

Supplementary Information

Reverting the mode of action of the mitochondrial FoF₁-ATPase by *Legionella pneumophila* preserves its replication niche

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Figure S1: OXPHOS and $\Delta\psi_m$ during infection by *L. pneumophila*

Figure S2: Basal respiration of hMDMs infected with *L. pneumophila* Δ spl mutant and validation of the model in HEK-293 cells.

Figure S3: Inhibition of Fo-F₁ ATPase “reverse mode” delays cell death in *L. pneumophila*-infected hMDMs, while transfection of *LpSpl* into HEK-293 cells protected transfected cells from Staurosporine (STS)-induced cell death.

Figure S4: BTB treatment, $\Delta\psi_m$ and cell death of *L. pneumophila*-infected hMDMs.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. OXPHOS and $\Delta\psi_m$ during infection by *L. pneumophila*. **(A)** Bioenergetic profiles of the key parameters of mitochondrial respiration during a mitochondrial respiratory control assay using the Seahorse XF Mitostress kit. Sequential compound injections measure basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration (Source: Seahorse Bioscience). **(B)** hMDMs were infected with *L. pneumophila* strain Paris (Lpp) wild-type (WT), a T4SS-deficient $\Delta dotA$ mutant, or left uninfected (Non-infected). At 6 hours post-infection (hpi), a cellular respiratory control assay (Seahorse) was performed by measuring oxygen consumption rate (OCR) during the sequential addition of mitochondrial respiratory inhibitors. Quantification of key parameters of mitochondrial functioning, derived from this assay (Figure 1A), are shown. **(C)** hMDMs were infected as in (B) with GFP-expressing bacteria, nuclei of host cells were stained with Hoechst and $\Delta\psi_m$ was monitored using TMRM dye in non-quenching conditions (10 nM). Single-cell analysis of TMRM intensity at 1-10 hpi (expressed as SD/Mean) is shown. **(D)** hMDMs were infected with GFP-expressing bacteria or left uninfected (Non-infected). At 6 hours post-infection (hpi), nuclei of host cells were stained with Hoechst and the $\Delta\psi_m$ was measured using TMRM dye in non-quenching conditions (10 nM), and FCCP (10 μ M) was added or not to non-infected cells as a control to monitor complete mitochondrial depolarization. Single-cell analysis are shown from assays performed from hMDMs purified from three different donors.

Figure S2. Basal respiration of hMDMs infected with *L. pneumophila* Δspl mutant and validation of the model in HEK-293 cells. **(A)** hMDMs were infected with *L. pneumophila* strain Paris WT (Lpp-WT), a T4SS-deficient $\Delta dotA$ mutant, a *LpSpl*-deficient Δspl mutant, or left uninfected (Non-infected). At 6 hours post-infection basal oxygen consumption rate (OCR) was measured using the Seahorse technology. Each dot represents the value of one replicate from a representative experiment. **(B)** HEK-293 cells stably expressing the Fc γ RII receptor to efficiently internalize IgG-opsonized *L. pneumophila* were labelled with Hoechst to identify the cell nucleus (Nuc, blue) and TMRM (red) to quantify $\Delta\psi_m$. Addition of medium (no changes) or FCCP (complete depolarization) to non-infected (NI) HEK-293 cells were used as controls. Representative confocal images at 5 min before the addition of medium (top) or FCCP (bottom), and at 50 min after addition of medium or FCCP. Bar: 20 μ m. **(C)** Quantification of (B) before (baseline) and after the addition of medium/FCCP (dotted line). Each dot represents mean \pm SD of 3 independent experiments. **(D)** HEK-293 cells were infected with GFP-expressing bacteria (WT, $\Delta dotA$ mutant) opsonized using an anti-flagellin antibody, or left uninfected (Non-infected). At 6 h p.i., DCCD was added to the cells and changes $\Delta\psi_m$ were monitored. **(E)**

