Expanded View Figures

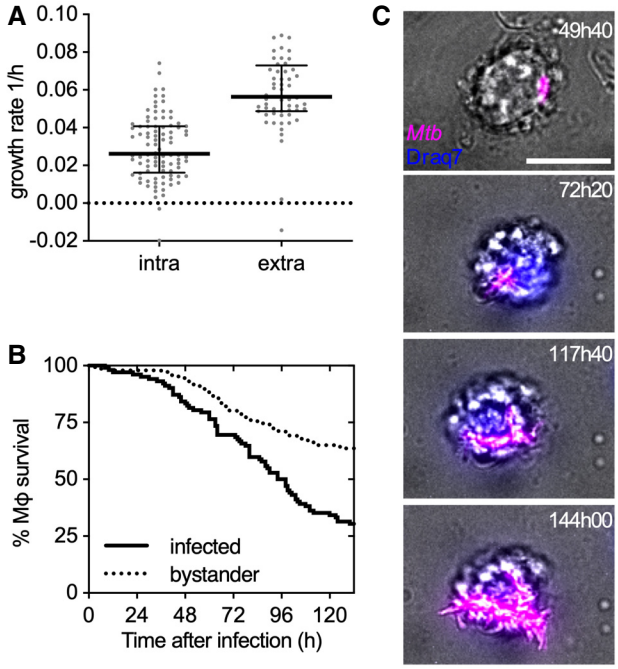

Figure EV1. Intracellular growth of *Mtb* results in death and lysis of the infected macrophage, rapid extracellular growth on the host-cell debris, and formation of large extracellular *Mtb* aggregates.

BMDMs were infected with *Mtb* Erd-tdTomato and imaged by time-lapse microscopy at 1- or 2-h intervals for up to 166 h.

A Growth rate of individual *Mtb* microcolonies growing inside a macrophage (intra) or on the debris of a lysed macrophage (extra). Black lines indicate median values and interquartile ranges. ($n = 96$, 47 bacterial microcolonies, respectively)

B Percentage survival over time of infected versus uninfected bystander macrophages. ($n = 110$ and 102, respectively)

C Representative example of an intracellular *Mtb* microcolony that after lysis of the host macrophage (Draq7 positive cell) grows on the debris of the dead cell. Scale bar, 10 μm. Representative snapshots from Movie EV2.

Source data are available online for this figure.

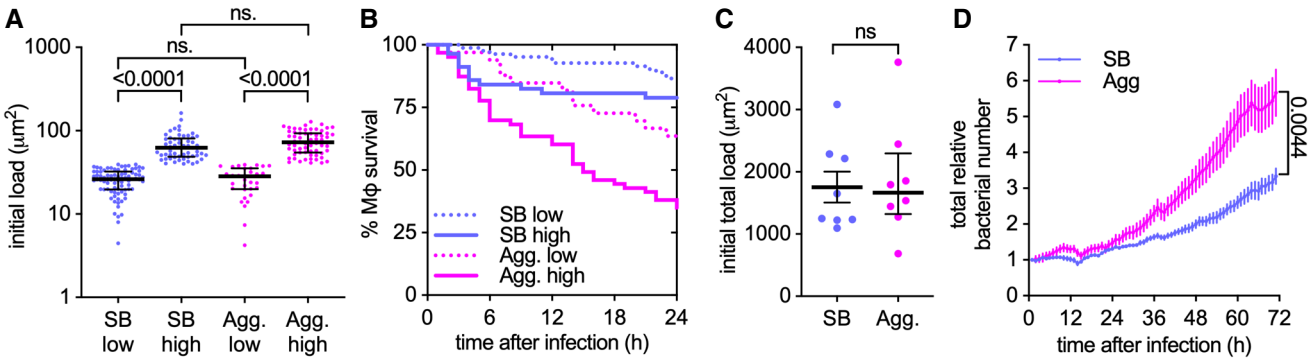

Figure EV2. Bacterial aggregation enhances the uptake-dependent killing of macrophages and bacteria propagation.

