Lymphatic endothelial cells are a replicative niche for *Mycobacterium tuberculosis,* Lerner et al.

Supplemental Information

Supplemental material and methods

Primary human macrophages culture

Primary human macrophages (HMDMs) were derived from peripheral blood mononuclear cells (PBMCs) from healthy donors and were isolated by a standard Ficoll-Paque PREMIUM gradient (GE healthcare, UK, #17-5442-02). Monocytes were isolated from PBMC using a magnetic cell separation system using CD14 magnetic beads (Miltenyi Biotec, Germany, #130-050-201). CD14+ monocytes were plated in standard 90 mm petri dishes at a concentration of 8 x 10^6 cells per dish in complete RPMI 1640 medium (Life Technologies, USA, #22400-089) supplemented with 10% heat-inactivated foetal calf serum and 10 ng/mL of recombinant human GM-CSF (Miltenyi Biotec, Germany, #130-093-862). Monocytes were differentiated into macrophages for 6 days at 37°C under a humidified 5% CO2 atmosphere. On day 6, the cells were detached with 0.5 mM EDTA/PBS and plated in 24-well plate at a concentration of 2 x 10^5 cells per well in fresh complete RPMI.

LDH Assay for cell viability

hLEC were infected with *Mtb* WT or left uninfected as previously described in 24-well plates for 2, 24, 48 and 72 h in the presence or absence of 200 ng/ml IFN-γ. In addition, at each time point, untreated and uninfected hLEC were treated with 1% (v/v) Triton X-100 (Santa Cruz Biotechnology, USA, #29112) for 5 minutes as a positive control for cell death. At the desired time point, 800 µl of the supernatant was removed, filtered twice through a 0.2 µm filter, and frozen at -20°C until required for the LDH assay. Lactate dehydrogenase concentration in the supernatant was measured using a LDH cytotoxicity assay (ScienCell

Research Laboratories, USA, #8078) according to the manufacturer's instructions.

Immunogold Labelling on Tokuyasu Thawed Cryo Sections

For immunogold labelling cells were fixed with 0.1% glutaraldehyde and 4% paraformaldehyde in 200 mM HEPES, pH 7.4, for the initial 30 min and thereafter with 4% PFA only overnight. Cells were scraped, cell pellet was embedded in 12% bovine gelatin and small cubes were infiltrated with 2.3 M sucrose. 100 nm sections were cut in a cryo ultramicrotome EM UC7 (Leica Microsystems, Austria) using trimtool 45° and cryo immuno diamond knives (Diatome, Switzerland). Cryo sections were thawed to do immunogold labelling. PBS with 1% fish skin gelatin (Sigma Aldrich) was used for a blocking step and to dilute labeling reagents. Primary antibodies, anti-CD63 (DSHB, USA; clone H5C6, undiluted hybridoma supernatant) or anti-LAMP-2 (DSHB, USA; clone H4B4, undiluted hybridoma supernatant) were incubated on sections for 30 min and subsequently washed on five drops of PBS. A bridging rabbit anti-mouse antibody DAKO, Denmark, #Z0259, 1:300) was incubated for 15 min and protein A-gold 10 nm (Center for Molecular Medicine, UMC, the Netherlands, 1:50) for 30 min. Labelled sections were embedded in 1.8% methyl cellulose and 0.2% uranyl acetate and examined as described for the ultrastructural analysis.

Antibodies

Antibodies used in immunofluorescence

The following primary antibodies were used: Anti-LC3B (Cell Signaling Technology, USA, #2775; 1:100 dilution, overnight at room temperature); anti-LAMP-2 (Developmental Studies Hybridoma Bank (DSHB), USA, #H4B4; 1:100 dilution, 2 hr at room temperature); anti-Cathepsin D (kindly provided by Dr. Bernard Hoflack (Dresden); 1:100 dilution, 2 hr at room temperature); anti-*Mycobacterium tuberculosis* (Acris Antibodies, Germany, #BP2027; 1:50, overnight at room temperature); anti-eNOS (Abcam, UK, #Ab5589; 1:100 dilution, 1 hr at room temperature); anti-iNOS (Novus Biologicals, USA, #NBP1-67618; 1:100 dilution, 1 hr at room temperature). The following secondary antibodies were all used at 1:800 dilutions for 1 hour at room temperature: goat

anti-rabbit AlexaFluor 546 (Life Technologies, USA, #A11010); goat anti-rabbit Cy3 (Alpha Diagnostic International, USA, #20334); goat anti-mouse AlexaFluor 546 (Life Technologies, USA, #A11003).