Representative confocal images at 5 min before the addition of DCCD, and at 50 min after addition of DCCD. Green: GFP-expressing bacteria. Bar: 20 μ m. **(F)** Same as (D) but infection was performed with opsonized Lpp-WT, Lpp- Δ *dotA*, or Lpp- Δ *lpSpl* strains. TMRM values (SD/Mean) at 50 min after DCCD addition are shown. Data from 3 experiments per strain with 6 or more replicates per strain. *p-value < 0.1; **p-value < 0.01. ***p-value < 0.001; ns = non-significant (Mann-Whitney U test)

Figure S3. Inhibition of Fo-F₁ ATPase “reverse mode” delays cell death in *L. pneumophila*-infected hMDMs, while transfection of *LpSpl* into HEK-293 cells protected transfected cells from Staurosporine (STS)-induced cell death. **(A)** Non-infected hMDMs were stained with Hoechst to identify their nuclei and challenged with FCCP (10 μ M) for 18 h, while Annexin-V Alexa Fluor 647 was added to the cell culture to monitor cell death. Percentage of living cells (Annexin-V negative) is shown. These values served as reference to interpret the results shown in Figure 4A. **(B)** hMDMs were infected with GFP-expressing bacteria or left uninfected (Non-infected), and then were treated or not with BTB (50 μ M). At 24 hpi, the nuclei of host cells were stained with Hoechst and Annexin-V Alexa Fluor 647 was added to the cell culture to monitor cell death. Percentage of Annexin-V+ cells at 24 hpi is shown. *p-value < 0.1; **p-value < 0.01. ***p-value < 0.0001; ns = non-significant (Mann-Whitney U test). **(C)** hMDMs were infected with Lpp-WT-GFP, the nuclei of host cells were stained with Hoechst and Annexin-V-647 was added to the cell culture to monitor early cell death from 1 to 18 hpi in non-treated or BTB-treated hMDMs. Single-cell analysis of Annexin-V intensity at 18 hpi is shown. **(D)** hMDMs were infected as in (A) and Hoechst intensity in the nucleus was analyzed in single cells at 12 hpi. **(E)** HEK-293 cells stably expressing the Fc γ RII receptor were transfected with a control plasmid (pGFPmax, Lonza), with a plasmid expressing *LpSpl* WT (harbouring an Xpress tag) or with a plasmid expressing a catalytically inactive *LpSpl* protein (*K366A, also harbouring an Xpress tag). At 24h post-transfection, cells were labelled with Hoechst and Annexin-V-647 to monitor cell death, and 1 μ M STS was added. At 6h, cells were fixed, permeabilized, blocked, and stained with primary mouse antibodies against Xpress tag and secondary anti-mouse Alexa Fluor 488 antibodies. Representative confocal images of transfected cells. Blue: Nucleus (Hoechst, Nuc), Yellow: Annexin-V-647, Green: GFP or anti-Xpress (i.e. *LpSpl*). Bar: 20 μ m. **(F)** Percentage of Annexin-V positive cells in each condition, upon addition or not of STS during 6 h. **(G)** Single cell data of Annexin-V mean fluoresce intensity. More than 400 transfected cells were analyzed in each condition. *p-value

< 0.1; **p-value < 0.01; ***p-value < 0.001; ****p-value < 0.0001; ns = non-significant (Mann-Whitney U test)

Figure S4: BTB treatment, $\Delta\psi_m$ and cell death of *L. pneumophila*-infected hMDMs. **(A)** hMDMs were infected with Lpp-WT-GFP (green), nuclei of host cells were stained with Hoechst (Nuc, blue), and TMRM (red) and Annexin-V Alexa Fluor 647 (yellow) were added to the cells to simultaneously monitor $\Delta\psi_m$ and early cell death, respectively, in non-treated or BTB-treated hMDMs, respectively. Confocal images of living infected cells were automatically acquired in 16 fields per well (4 wells per condition) using 60X magnification. Representative images of 16 stitched fields per condition are shown at 6, 12 and 18 hpi. Bar: 200 μ m. **(B)** hMDMs were infected with Lpp-WT-GFP, nuclei of host cells were stained with Hoechst, and TMRM and Annexin-V Alexa Fluor 647 were added to the cells to simultaneously monitor (1-18 hpi) $\Delta\psi_m$ and early cell death, respectively, in non-treated or BTB-treated hMDMs, respectively. Single-cell analyses (18 hpi) of $\Delta\psi_m$ (TMRM SD/Mean) and cell death (Annexin-V intensity) in more than 1600 cells per condition are shown. Single-cell data from one representative experiment; Green dots: Non-treated Lpp-WT-infected single cells. Orange dots: BTB-treated Lpp-WT-infected single cells.