BMDMs were infected with aggregated (Agg) or non-aggregated (SB) *Mtb* Erd-tdTomato and imaged by time-lapse microscopy at 1-h intervals for 72 h.

A Infected individual macrophages are binned into low and high initial loads according to the amount of single (SB) or aggregated (Agg) bacteria they internalize. The bacterial load is calculated as fluorescent area per macrophage and gates are set at < 40 μm^2 (low) or > 40.0 μm^2 (high) per macrophage. The area of one bacterium is included between 0.5 and 2 μm^2. Each symbol represents the bacterial load of one individual macrophage ($n = 82$, 57, 33, and 63 macrophages, respectively). Bars represent the median and interquartile range. *P*-values were calculated using a Krustal–Wallis test; ns, *P*-values > 0.05.

B Percentage survival over time for individual macrophages with an initial bacterial load as indicated in panel A ($n = 82$, 57, 33, and 63 macrophages, respectively).

C Total initial bacterial load per microscopy field of view (332.80 × 332.80 μm^2, approx. 100 cells/field of view). Each symbol represents one field of view ($n = 8$). Bars represent the mean and standard errors of the mean. *P*-value calculated using an unpaired *t*-test; *P*-values > 0.05.

D Total relative bacterial load over time per microscopy field of view. Symbols represent the average bacterial load ($n = 8$) and bars represent standard errors of the mean. *P*-values were calculated using an unpaired *t*-test.

Source data are available online for this figure.