Antibodies used in immunohistochemistry

The specificity of secondary antibodies were confirmed either by isotype or no-primary controls using the same staining and imaging protocol as sections containing stained with primary antibodies. We were unable to identify antibodies for Prox-1 that worked for immunohistochemistry in tissues (data not shown).

Under these conditions the following antibodies diluted in PBS were used: For double staining, rabbit anti-Mtb (Menapath # MP-140-CR05, 1:100 dilution) was used with one of the following: mouse anti-PDPN (Reliatech, #18H5, 1:200 dilution), rat anti-LYVE-1 (Abcam, UK, #ab56312, 1:100 dilution), mouse Anti-CD31 (Abcam, UK, #ab9498, 1:200 dilution) or mouse anti-CD14 (AbD Serotec, UK, #MEM-18, 1:100 dilution). For triple staining, rabbit anti-Mtb (Menapath, 1:100 dilution) was used in combination with rat anti-PDPN (BioLegend, USA, #337002, 1:200 dilution) and mouse anti-eNOS (BD Biosciences, USA, #610297, 1:250 dilution), or rat anti-PDPN (BioLegend, 1:200 dilution) and mouse anti-LYVE-1 (Novus, USA, #MAB20892, 1:200 dilution). Secondary antibodies were AlexaFluor 488, AlexaFluor 546 and AlexaFluor 633 conjugated from Life Technologies.

Antibodies used for Western blot

The following primary antibodies were used overnight at 4°C: anti-LC3B (Cell Signaling Technology, USA, #3868; 1:1000 dilution); anti-p62 (Novus Biologicals, USA, #NBP1-42822; 1:2000 dilution); anti-ATG5 (Sigma, USA, #A0731, 1:500 dilution), anti-eNOS (BD Biosciences, USA, #610297; 1:250 dilution); anti-iNOS (Novus Biologicals, USA, #NBP1-67618; 1:250 dilution); anti-IDO (New England Biolabs, USA, #12006; 1:1000 dilution); anti-CD31 (Abcam, UK, #Ab28364; 1:500 dilution); anti-podoplanin (Abcam, UK, #Ab10288; 1:500 dilution); anti-LYVE-1 (Abcam, UK, #Ab33682; 1:500 dilution); anti-β-actin (Cell Signalling Technologies, USA, #4967; 1:10000 dilution). The following horseradish

peroxidase (HRP) conjugated secondary antibodies were used at 1:10000 dilution for 1 hour at room temperature: anti-mouse HRP (Promega, USA, #W4021) and anti-rabbit HRP (Promega, USA, #W4011).

Supplemental Figure legends

Supplemental Figure 1 – Human lymph node resident lymphatic endothelial cells (hLECs) host *M. tuberculosis*

A- Tissue section of a granuloma present in a human lymph node with associated blood vessels (Bv) and lymphatic vessels (Lv) stained for H&E and acid fast bacilli (AFB). Zoomed region shows association of *M. tuberculosis* with endothelial cells (black arrows). **B-** Lymphatic vessel containing a myelocytic cell infected with *M. tuberculosis* (Mtb-488) and showing an infected PDPN+ cell lining the vessel (white arrows). Scale bar is 10 μm.

Supplemental Figure 2 – Phenotypic analysis of commercially obtained hLECs

A– Western blots were performed on whole cell lysates of hLEC, primary human umbilical vein endothelial cells (HUVEC) and primary human monocyte derived macrophages (HMDM) to check that hLEC were positive for the specific LEC markers PDP and LYVE-1. β-actin was used as a loading control. **B-** i) Western blot of the IFN-γ induced protein indoleamine 2,3-dioxygenase (IDO) on samples from whole cell lysates of uninfected or Mtb WT hLEC in the presence (red bars) or absence (blue bars) of IFN-γ. ii) Measurement of the IFN-γ induced cytokine CXCL10 (IP-10) from supernatants of uninfected hLEC in the presence (red squares) or absence (blue circles) of IFN-γ. Data are from five biological repeats and the means ± SEM are shown.

