Example of a macrophage (brightfield) interacting with an extracellular *Mtb* aggregate (magenta). Annexin V-positive plasma membrane domains (yellow). Cytosolic Ca^{2+} stained with Oregon Green 488 Bapta-1 AM (green). Annexin V and Oregon Green 488 Bapta-1 AM staining increase over time until cell death at 10h40 after first contact. **(C)** Example of a macrophage (brightfield) interacting with an extracellular *Mtb* aggregate (magenta). Cytosolic Ca^{2+} stained with Oregon Green 488 Bapta-1 AM (green). Nucleus of dead cell stained with Draq7 (blue). Oregon Green 488 Bapta-1 AM staining increases over time until cell death at 4h00 after first contact. **(D)** Oregon Green 488 Bapta-1 AM fluorescence in uninfected bystander cells and for infected cells with (+) or without (–) Annexin V-positive plasma membrane domains at the site of contact with an *Mtb* aggregate. Oregon Green 488 Bapta-1 AM fluorescence values for infected cells correspond to the time of death after first contact or 16 hours post-contact for cells that survive. Values for uninfected bystander cells correspond to the time of death or 16 hours. Each symbol represents a single macrophage. Black bars represent median and interquartile range. *P*-values were calculated using a Krustal-Wallis test (n = 18, 46, 52 respectively). ns, *P* values > 0.05.



Appendix figure S8. Calcium chelation does not affect formation of Annexin V-positive local membrane domains in macrophages in contact with *Mtb* aggregates.

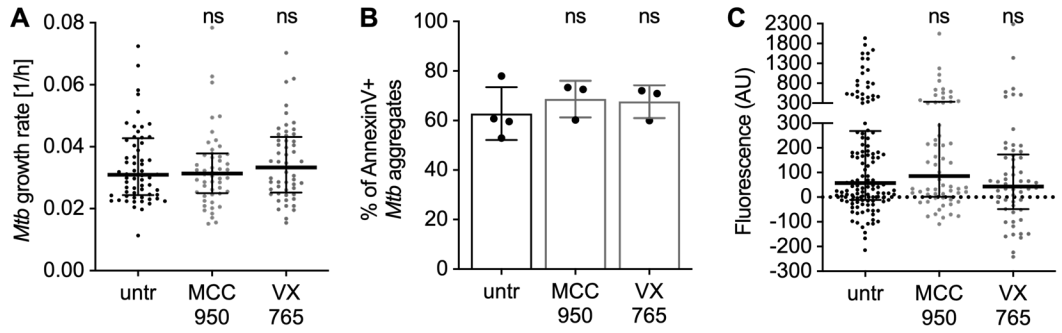
BMDMs treated with cytochalasin D were infected with aggregates of *Mtb*, incubated with Annexin V without or with BAPTA-AM and imaged by time-lapse microscopy at 1-hour intervals for 60 hours. The plot represents the percentage of macrophages that show Annexin V-positive membrane domains within the first 12 hours after entering in contact with *Mtb* aggregates. Each symbol represents a single biological replicate. At least 100 individual macrophage-*Mtb* aggregate interactions were tracked for each replicate. Bars represent average and standard deviation. *P*-value calculated using an unpaired t test; ns, *P* value > 0.05.



Appendix figure S9. Apoptosis, pyroptosis and necroptosis markers can be induced chemically, but only pyroptosis markers are induced by contact with *Mtb*.

(A,B,E-I,L-N,Q-S) Cytochalasin D-treated BMDMs were incubated without **(A,E,G,I,L,N,Q,S)** or with apoptosis inducers (20 ng/ml TNFα, cycloheximide 10 µg/ml) (Zhang *et al*, 2020) **(B)**, pyroptosis inducers (50 ng/ml LPS, 0.5 mM ATP) (Rastogi *et al*, 2021) **(F,H,I)** or necroptosis inducers (5 µM zVAD, 20 ng/ml TNFα, 50 nM SM164) (Zhang *et al*, 2020) **(M,N,R,S)** for 2 hours, fixed and processed for immunofluorescence with a anti-cleaved Caspase-8 antibody (cCasp8) **(A,B)**, a anti-ASC antibody **(E,F)**, a anti-cleaved Caspase-1 antibody (cCasp1) **(G-I)**, a anti-phosphorylated RIP3 antibody (pRIP3) **(L-N)** or a anti-phosphorylated MLKL