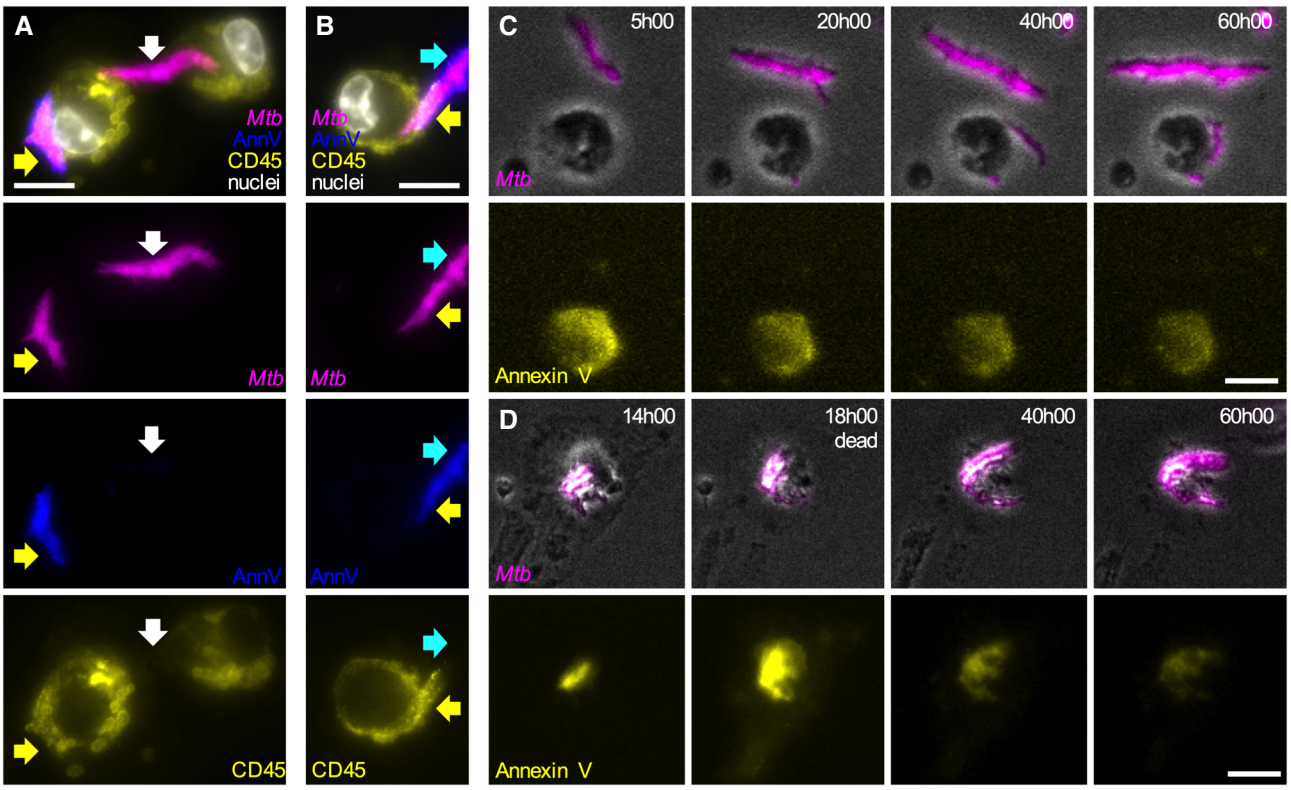

Figure EV3. Formation of Annexin V-positive membrane domains requires physical contact between *Mtb* aggregates and live macrophages.

A, B Representative fluorescence microscopy images of cytochalasin D-treated BMDMs infected with aggregates of *Mtb* Erd-tdTomato in the presence of Annexin V-FITC and fixed at 8 h post-infection. The plasma membrane of the cells was stained with an anti-CD-45 antibody and nuclei were stained with Hoechst (white). Yellow arrows point at *Mtb* aggregates (magenta) overlapping with areas that stain positive for Annexin V (blue) and macrophages plasma membrane (yellow). White arrows indicate an *Mtb* aggregate that do not colocalize neither with the macrophages plasma membrane now with an Annexin V area. Cyan arrows indicate the distal area of an *Mtb* aggregate that stains positive for Annexin V but does not colocalize with the macrophages plasma membrane. Scale bars, 20 μm.

C, D BMDMs treated with cytochalasin D were infected with aggregates of *Mtb*, incubated with Annexin V, and imaged by time-lapse microscopy at 1-h intervals for 60 h. (C) Example of bacterial aggregates (magenta, top panels) that do not interact with macrophages and never become Annexin V-positive (yellow, bottom panel) during the course of the experiment. (D) Example of bacterial aggregate (magenta, top panels) that induces the formation of a local Annexin V-positive membrane domain in the interacting macrophage (yellow, bottom panel). After the death of the macrophage (at 18:00 h) the bacterial aggregates gradually lose fluorescence (40:00 h–60:00 h). Scale bars, 20 μm.

Figure EV4. Expression and secretion patterns of ESX-1 proteins in the different mutant strains used in this study.

A Representation of the *espACD* and *esx-1* loci in the *Mtb* genome.

B, C Expression levels of selected genes of the *espACD* and *esx-1* loci in different mutants. Relative expression (fold changes) was normalized to the WT strain (WT expression = 1, indicated by the dotted line). Bars represent mean values ($n = 3$ biological replicates) and error bars represent standard deviation.

D–H Representative immunoblots of cell lysates and culture filtrates from different *Mtb* strains were probed with the indicated antibodies. In culture filtrates EspB shows a full-length 60-kDa isoform and a truncated 50-kDa isoform as previously reported (Ohol *et al*, 2010; Chen *et al*, 2013a, 2013b). GroEL2 and Ag85 were respectively used as loading controls for cell lysates and culture filtrates.