Supplemental Figure 3- *M. tuberculosis* replicates in hLECs without affecting cell viability

A- Measurement of the secreted LDH concentration from uninfected and Mtb WT infected hLEC at 2 h, 24 h, 48 h and 72 h. Cells were also lysed at each time point using Triton X-100 as a positive control. Data are from three biological replicates and show means ± SEM. **B-** Control experiment for Figure 2E showing the CFU

recovered from the supernatant in the wells of Mtb WT infected hLEC at 2, 24, 48 and 72 h post-infection, in the presence (blue line) or absence (red dashed line) of IFN-γ in the supernatant. Growth is expressed as percentage change from the 2 h time point. Data are means ± SEM of four biological replicates. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test; ns = $p>0.05$. **C-** Correlation between hLEC cell surface area and intracellular fluorescence intensity of Mtb WT at 2 h, 24 h, 48 h and 72 h in the presence or absence of IFN-γ. **D**– Growth of Mtb WT in hLEC (red circles) and HMDM (black circles). Data are means ± SEM of CFU from three technical replicates from one biological experiment. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test; ns = $p>0.05$, * = $p<0.05$. **E**- 10μg/ml gentamycin (gent) present in the infection medium (consisting of endothelial cell medium (ECM)) is sufficient to prevent growth of *M. tuberculosis* (as measured by OD_{600}).

Supplemental Figure 4- A subpopulation of intracellular *M. tuberculosis* is associated with the late endocytic marker LAMP-2

A- Representative images of hLEC infected with Mtb WT taken using confocal microscopy at 2 h, 24 h, 48 h and 72 h post-infection in the presence or absence of IFN-γ. Images show bacteria expressing EGFP, endogenous LAMP-2 labelled with AlexaFluor-546 and DNA labelled with DAPI. **B**- Quantification of the association of LAMP-2 with Mtb WT from images such as those in panel A (see materials and methods for explanation). Quantification is from images taken at 2 h, 24 h, 48 h and 72 h post-infection, with at least six images taken from each condition, from 3 biological replicates. N is the total number of individual bacterial entities measured in each condition, and the displayed percentage refers to the proportion of LAMP-2 positive population (this is the population within the dotted black box). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test; ns = $p>0.05$, *** = $p<0.001$.

Supplemental Figure 5- Subcellular localisation of *M. tuberculosis* in hLECs

A- CD63 and LAMP-2 immunogold labelling on Tokuyasu thawed cryo sections of resting hLEC infected with Mtb WT for 48 h. * are cytosolic bacteria, yellow lines

are host membranes. Scale bars are 500 nm. **B-** Example TEM images of hLECs infected with *M. tuberculosis* showing the variety of subcellular localisations in which they can be found. A' and A'' are in phagosomes, B' and B'' are in the cytosol, C' and C'' are in autophagosomes, D' and D'' are in late endocytic/lysosomal compartments. See materials and methods for the defined rules of categorisation. All scale bars are 500 nm.

Supplemental Figure 6- IFN-γ modulates autophagy in hLEC

A- Quantification of the number of LC3 positive vesicles per cell in uninfected hLEC in the presence (red dots) or absence (blue dots) of IFN-γ at 48 h. Data are means ± SEM of at least three biological replicates. The fold change between the means are displayed; *** = $p<0.001$. **B**- The data from **Figure 4B** was normalised to –IFN-γ for each infection condition and replotted. **C**- Western blot with quantification showing the siRNA-mediated knockdown of ATG5 protein in resting and activated hLEC that had been infected with Mtb WT for 48 h. Lanes were run on the same gel but were non-contiguous. The amount of protein knockdown is expressed relative to the level found in control cells treated with a random siRNA sequence (SCRAMBLED). **D-** Activated (red dots) or resting (blue dots) uninfected hLECs were treated with or without 250 μM of the NOS inhibitor L-NMMA for 48 h and then exposed to 10 μM DAF-FM which becomes fluorescent after reacting with nitric oxide. The fluorescence intensity per cell was plotted. Bars are means ± SEM of two biological replicates; ns = $p>0.05$, * = $p<0.05$, *** = $p<0.001$, Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test.

Supplemental Movie 1- *M. tuberculosis* replicates intracellularly in hLEC

Live cell imaging of hLEC infected with Mtb WT (tagged with EGFP) over the course of 6.5 days. Frames were taken every five minutes (see materials and methods) and the movie was analysed to quantify *M. tuberculosis* growth (**Figure 2D**).