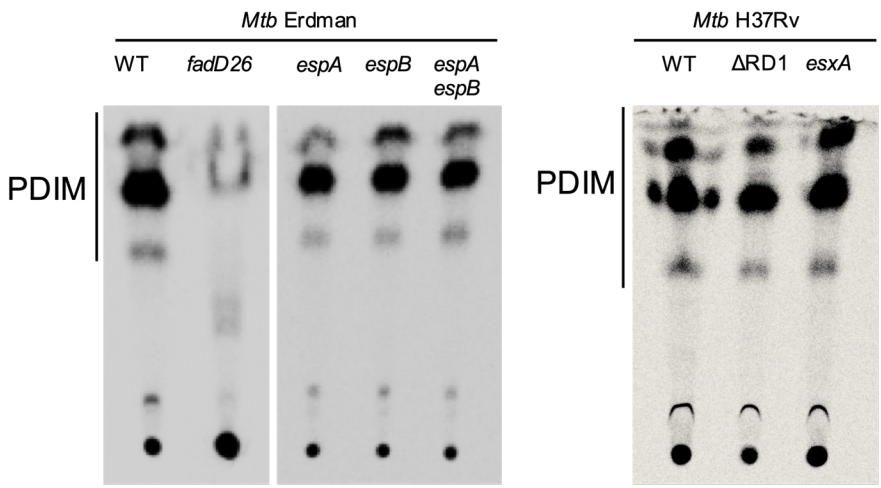
antibody (pMLKL) **(Q-S)**. **(A,B,E,F,G,H,L,M,Q,R)** Representative microscopy images of BMDMs treated without (left panels-untreated) or with (right panels-induced) death pathways inducers. Antibody staining in yellow, nuclei in blue (stained with Hoechst). Scale bars, 10 μm. **(I,N,S)** Median fluorescence values for BMDMs treated without (-) or with (ind) death pathways inducers. Each symbol represents a single macrophage. Black bars represent median and interquartile range. P-value were calculated using an unpaired Mann-Whitney test. (**I:** n=108, 109 respectively; **N:** n=103, 94 respectively; **S:** n=110, 109 respectively). **(C,D,J,K,O,P,T,U)** Representative microscopy images of cytochalasin D-treated BMDMs infected with aggregates of *Mtb* Erd-tdTomato, fixed at 24 hours post infection and processed for immunofluorescence with a anti-cleaved Caspase-8 antibody (cCasp8) **(C,D)**, a anti-cleaved Caspase-1 antibody (cCasp1) **(J,K)**, a anti-phosphorylated RIP3 antibody (pRIP3) **(O,P)** or a anti-phosphorylated MLKL antibody (pMLKL) **(T,U)**. White arrows indicate the cells that are considered as "in contact" for quantitative fluorescence analysis, the other cells are considered as "bystander". **(C,J,O,T)** Representative microscopy images showing the cells (brightfield) overlapped with the fluorescence channel corresponding to the bacteria (magenta). **(D,K,P,U)** Fluorescence channels showing the indicated antibody staining (yellow) and the nuclei (blue, stained with Hoechst). Scale bars, 10 μm. In **C** and **D** we show that both dying cells with intact nuclei (white arrow) and dead cells with disrupted nuclei (cyan arrow) show signs of Caspase-8 activation.



Appendix figure S10. Treatment with pyroptosis inhibitors does not affect *Mtb* growth, formation of Annexin V-positive local membrane domains and intracellular calcium accumulation in macrophages in contact with *Mtb* aggregates.

