Supplementary information

Material and Methods

Antibodies and phalloidin

Antibodies against the following antigens were obtained from: PM4C4 from Dr. J. Garin (CEA, Grenoble, France); vacuolins (221-1-1) from Dr. M. Maniak (Kassel University, Germany); p80 from Dr. P. Cosson (University of Geneva, Switzerland). Secondary antibodies were goat anti-mouse or goat anti-rabbit IgG coupled to Alexa488 or Alexa594 (Molecular Probes) and Cy5 (Rockland). Phalloidin staining was performed with Alexafluor568-phalloidin or Alexafluor633-phalloidin (Molecular Probes). The monoclonal anti-ESAT-6 HYB076-08 antibody was obtained from Biotrend and used at a dilution 1:500 to detect expression of ESAT-6 on western blots. To generate anti-mycobacterium serum, *M. marinum* wild type was cultured in 7H9 plus OADC until OD_{600} 1. Bacteria were killed by incubation with 4% paraformaldehyde, washed and resuspended in PBS. Rabbits were immunized with three injections of 10^7 cfus (Eurogentec).

Dictyostelium cell culture

Wild type *Dictyostelium discoideum* (Ax2) was cultured axenically at 22°C in HL5c medium (Formedium). *Dictyostelium* mutants were kindly provided by Drs. M. Maniak (Kassel University, Germany; *vacA*- and *vacB*- strains) and F. Rivero (Cologne University, Germany; *racH*- strain).

Mycobacteria strains, plasmids and culture

Mycobacteria were cultured in 7H9 + OADC supplement (oleic acid, albumin, dextrose, catalase) (Difco) as described (*1*). Dr. L. Ramakrishnan (Washington University, Seattle, USA) provided *M. marinum* wild type and *M. marinum* ΔRD1. *M. avium* (MAC101, (*2*)) was kindly

provided by Dr. G Griffiths (EBML, Heidelberg, Germany). GFP- and DsRed-expressing bacteria were obtained by transformation with msp12::GFP or pR2Hyg vectors respectively into *M. marinum* wild type and *M. avium*. Cultivation was in the presence of 20 µg/ml kanamycin for GFP-expressing bacteria and 30 µg/ml hygromycin for DsRed-expressing *M. marinum*. The *M. tuberculosis* strain CDC1551 expressing a GFP variant roGFP1-12 (*3*) from the hsp60 promoter of plasmid pVV16 was grown in static cultures to mid-log phase (OD600=0.5-0.6) in 7H9 + OADC containing 25 mg/ml kanamycin.

Culture and infection of microglia cells (BV-2)

BV-2 cells (*4*) were maintained in RPMI1460 medium with GlutamaxI (Gibco) and 10% fetal calf serum at 37°C and 5% CO_2. For infection, cells were allowed to adhere to µ-Dishes (Ibidi) at 32°C and 5% CO_2 for 6 hours. GFP-expressing *M. marinum* were washed twice with RPMI1460-GlutamaxI medium and added to the cells at an MOI (multiplicity of infection) of 5. Bacteria were centrifuged onto the attached cells for 15 min at 500 g. The cells were allowed to phagocytose for an additional 2 hours at 32 °C before extracellular bacteria were washed off by rinsing attached cells with medium. Finally, cells were overlayed with medium plus 10% FCS and 5 µg/ml streptomycin. At 48 and 60 hpi, attached cells were fixed with 4% PFA in PBS for 1 hour and processed for fluorescence microscopy.

Generation of ESAT-6 expressing *Dictyostelium*

M. marinum genomic DNA was isolated form *M. marinum* wild type bacteria and ESAT-6 encoding DNA amplified by PCR with primers specific for the ESAT-6 encoding gene MM5450 (esat6for: 5′-TCCAAGCTTAAAAAATGACAGAACAGCAG-3 and esat6rev: 5′-GGATCTAGACTAAGCAAACATCCCCGTG-3). The 309 bp product was cloned into pGEM-

T (Promega) and sequenced (Eurogentec). Subsequently it was cloned into the construct MB38 (*5*) and transformed into *Dictyostelium* cells, which stably express the tetracycline-sensitive transactivator (*5*). Transformants were selected and maintained under blasticidin (20 µg/ml), G418 (10 µg/ml), as well as tetracycline (10 µg/ml) to suppress the expression of ESAT-6. Induction of ESAT-6 expression by tetracycline removal was confirmed by western blotting (Fig. 4C).