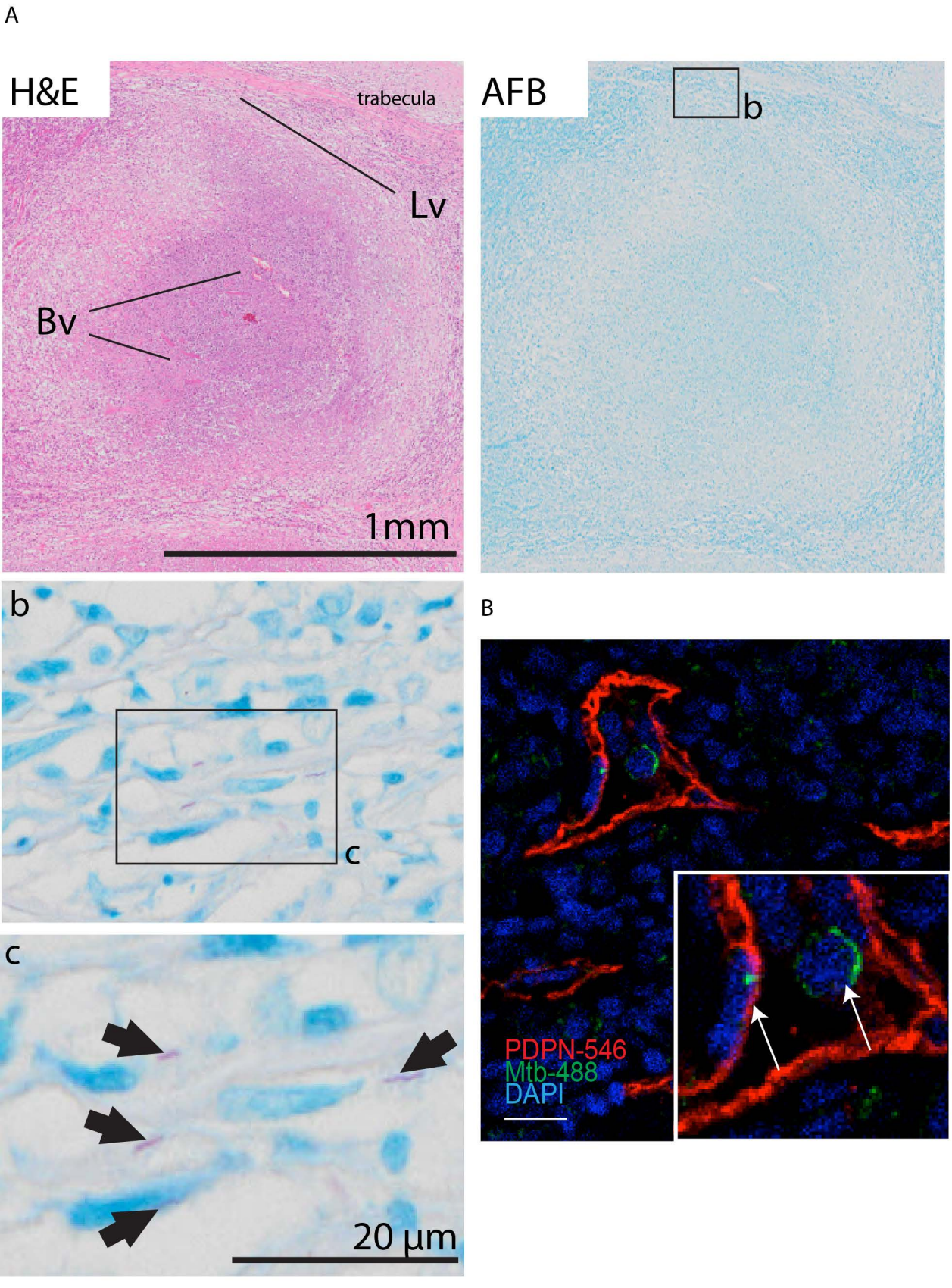
Supplemental Movie 2– *M. tuberculosis* can replicate in an LC3+ membrane-bound compartment

Live cell imaging of hLEC transduced with LC3-RFP and infected with Mtb WT (tagged with EGFP) of the course of 5.2 days. Frames were taken every two hours for the first 12 hours and every 30 minutes thereafter. The sample was used for correlative light electron microscopy (CLEM) (see materials and methods and **Figure 5D**).

Supplemental Movie 3- SBF SEM images and 3D Reconstruction of *M. tuberculosis* in an LC3+ membrane-bound compartment

Movie showing the SBF SEM serial images and 3D models of Mtb WT (green) and LC3-positive membranes (red) in **Figure 5D**.