Figure S1

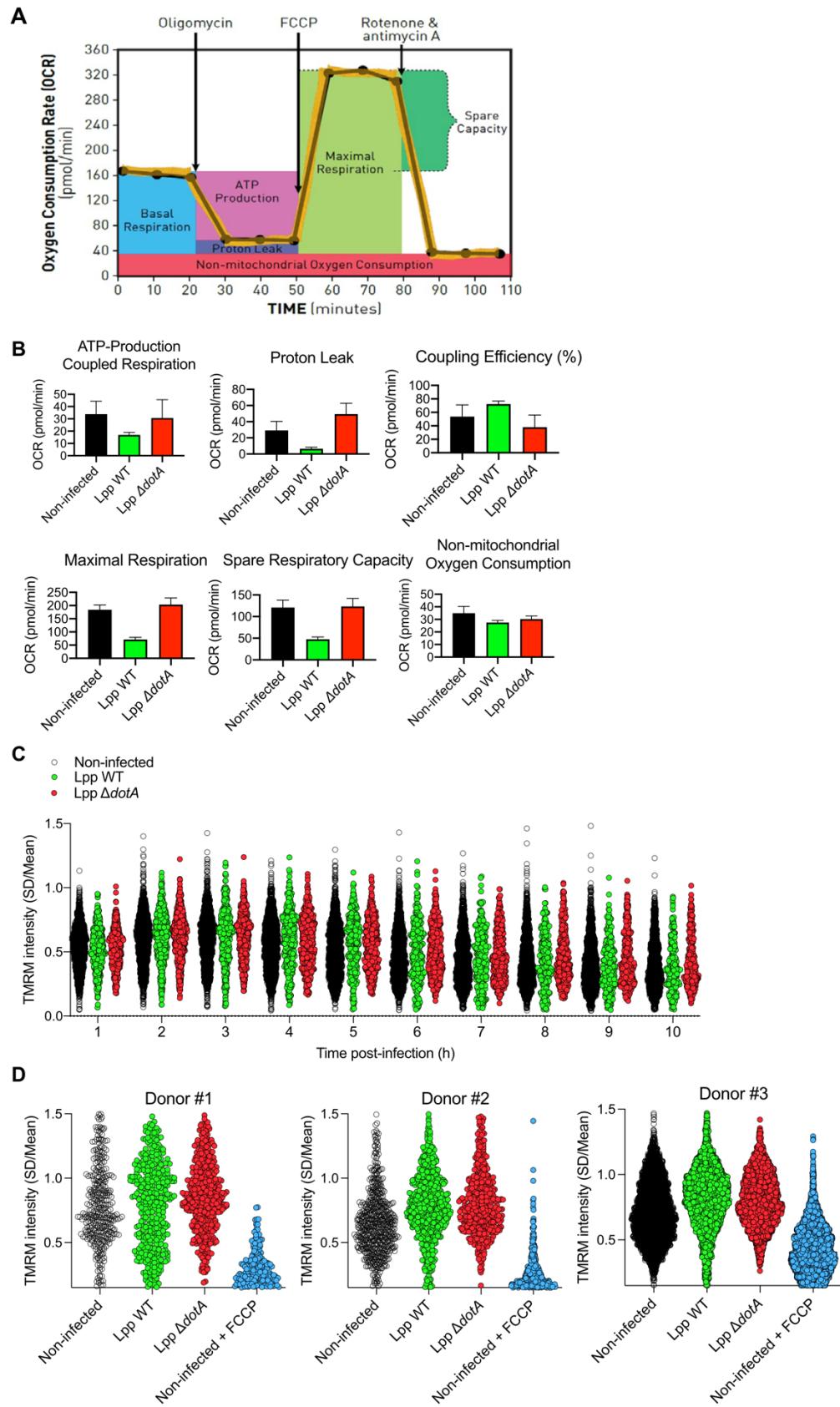