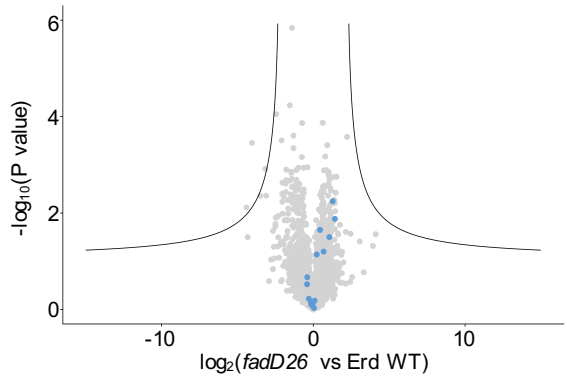
(A) BMDMs treated with cytochalasin D were infected with aggregates of *Mtb,* incubated with the indicated pyroptosis inhibitors and imaged by time-lapse microscopy at 1-hour intervals for 60 hours. The bacterial growth rate was calculated from microscopy time-series by measuring the fluorescent area over time of individual *Mtb* microcolonies. Each symbol represents an *Mtb* microcolony (n ≥ 50 bacterial aggregates per condition). Black lines indicate median values and interquartile ranges. P-values were calculated using a Krustal-Wallis test; ns, *P* values > 0.05. **(B)** BMDMs treated with cytochalasin D were infected with aggregates of *Mtb*, incubated with Annexin V and the indicated pyroptosis inhibitors and imaged by time-lapse microscopy at 1-hour intervals for 60 hours. The plot represents the percentage of macrophages that show Annexin V-positive membrane domains within the first 12 hours after entering in contact with *Mtb* aggregates. Each symbol represents a single biological replicate. At least 70 individual macrophage-*Mtb* aggregate interactions were tracked for each replicate. Bars represent average and standard deviation. *P* values were calculated using a one-way ANOVA test comparing treated samples with the untreated control; ns, *P* values > 0.05.
(C) Cytochalasin D-treated BMDMs were stained with the membrane-permeable dye Oregon Green 488 Bapta-1 AM to visualize cytosolic Ca^{2+}, infected with aggregates of *Mtb* and incubated with the indicated death inhibitors. Samples were imaged by time-lapse microscopy at 20-minute intervals for 24 hours. Oregon Green 488 Bapta-1 AM fluorescence values at each time point were normalized to the time of first contact with an *Mtb* aggregate. Plotted values represent relative Oregon Green 488 Bapta-1 AM fluorescence values at the time of death (or to 16 hours post-

contact for cells that survive) in untreated (untr.; n=124) infected macrophages and in infected macrophages treated with MCC950 (n=64) or VX765 (n=62). Each symbol represents a single macrophage. Black bars represent median and interquartile range. *P*-values were calculated using a Krustal-Wallis test comparing treated samples with the untreated control; ns, *P* values > 0.05.



Appendix figure S11. TLC analysis of PDIM production by wild-type and mutant *Mtb* strains used in this study.

C^{14}-labelled surface-exposed lipids were collected from *Mtb* pellets by petroleum ether extraction and spotted on a TLC silica gel plate. The plate was resolved in a mobile phase of 9:1 petroleum ether-diethyl ether.



Appendix figure S12. Mass spectrometry analysis of the bacterial secretome of the *fadD26* mutant.

Volcano plot showing changes in secreted protein abundances in the *fadD26* mutant compared to the Erdman WT parental strain. Black lines correspond to a false discovery rate (FDR) of 0.05, S0=1. Light blue symbols represent proteins that are part of the ESX-1 operon.