Supplemental Figure 1



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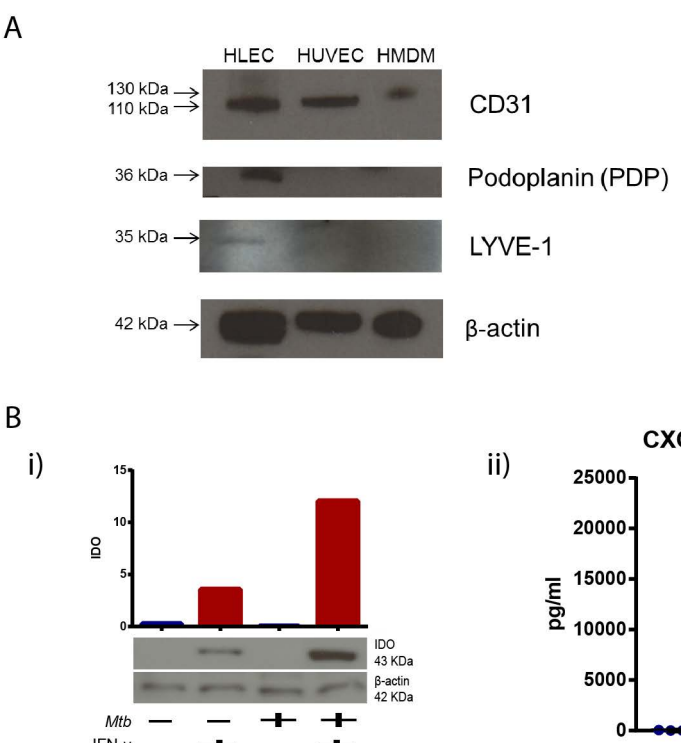

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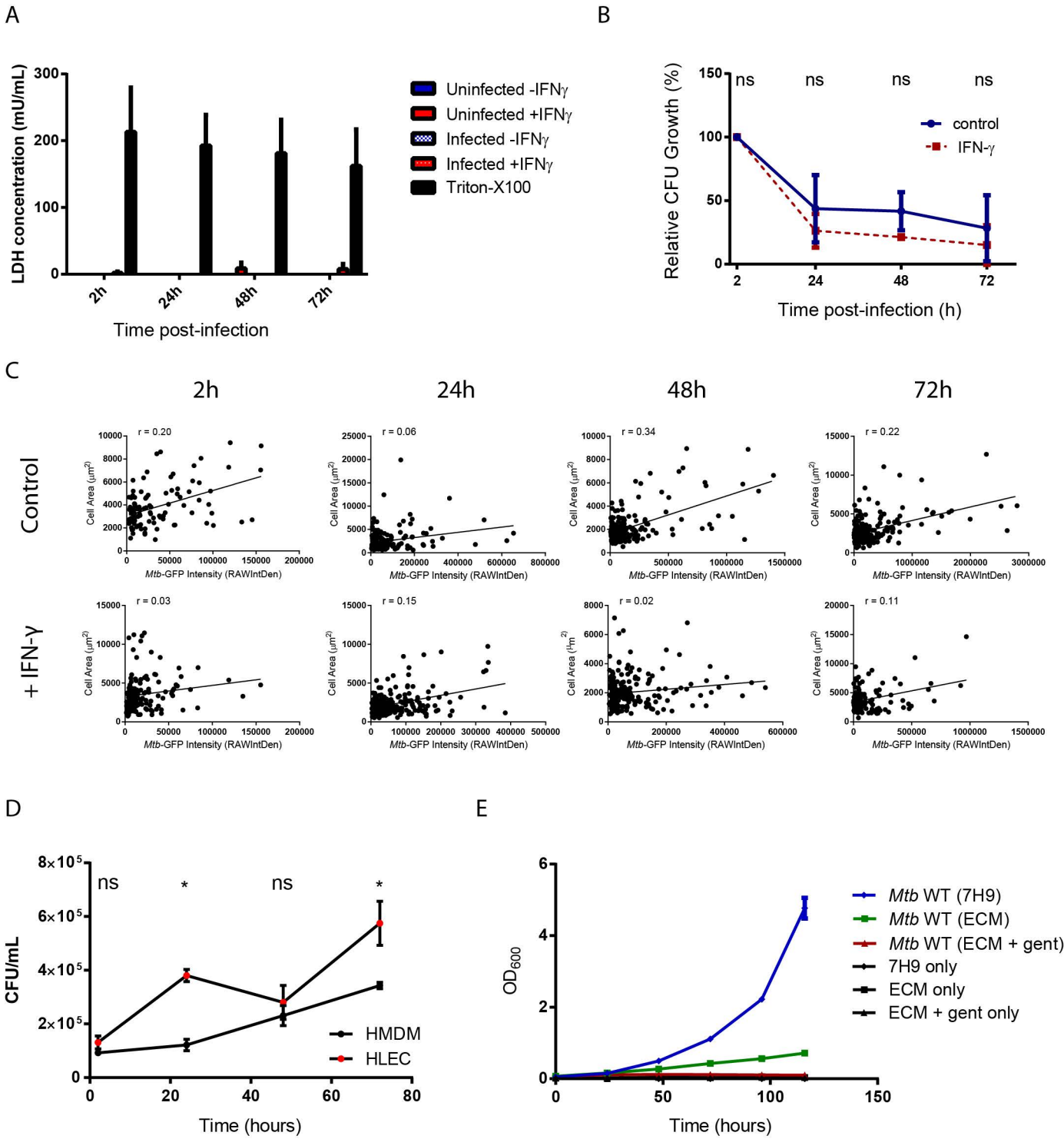