Infection assay

All infections were performed as previously described (*1*). In brief, mycobacteria were centrifuged onto attached *Dictyostelium* cells at an MOI of 10 to promote efficient and synchronous uptake. Centrifugation was performed at RT at 500 g for two periods of 10 min. After an additional 10-20 min, uningested bacteria were washed off with medium HL5c and infected cells resuspended in HL5c containing 5 µg/ml streptomycin. The infected culture was incubated with shaking (130 rpm) at 25°C for up to 48 hours. The first sample was taken as soon as the infected cells were transferred into HL5c containing 5 µg/ml streptomycin and is referred to as 0.5 hpi. For infections with *M. tuberculosis*, infections were not kept under shaking conditions, but maintained adherent on coverslips at 25 °C for up to 48 hr.

Flow cytometry assay (FACS)

To monitor infection dynamics by FACS, *Dictyostelium* cells were infected with GFP-expressing bacteria as described above. Aliquots were taken at 0.5, 12, 21 37 and 43 hpi and diluted with 500 µl Soerensen buffer (14.7 mM KH_2PO_4, 2.5 mM $NaHPO_4$, pH 6.3) containing 5 mM sodium azide to strip the cell surface of loosely attached bacteria. After pelleting the cells (4 min, 14000 rpm), they were resuspended in 500 µl Soerensen with 20 mM sorbitol. Cells were kept on ice

and, immediately before analysis, fluorescent latex beads (4.5 µm YG beads, Molecular probes, 100 beads/µl) were added as an internal concentration standard. Flow cytometry was performed with a FACScalibur (Beckton Dickinson) and data analyzed with FlowJo (TreeStar, USA).

Dissemination assay

This assay is adapted from the commonly used microplaque assay to monitor and quantitate cell-to-cell spreading of bacteria via fluorescence microscopy. A so called "donor strain" (either *Dictyostelium* wild type or *racH*- strain) is infected as described above at an MOI of 100 with DsRed-expressing *M. marinum*. At 12 hours post infection, green fluorescent wild type *Dictyostelium* (acceptor strain, GFP-ABD-expressing) is added to the infection at a donor:acceptor ratio of 1:5. At the indicated times, cells were fixed for fluorescence microscopy as described for immunofluorescence. At each time point, infected cells were counted and scored into 3 groups according to the number of bacteria present (1-3, 4-10 and >10 bacteria).

Cell integrity assay

Dictyostelium cells were infected as above with GFP-expressing wild type *M. marinum*. At 37 hpi, $2x10^6$ infected cells were plated on plastic dishes (µ-Dish, 3 cm diameter, Ibidi) in HL5c. After 10 min, DAPI was added to a final concentration of 0.3 µM and cells were monitored for two hours by phase-contrast and fluorescence microscopy using a water-immersion objective (Apochroplan 100x NA 1.0 Ph3, Zeiss, Jena, Germany), and recorded with a CCD camera (Sensicam, PCO AG, Kelheim, Germany). In order to generate a positive control for DNA staining resulting from cell leakage, we generated transient membrane pores by electroporation. Approximately $2x10^6$ infected cells were spun 5 sec in a tabletop centrifuge. The cell pellet was resuspended in 100 µl cold electroporation buffer (10 mM K_2HPO_4/ KH_2PO_4, 50 mM sucrose, 1

mM $MgSO_4$, 1 mM $NaHCO_3$, 1 mM ATP, 1 μM $CaCl_2$, pH 6.2) with 0.3 μM DAPI. The cells were electroporated in precooled BTX electroporation cuvettes (2 mm gap) with a BTX square electroporator (settings: 300 V, pulse duration 2 ms, 5 unipolar pulses at 1 sec intervals). Electroporated cells were allowed to recover on ice for 10 minutes before being transferred into plastic dishes (μ-Dish) in HL5c. After an additional 10 minutes, the majority of cells had attached and were monitored with identical settings as for non-electroporated cells.