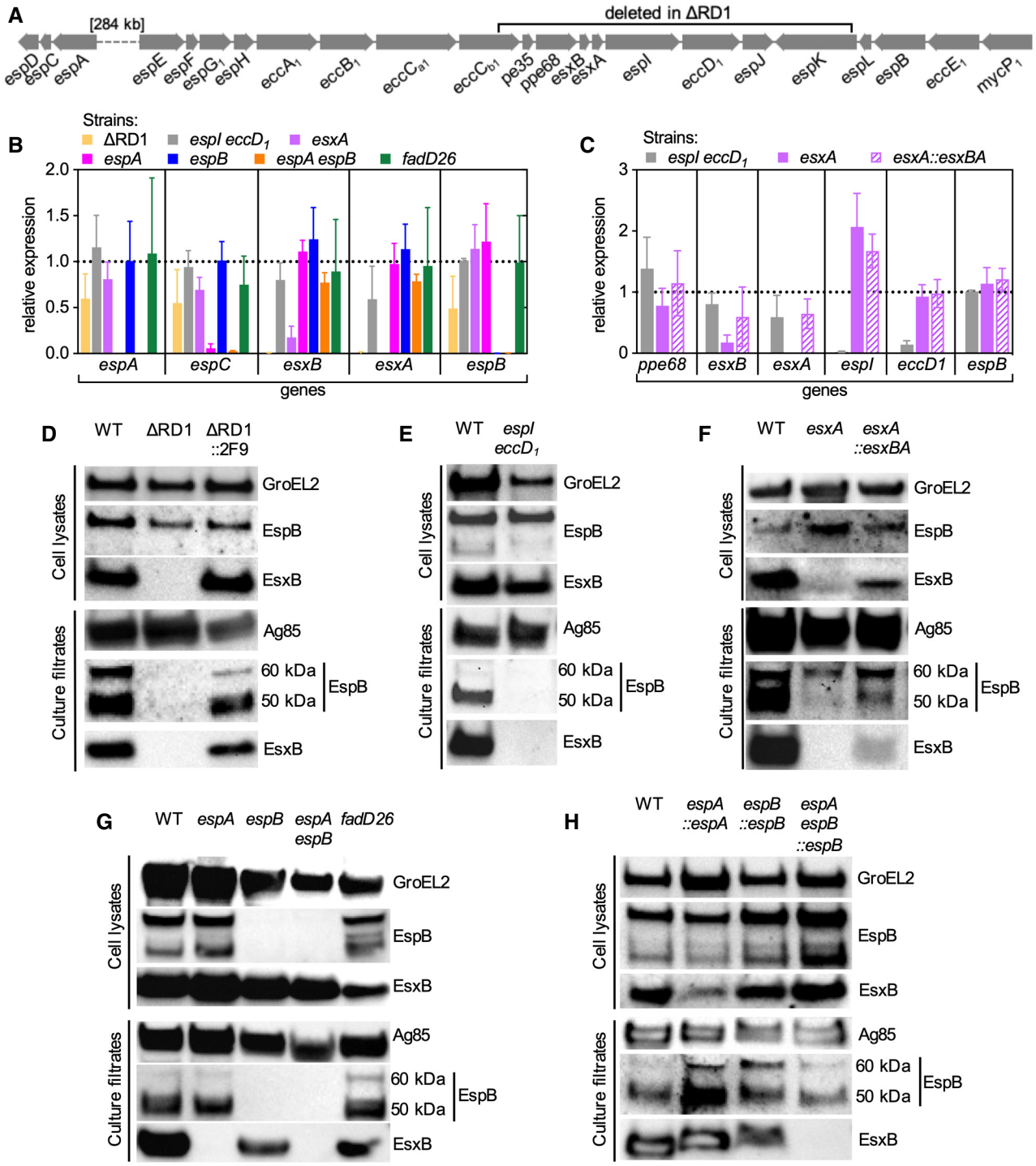
A
[284 kb]
deleted in ΔRD1
espD espC espA espE espF espG1 espH eccA1 eccB1 eccCa1 eccCb1 pe35 ppe68 esxB esxA espI eccD1 espJ espK espL espB eccE1 mycP1
B
Strains:
ΔRD1
espI eccD1
esxA
espA
espB
espA espB
fadD26
relative expression
espA espC esxB esxA espB
genes
C
Strains:
espI eccD1
esxA
esxA::esxBA
relative expression
ppe68 esxB esxA espI eccD1 espB
genes
D
WT ΔRD1 ΔRD1 ::2F9
Cell lysates
Culture filtrates
GroEL2
EspB
EsxB
Ag85
60 kDa
50 kDa
E
WT espI eccD1
F
WT esxA esxA ::esxBA
G
WT espA espB espA espB fadD26
H
WT espA ::espA espB ::espB espA espB ::espB

Figure EV4.

Figure EV5. BTP15 treatment reduces ESX-1 secretion and local plasma membrane perturbation in macrophages in contact with *Mtb* aggregates, without affecting ESX-1 expression, aggregates growth dynamics and morphology in *Mtb*.