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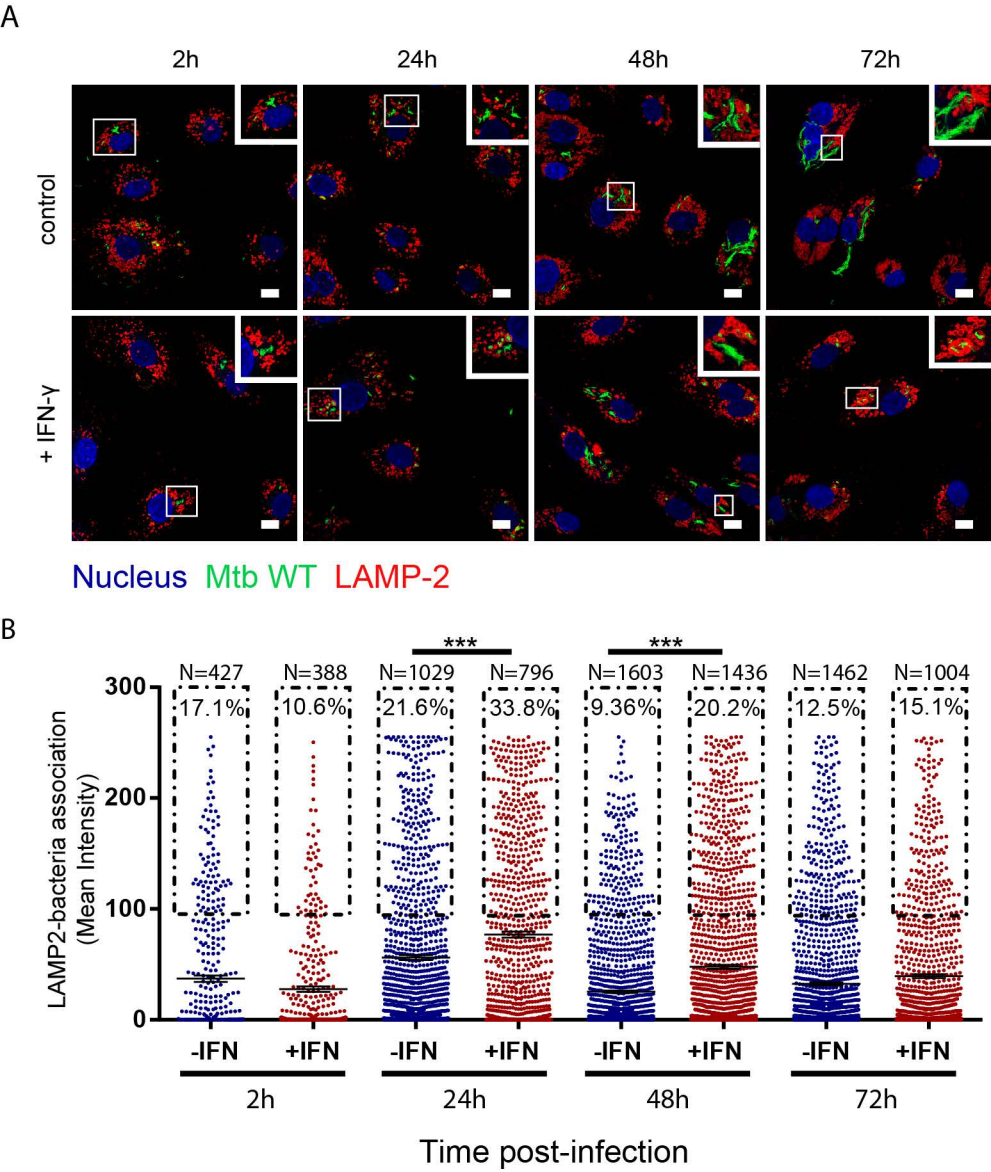