Antibody accessibility assay

Dictyostelium cells were infected with GFP-expressing *M. marinum* as described above. At 32 hpi an anti *M. marinum* serum was added to the cells in suspension at a dilution of 1:100 for 20 min. Subsequently, aliquots of infected cells were centrifuged onto coverslips and allowed to attach. Unbound antibody was removed by washing cells with medium. Finally, the cells were fixed with 4% paraformaldehyde for 45 min. Immunofluorescence was performed as described (*6*), visualizing the *M. marinum* antiserum with a secondary antibody directed against rabbit IgG.

Immunofluorescence and phalloidin staining

Dictyostelium cells were infected as above with either GFP- or DsRed-expressing mycobacteria. At the indicated time points, an aliquot of cells was centrifuged onto poly-lysine coated coverslips (500 g, 2 min) and allowed to settle for 2 min. Cells were then either fixed by rapid freezing in cold methanol (*6*) or in Soerensen buffer containing 4% paraformaldehyde. Immunofluorescence and phalloidin labeling was performed as described (*6*). Fluorescence images were documented using a Leica SP2 confocal microscope with a 100x 1.4 NA oil-immersion objective. Recording parameters for fields of 1024 x 1024 pixels with appropriate electronic zoom (2-8x) were 2x line and 2x frame averaging with 0.1-0.32 μm vertical steps.

Time-lapse fluorescence microscopy

GFP-ABD-expressing *Dictyostelium* cells were infected with DsRed-expressing *M. marinum* as described above. At 35 hpi aliquots of cells were transferred into 35 mm glass-bottom dishes (Matek) and allowed to attach for 10 min. Recordings were performed using a Leica LF6000LX microscope (100x 1.4 NA oil immersion objective) with time intervals of 1 min over a total length of up to 20 min. Z-stacks of 4 µm depths (600 nm steps) for each channel (GFP, DsRed and phase-contrast) were documented for each field with 2x2 binning. Using ImageJ, maximum projections were generated from 2 selected sections for each time-point and combined to image series (Fig. 1 C-E, fig. S5A, B) or time-lapse movies (Movies S1-S7).

Scanning electron microscopy

For ultrastructural analysis by scanning electron microscopy, infected cells were fixed on coverslips by rapid freezing (*6*) and washed in buffer (PBS). Samples were subsequently dehydrated in a graded series of propanol, and critical point dried using carbon dioxide. The cells were then rotary coated by electron beam evaporation with platinum-carbon (3 nm) in a Balzers 300 freeze etching device (Bal-Tec, Principality of Lichtenstein). Samples were viewed in a field emission scanning electron microscope (Hitachi S-5200) at an accelerating voltage of 10 kV using the secondary electron signal.

Transmission electron microscopy

To prepare samples for transmission electron microscopy, infected cells in suspension were fixed for 2 hours with 0.2% OsO4 and glutaraldehyde in HL5c, according to (*7*). Subsequently, fixed samples were dehydrated, embedded in Epon resin and processed for conventional electron

microscopy as described previously (*8*). Grids were examined with a Tecnai transmission electron microscope (FEI, Eindhoven, The Netherlands).

Supplemental Figures and Movies legends.

The movies correspond to low (S1) and high (S2-S7) magnification time-lapse microscopy of GFP-ABD-expressing *Dictyostelium* (green) infected with DsRed expressing *M. marinum* (red). Movies S3-S7 correspond to galleries of still frames in Fig.1C-E and fig. S5A, B.

Movie S1: Infected cells can be captured in a wide variety of situations, performing normal motility, cell division or bacteria ejection.

Movie S2: Infected *Dictyostelium* cells can undergo apparently normal cell division.

Movie S3: An actin-flash is produced at a point of contact between *M. marinum* (red) and the plasma membrane.

Movie S4: Nonlytic ejection of *M. marinum* (red) occurs through an F-actin dense structure (bright green) termed ejectosome.

Movie S5: *M. marinum* (red) can be seen spanning the host cell plasma membrane through an ejectosome (bright green), while the cell maintains normal motility over the recording time of 17 minutes.

Movie S6: Multiple *M. marinum* (red) attached to the plasma membrane of a *Dictyostelium* cell via ejectosomes (bright green structures).

Movie S7: Direct cell-to-cell transmission from a donor cell into an acceptor cell, through an ejectosome and via a phagocytic cup.