A Western blot showing EspB and EsxB expression (cell lysates) and secretion (culture filtrates) pattern of *Mtb* Erdman WT cultures treated with different concentrations of BTP15 (0, 2, 10, and 50 μM). GroEL2 and Ag85 were respectively used as loading controls for cell lysates and culture filtrates.

B EspB (whole or 50 kDa isoform) and EsxB quantification from the Western blot images. Values were normalized to the loading control first and then to the untreated samples.

C–H BMDMs treated with cytochalasin D were infected with aggregates of *Mtb* and incubated with or without BTP15. Infected cells were imaged by time-lapse microscopy at 1-h intervals for 60 h (C, D, F–H), or incubated at 37°C with 5% CO_2 for quantification of colony-forming units (CFU) at 0, 24, and 48 h post-infection (E). (C) Percentage of macrophages that die within the first 12 h after interaction with an *Mtb* aggregate. Bacteria are incubated with different concentrations of BTP15 (0, 2, 10, and 50 μM) for 48 h before infection. BTP15 is not added to the medium of the cells during the course of the experiment. Each symbol represents a single biological replicate (> 70 macrophage-*Mtb* aggregate interactions per replicate). Bars represent average and standard deviation. *P*-values were calculated using a one-way ANOVA test. (D) Growth rate of individual untreated and BTP15-treated (50 μM) *Mtb* aggregates. Each symbol represents a micro-colony. Black lines indicate median values and interquartile ranges ($n \geq 40$ bacterial aggregates per condition). *P*-value calculated using an unpaired Mann–Whitney test; ns, *P*-value > 0.05. (E) Total CFU per well at different time points from untreated and BTP15-treated (50 μM) bacterial cultures. Symbols and bars represent means and standard deviations ($n = 4$ biological replicates). *P*-value calculated using an unpaired *t*-test; ns, *P*-value > 0.05. (F, G) Representative examples of untreated (F) or BTP15-treated (50 μM) (G) aggregates of fluorescent *Mtb* in contact with cytochalasin D-treated BMDMs at different time-points post-infection. Scale bars, 20 μm. (H) BMDMs treated with cytochalasin D were infected with aggregates of different *Mtb* strains, incubated with Annexin V, and imaged by time-lapse microscopy at 1-h intervals for 60 h. Percentage of macrophages that show Annexin V—positive membrane domains within the first 12 h after entering in contact with *Mtb* aggregates without (–) or with (+) BTP15 treatment (50 μM). Each symbol represents a single biological replicate (> 90 macrophage-*Mtb* aggregate interactions per replicate). Bars represent average and standard deviation. *P*-values were calculated using an unpaired *t*-test comparing the treated samples with their untreated reference.

I BMDMs were infected with aggregates of *Mtb* strains, incubated without (untr) or with (BTP15) 50 μM BTP15 and imaged by time-lapse microscopy at 1-h intervals for 60 h. Percentage of macrophages that die within the first 12 h after interaction with an *Mtb* aggregate. Each symbol represents a single biological replicate (> 100 macrophage-*Mtb* aggregate interactions per replicate). Bars represent average and standard deviation. *P*-values were calculated using an unpaired *t*-test.

Source data are available online for this figure.