Figure S2

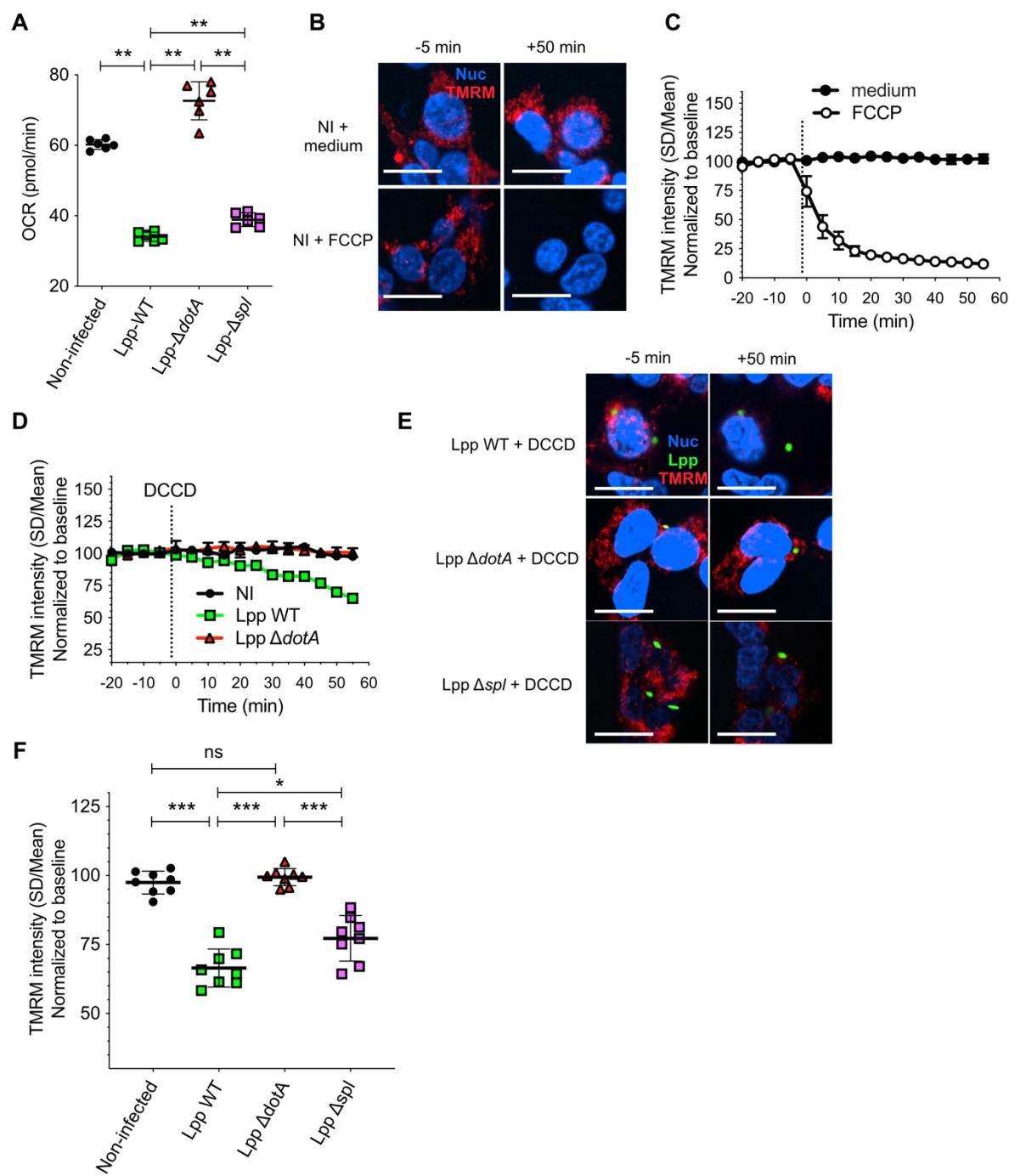


Figure S3