Figure S1. Schematic representation of the fate of pathogenic *M. marinum* in *Dictyostelium* during uptake, establishment of the niche, proliferation and ejection. Bacteria are taken up by phagocytosis (phagocytic cup indicated in red). Most bacteria successfully prevent the delivery of the vacuolar-H^+-ATPase and hydrolases, such as Cathepsin D, inducing the phagosome to bifurcate from normal maturation. Pathogens proliferate in a compartement that strongly accumulates *Dictyostelium* flotillin (vacuolin), ruptures and releases bacteria into the cytosol. Cytosolic bacteria are ejected from the host cell in a nonlytic fashion via a dense F-actin structure (ejectosome; indicated as a red barrel) starting a new cycle of uptake/proliferation/ejection. Host factors that play a role in efficiency of establishment of a niche (VacB and RacH) and ejection (RacH) are indicated with a blue background, bacterial factors (the RD1-locus and the secreted peptide ESAT-6) are shown on a grey background. Based on the scheme from (*1*).

Figure S2. FACS analysis of an infected culture and bacterial inoculum. **(A)** An FSC-SSC plot allows for clear distinction between live cells and smaller debris and extracellular bacteria. An FL1-SSC plot allows to further separate the live cell population in non-infected and infected cells, and the cell debris from extracellular bacteria based on their fluorescence. **(B)** The FACS patterns of the inoculum confirmed the identity and distribution of the extracellular bacteria (FSC-SSC and FL1-SSC plots). The addition of a reference number of fluorescent beads (4.5 µm YG beads, Molecular probes; indicated in the FSC-SSC and FL1-SSC plots) allows for quantification of the FACS-data per volume of infected culture.

Figure S3. *Dictyostelium racH-* cells are deficient at spreading of an *M. marinum* infection.
(A) A representative experiment of quantitative FACS-analysis of wild type and *racH-* cells infected with GFP-expressing *M. marinum* at the indicated times (0.5, 21 and 37 hpi) is shown.

In SSC versus FL1 plots, three populations can be distinguished: uninfected cells (1), infected cells (2) and extracellular bacteria (3). Histograms show the distribution of fluorescence of infected cells (FL1). **(B)** Evolution of extracellular bacteria counts over time. **(C)** Counts of extracellular bacteria at 37 hpi. **(D)** Mean fluorescence (FL1) of the infected cells at 37 hpi.

Figure S4. Induction of F-actin structures in microglial cells (BV-2) during late stages of *M. marinum* infection

Adherent microglial cells, BV-2, were infected with GFP-expressing wild type *M. marinum* and incubated at 32°C. At 48 and 60 hpi, infected cells were fixed and stained for F-actin. **(A)** At this late stage of infection, cytosolic bacteria were often observed to form long, curved actin-tails (white arrowheads). **(B)** Infected cells form a variety of actin-rich structures, including densities at the level of the plasma membrane and long cytoplasmic bridges (white arrowheads). **(C, D)** In many cases, bacteria induced actin-dense filopodia-like structures (white arrowheads) that can apparently be captured by neighboring cells (black arrowheads), representing a form of direct cell-to-cell transmission. Scale bars 1 μm.

Figure S5. Time-lapse microscopy monitors that nonlytic release of *M. marinum* from infected cells occurs through an ejectosome.

Live GFP-ABD (*9*) expressing *Dictyostelium* cells (green) infected with DsRed-expressing *M. marinum* (red) were imaged at 35 hpi for the indicated times (upper left corner). **(A)** Local and transient F-actin accumulations (circle) are induced at points of contact between cytosolic mycobacteria and the plasma membrane. **(B)** Multiple bacteria (asterisks) attached to a host cell via ejectosomes (white arrowhead). **(C)** Movement of cytosolic bacteria (blue) through an ejectosome (F-actin, green) induces an outward plasma membrane deformation decorated by anti-PM4C4 labeling (small arrows), but the intracellular part of the ejecting bacterium is devoid