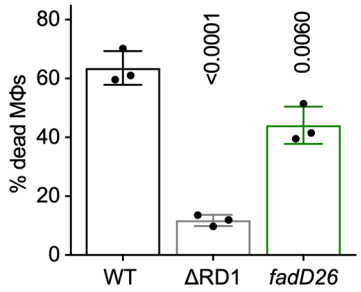
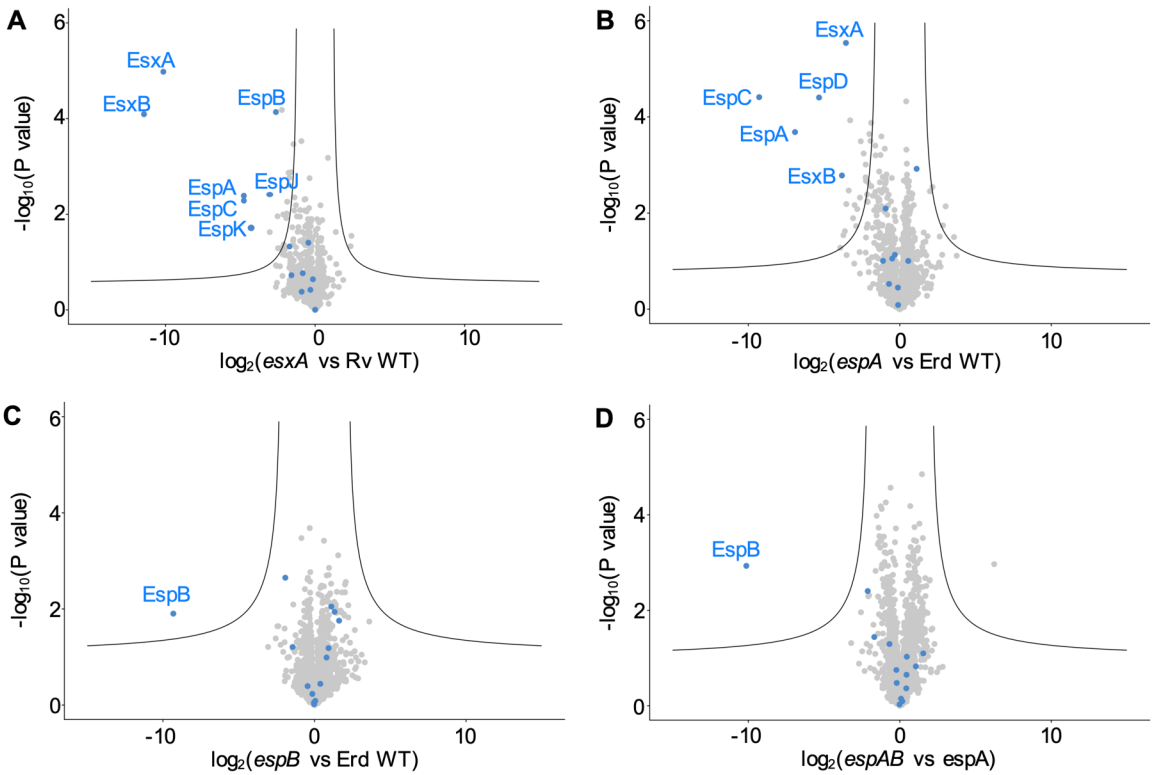
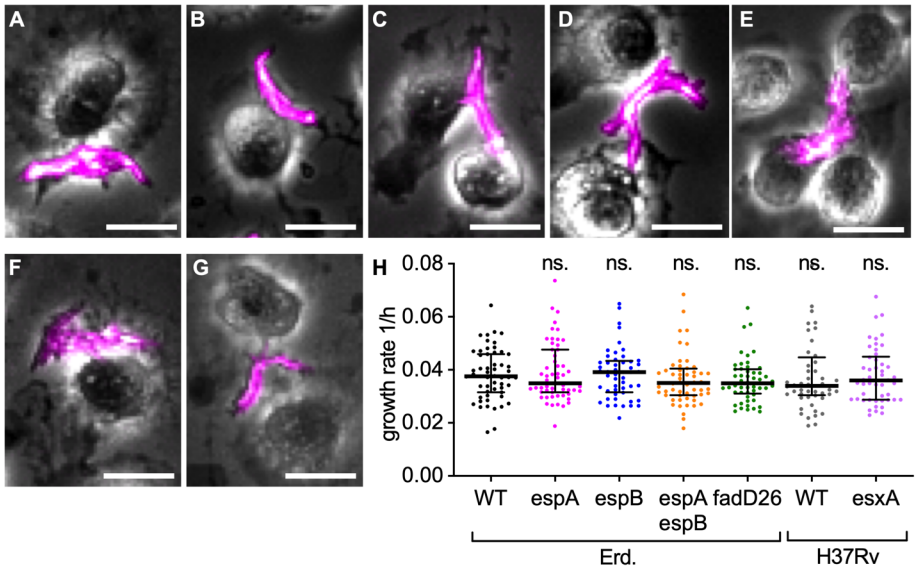