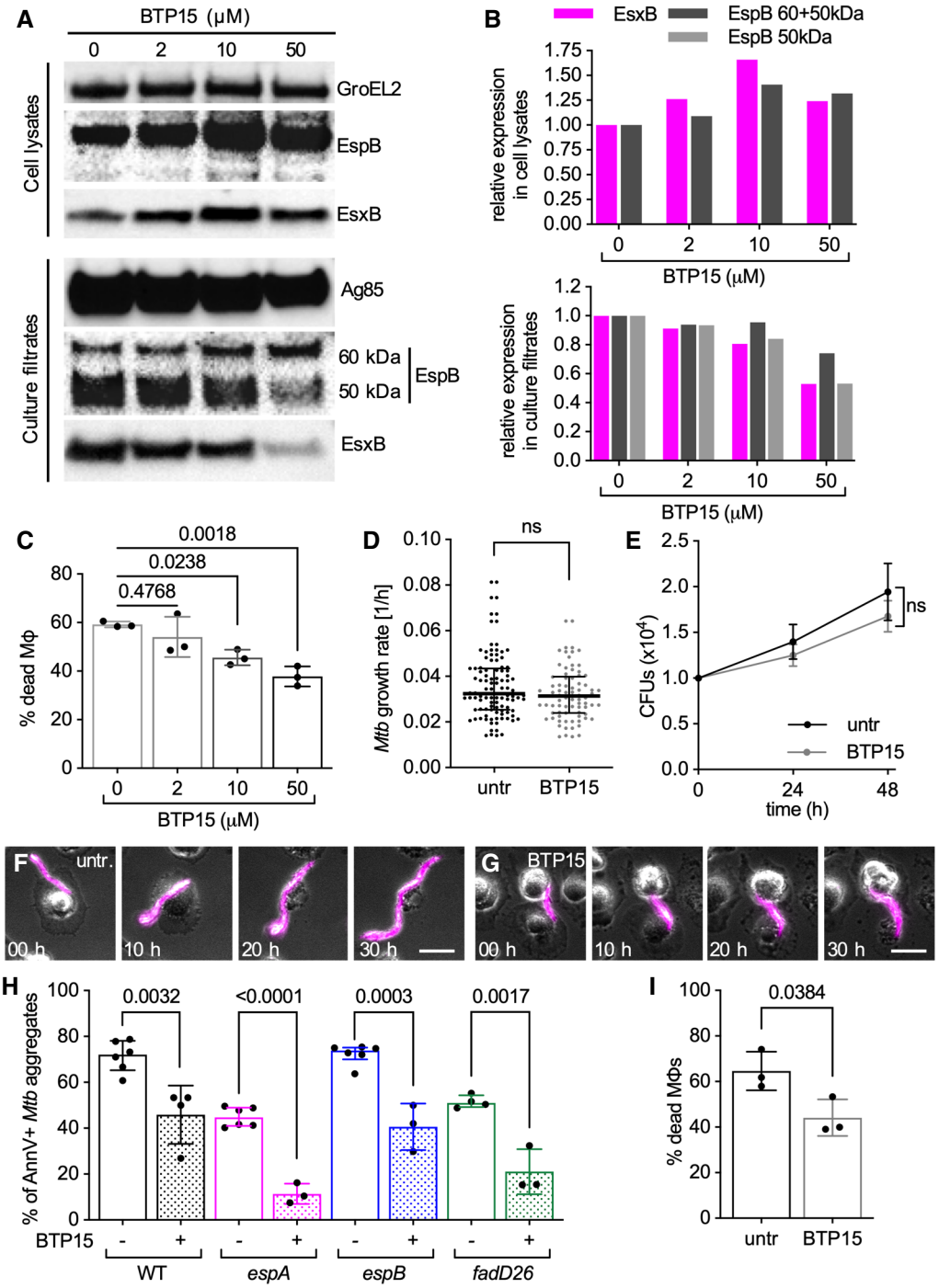
A
BTP15 (μM)
0 2 10 50
Cell lysates
GroEL2
EspB
EsxB
Culture filtrates
Ag85
60 kDa
50 kDa
EspB
EsxB
B
EsxB
EspB 60+50kDa
EspB 50kDa
relative expression in cell lysates
BTP15 (μM)
relative expression in culture filtrates
BTP15 (μM)
C
0.0018
0.0238
0.4768
% dead Mϕ
BTP15 (μM)
D
ns
Mtb growth rate [1/h]
untr
BTP15
E
ns
CFUs (x10^4)
untr
BTP15
time (h)
F
untr.
00 h
10 h
20 h
30 h
G
BTP15
00 h
10 h
20 h
30 h
H
0.0032
<0.0001
0.0003
0.0017
% of AnnV+ Mtb aggregates
BTP15
WT
espA
espB
fadD26
I
0.0384
% dead Mϕs
untr
BTP15

Figure EV5.