of labeling (black arrowhead). The membrane is ruptured at the tip of the ejecting bacterium (asterisk). **(D)** Some extracellular bacteria were positive for PM4C4 (small arrows). **(E)** Live cells were monitored by phase-contrast (red) at 37 hours after infection by *M. marinum* (green). Prolonged incubation in the presence of a membrane impermeant DNA dye (DAPI), did not result in nuclear staining, even in cells undergoing bacteria ejections (dashed circles). **(F)** As a control, generation of transient plasma membrane disruptions by electroporation resulted in nuclear staining (blue). Note that both infected (arrowhead) and uninfected cells (arrow) can be transiently permeabilised. Only few cells are lysed by the procedure (asterisk), with maybe a small bias for heavily infectecd cells, as judged by the increase of extracellular bacteria compared to **(E)**. Immunofluorescence of fixed cells showed that ejectosomes (circle) are enriched for specific cytoskeletal proteins, such as Myosin IB **(G)** and coronin **(H)**, but apparently not others such as myosin II **(I)** and Arp3 **(J)**. Scale bars 1 μm **(A-D, G-J)** and 10 μm **(E, F)**. Scale bars 1 μm.

Figure S6. In *Dictyostelium, M. tuberculosis* translocates into the cytosol and forms ejectosomes.

Dictyostelium cells were infected with GFP-expressing *M. tuberculosis* as described and incubated in adherent conditions, on coverslips, at 25 °C. At 24 and 48 hpi, cells were fixed with cold methanol **(A)** or 4% paraformaldehyde **(B-D)** and stained for vacuolin **(A)** or the plasma membrane markers p80 **(B)** and 4C4 **(C, D)**, together with actin **(B-D)**. **(A)** *M. tuberculosis* was found in spacious vacuoles strongly decorated with vacuolin. Infrequently, but in a clearly identifiable process, the *M. tuberculosis*-containing vacuole appeared to break, releasing the bacteria into the cytosol (black arrowheads). Overall, this pattern of events very closely resembles the sequence observed for *M. marinum* infection of *Dictyostelium* and macrophages

(*1*). **(B-D)** Like *M. marinum*, but at a lower frequency, *M. tuberculosis* also induced ejectosomes in infected *Dictyostelium*. Ejecting bacteria were found to pass through the plasma membrane at a region of more or less prominent F-actin staining (white arrowheads). The extracellular part of bacteria was labeled with the plasma membrane markers p80 (arrows, **B**) or PM4C4 (arrows, **C**), rupturing at the tip (asterisk, **B**), in contrast to the intracellular part, that was free of any staining (black arrowhead, **B**). Sometimes, extracellular *M. tuberculosis* were found to be wrapped in plasma membrane as indicated by labeling against PM4C4 (arrows, **D**). Scale bars 1 μm.

Figure S7. Transmission electron micrographs of *Dictyostelium* cells at late stages of infection by *M. marinum* and *M. tuberculosis.*

Both pathogens, wild type *M. marinum* and *M. tuberculosis*, were found in spacious, membranous compartments (asterisk). Very frequently for *M. marinum* and much less so for *M. tuberculosis,* these compartments were observed to rupture (black arrowheads) and bacteria were released into the cytosol (white arrowheads). Scale bars 1 μm.

Figure S8. The success of infection correlates with ejectosome formation

(A) In both *Dictyostelium* mutants, *vacA*- and *vacB*-, *M. marinum* was observed to break the replication compartment and translocate into the cytosol (upper row). In both cases formation of ejectosomes was observed (lower row, white arrowheads), with a topology of plasma membrane marker and rupture at the tip of ejecting baceria as in Fig. 3. **(B)** To test, if the outcome of an infection correlates with ejectosome formation, *Dictyostelium* wild type and mutants were infected with GFP-expressing *M. marinum* wild type and *M. marinum* ΔRD1. At 37 hpi, infected cells with fixed, stained for F-actin and the number of ejectosomes quantitated by visual inspection. **(C)** Expression of GFP-RacH in *racH*- cells partially restore ejectosome formation (white arrowheads) and spreading of the infection. **(D)** Expression of *M. marinum* ESAT-6

directly in the cytosol of *Dictyostelium* partially complements the infection defect of *M. marinum* ΔRD1. Contrary to the situation in wild type *Dictyostelium*, a population of cells highly infected with *M. marinum* ΔRD1 persists longer in ESAT-6 expressers. (see also Fig. 4C-E). Scale bars 1 μm.

Figure S9. Transmission electron micrographs of *racH*- cells infected with wild type *M. marinum* and wild type cells infected by *M. marinum* ΔRD1.