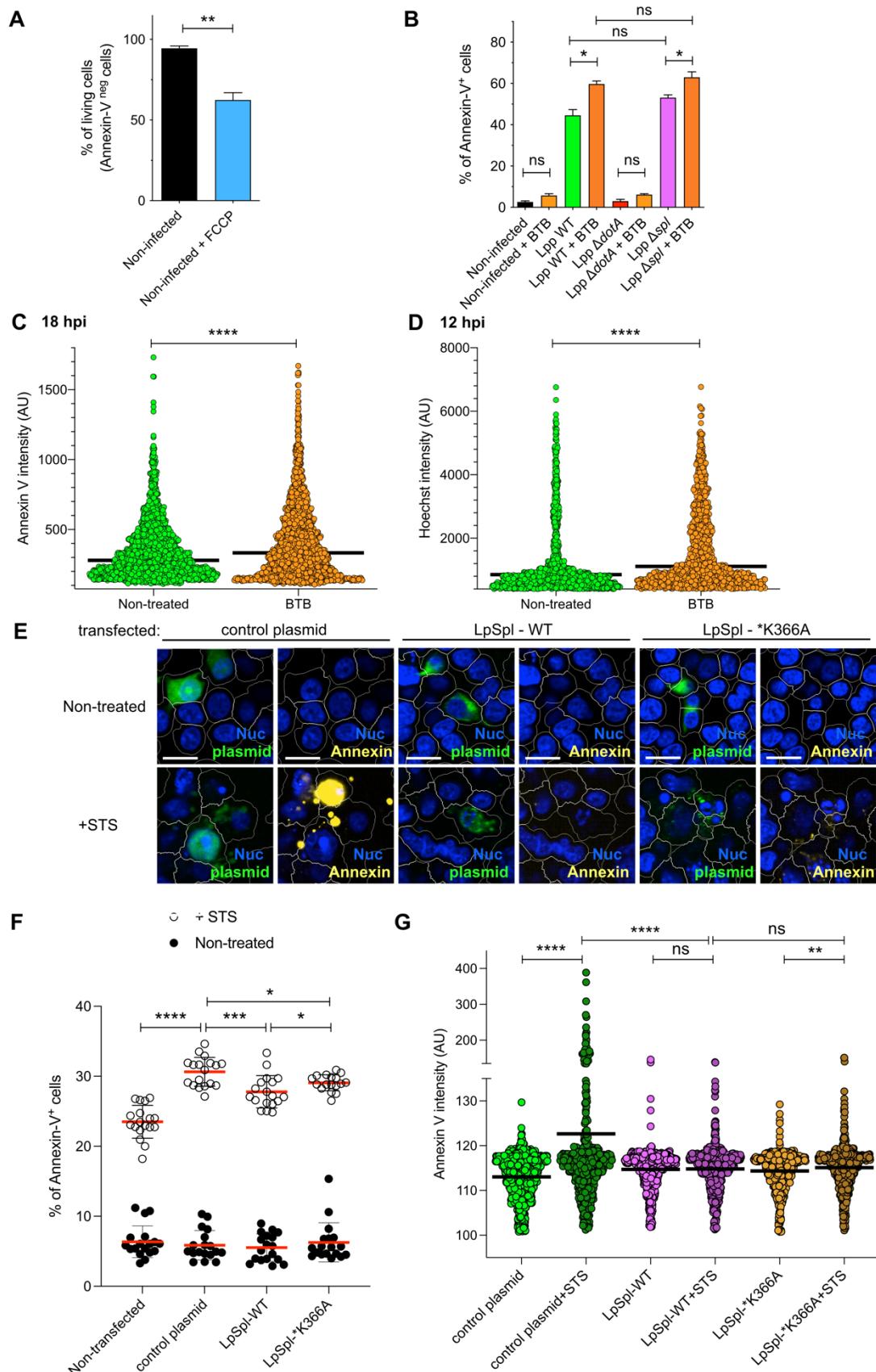


Figure S4