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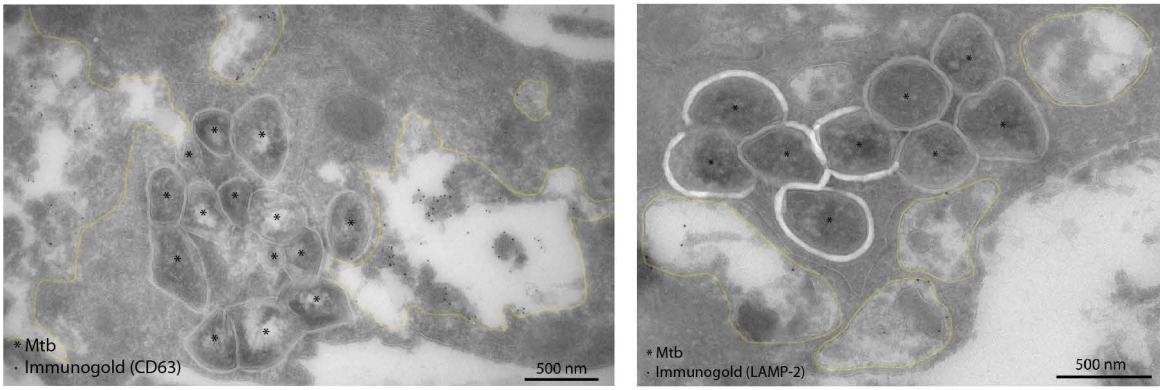