Figure S13. Lack of PDIM has a minor effect on uptake-dependent killing of macrophages by *Mtb* aggregates

BMDMs were infected with aggregates of different *Mtb* strains and imaged by time-lapse microscopy at 1-hour intervals for 60 hours. The plots represent the percentage of macrophages that die within the first 12 hours after interaction with an *Mtb* aggregate. Each symbol represents the percentage of dead macrophages for a single biological replicate ($n \geq 3$ replicates with ≥ 70 cells per replicate). Bars represent means and standard deviations. *P*-values were calculated using a one-way ANOVA test.

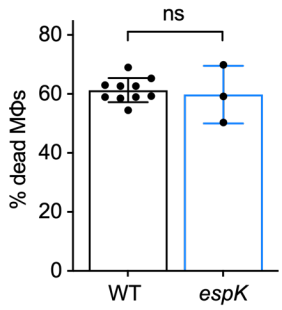


Appendix figure S14. Mass spectrometry analysis of the bacterial secretome. Volcano plots showing changes in secreted protein abundances between couples of *Mtb* strains. Black lines correspond to a false discovery rate (FDR) of 0.05, S0=1. Light blue symbols represent proteins that are part of the ESX-1 operon. The names of the ESX-1 operon proteins showing a significant change are indicated. **(A)** *esxA* mutant strain compared to the Rv WT parental strain; **(B)** *espA* mutant strain compared to the Erdman WT parental strain; **(C)** *espB* mutant strain compared to the Erdman WT parental strain; **(D)** *espA espB* mutant strain compared to the *espA* mutant parental strain.



Appendix figure S15. Bacterial aggregates from different *Mtb* strains have comparable morphology and show similar growth dynamics.

(A-G) Representative examples of aggregates of different fluorescent *Mtb* strains in contact with cytochalasin D-treated BMDMs at 5 hours post infection. Scale bars, 20 µm. **(A)** *Mtb* Erdman WT expressing tdTomato; **(B)** *Mtb espA* expressing tdTomato; **(C)** *Mtb espB* expressing tdTomato; **(D)** *Mtb espA espB* expressing tdTomato; **(E)** *Mtb fadD26* expressing tdTomato; **(F)** *Mtb* H37Rv WT expressing GFP; **(G)** *Mtb esxA* expressing GFP. **(H)** The bacterial growth rate was calculated from microscopy time-series by measuring the fluorescent area over time of individual *Mtb* microcolonies. Each symbol represents an *Mtb* microcolony ($n \geq 50$ bacterial aggregates per condition). Black lines indicate median values and interquartile ranges. *P*-values were calculated using a Krustal-Wallis test comparing each strain with Erdman (Erd.) WT; ns, *P* values > 0.05.



Appendix figure S16. EspK is not required for uptake-independent killing of macrophages by *Mtb* aggregates

BMDMs treated with cytochalasin D were infected with aggregates of wild-type *Mtb*, or with aggregates of the *espK* mutant and imaged by time-lapse microscopy at 1-hour intervals for 60 hours. Plots represent the percentage of BMDMs that die within the first 12 hours after stable contact with an aggregate of *Mtb.* Each symbol represents the percentage of dead macrophages for a single biological replicate ($n \geq 3$ replicates with ≥ 70 cells per replicate). Bars represent means and standard deviations. *P*-value calculated using an unpaired t test comparing the mutant strain to the wild-type control; ns, *P* value > 0.05.

References

Rastogi S, Ellinwood S, Augenstreich J, Mayer-Barber KD & Briken V (2021) Mycobacterium tuberculosis inhibits the NLRP3 inflammasome activation via its phosphokinase PknF. *PLOS Pathogens* 17: e1009712

Zhang L, Jiang X, Pfau D, Ling Y & Nathan CF (2020) Type I interferon signaling mediates Mycobacterium tuberculosis–induced macrophage death. *Journal of Experimental Medicine* 218