In both combinations, spacious, bacteria-containing vacuoles were observed (asterisk). Very frequently for *M. marinum* wild-type and much less so for *M. marinum* ΔRD1, these compartments were observed to rupture (black arrowheads) and bacteria were released into the cytosol (white arrowheads). In agreement with the observed high susceptibility to infection (*1*), in *racH*- cells, bacteria were more often found in the process of division, indicated by the presence of septa. Scale bars 1 μm.

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Figure S1
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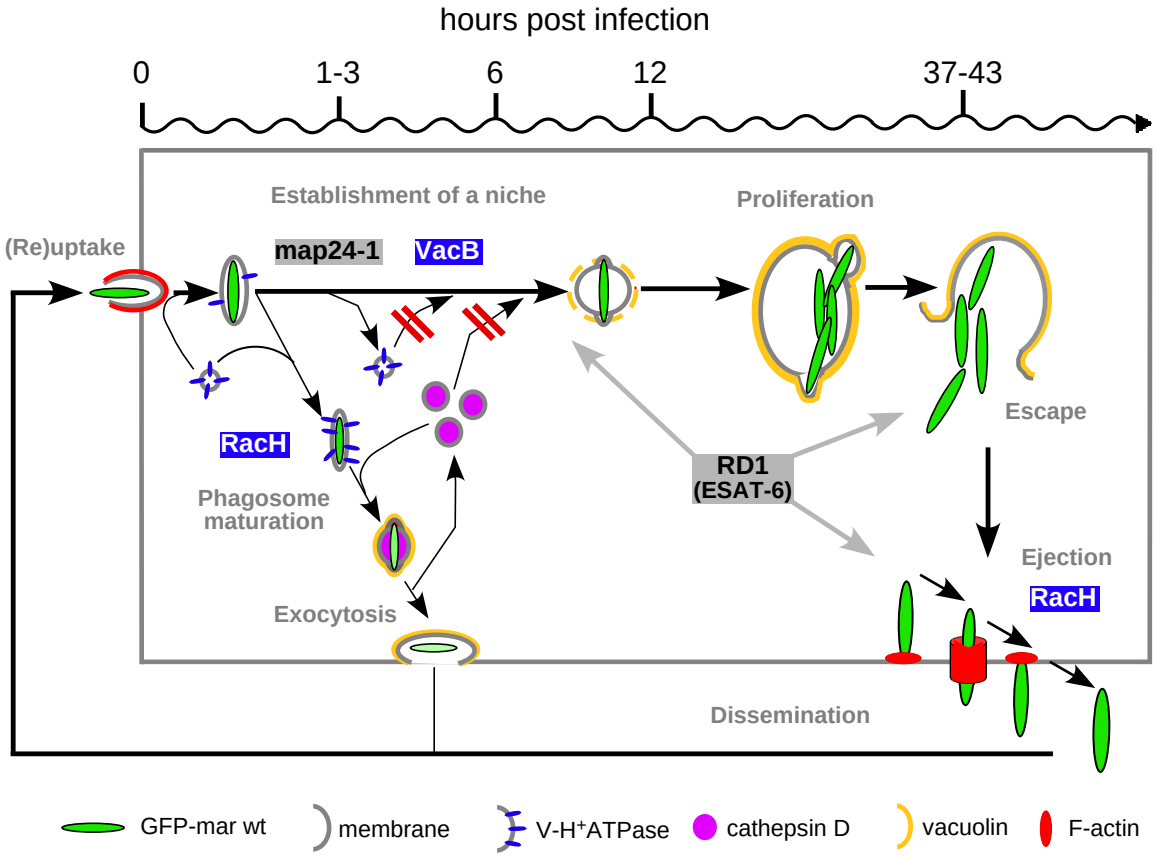