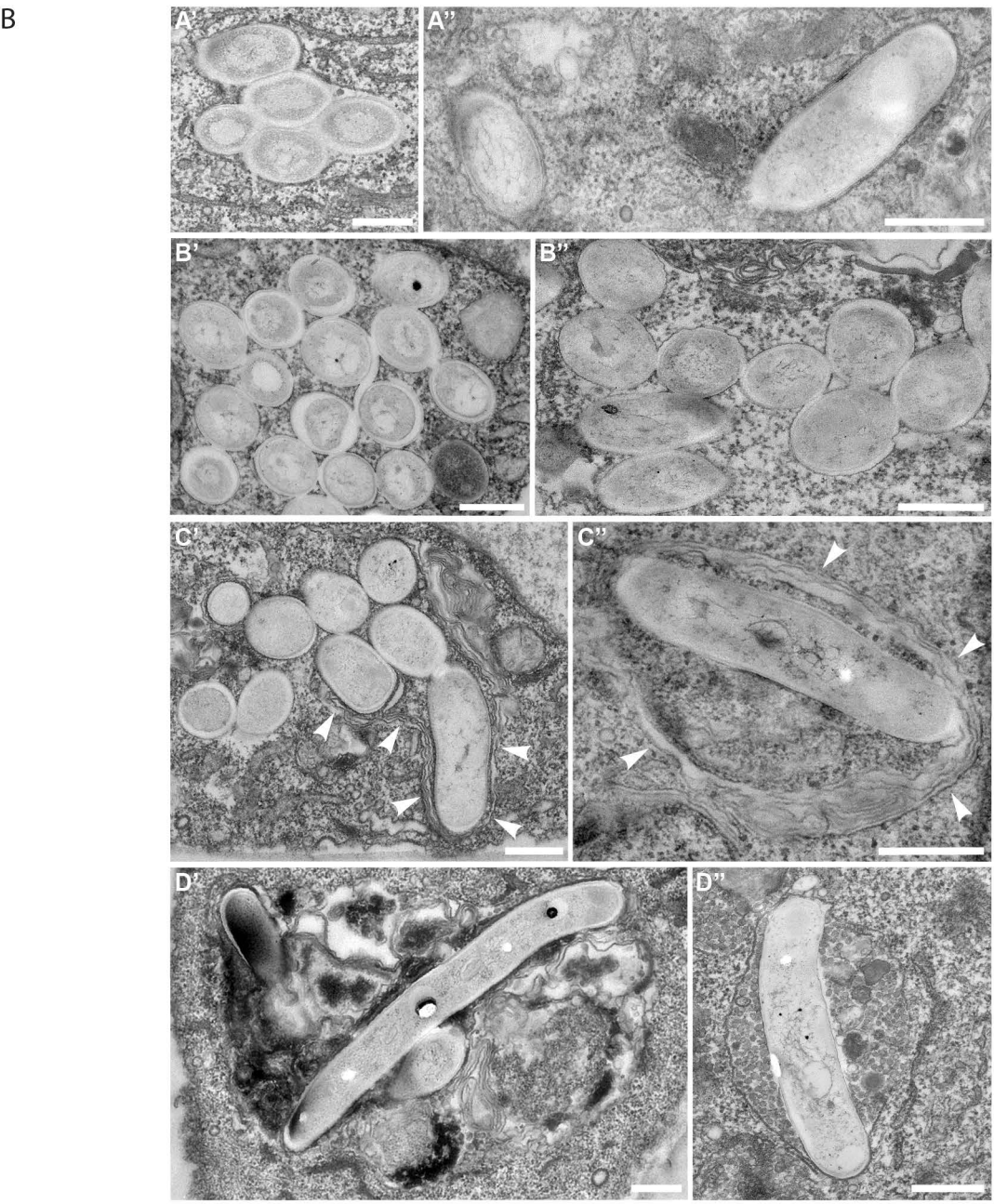

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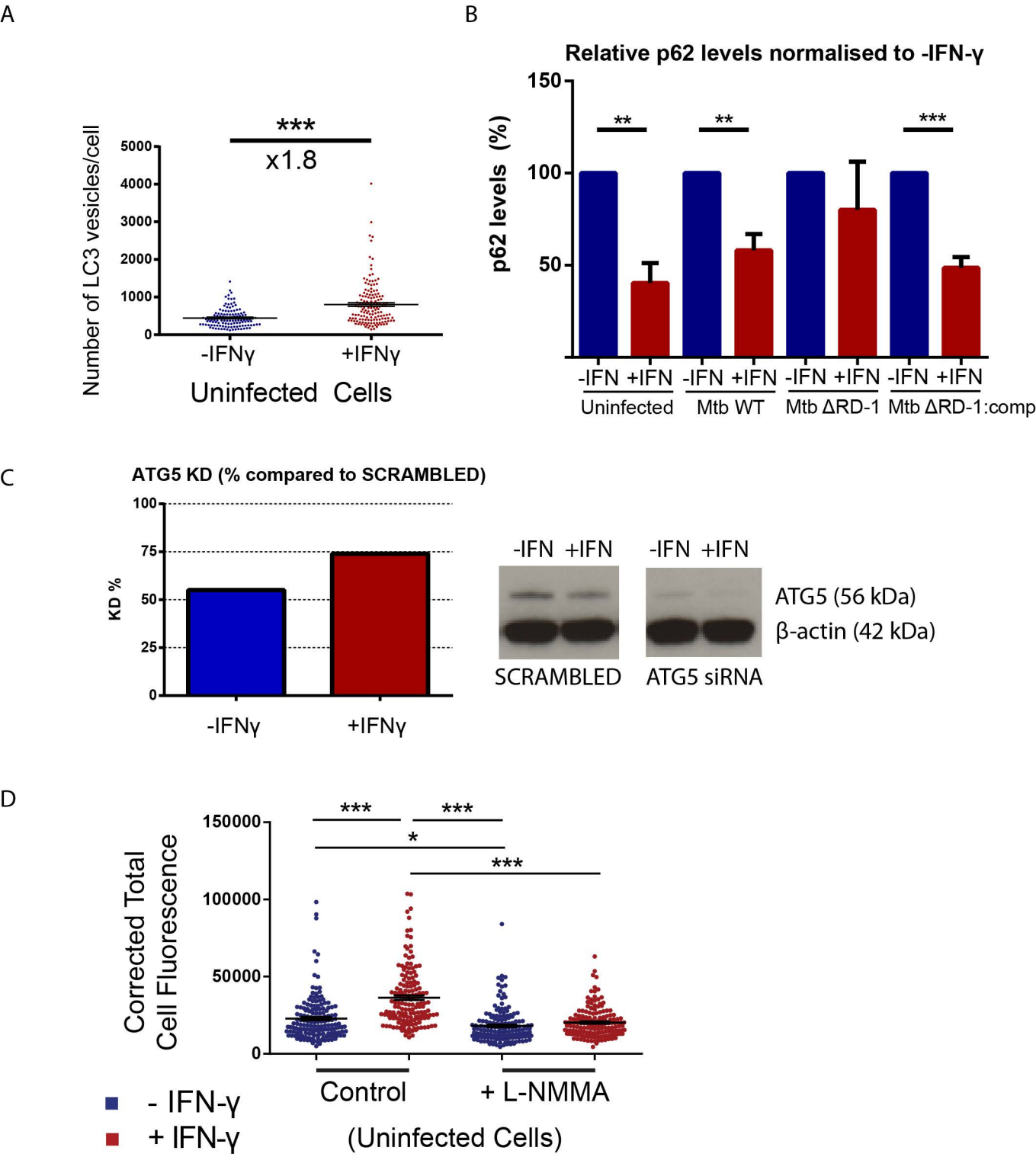

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