Figure S2
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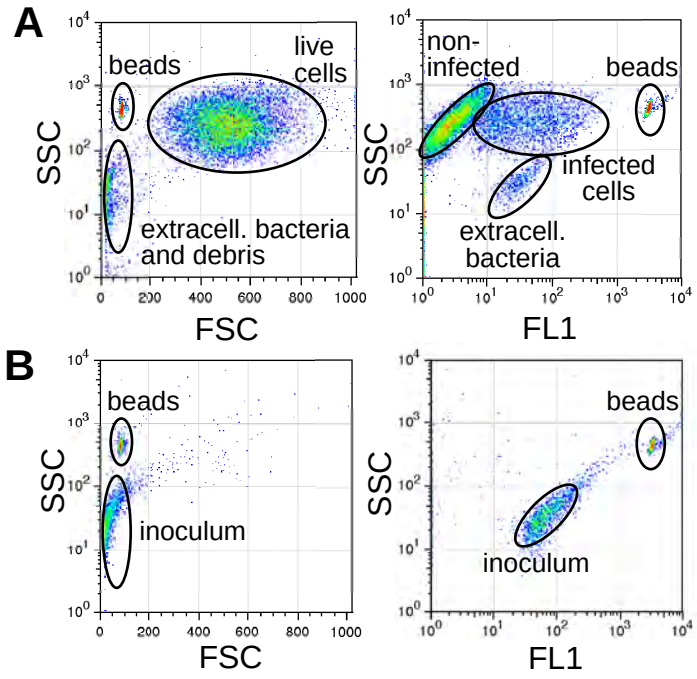

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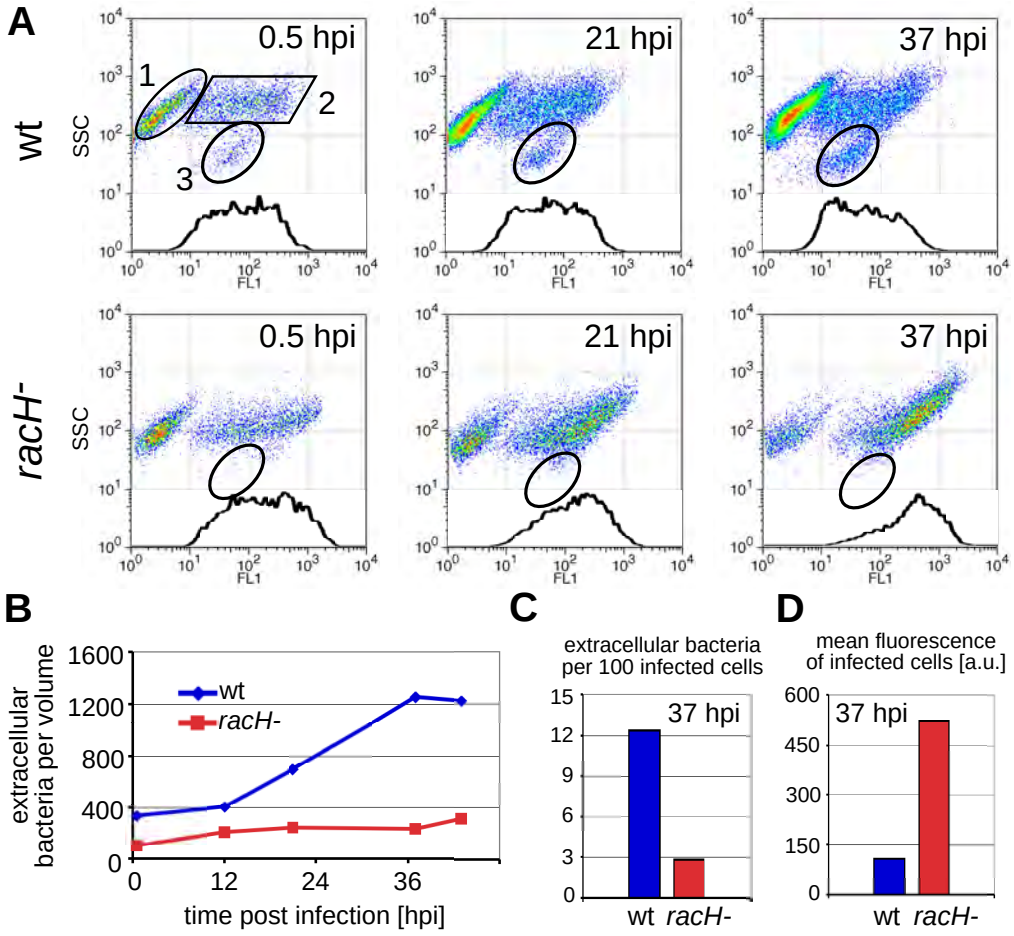

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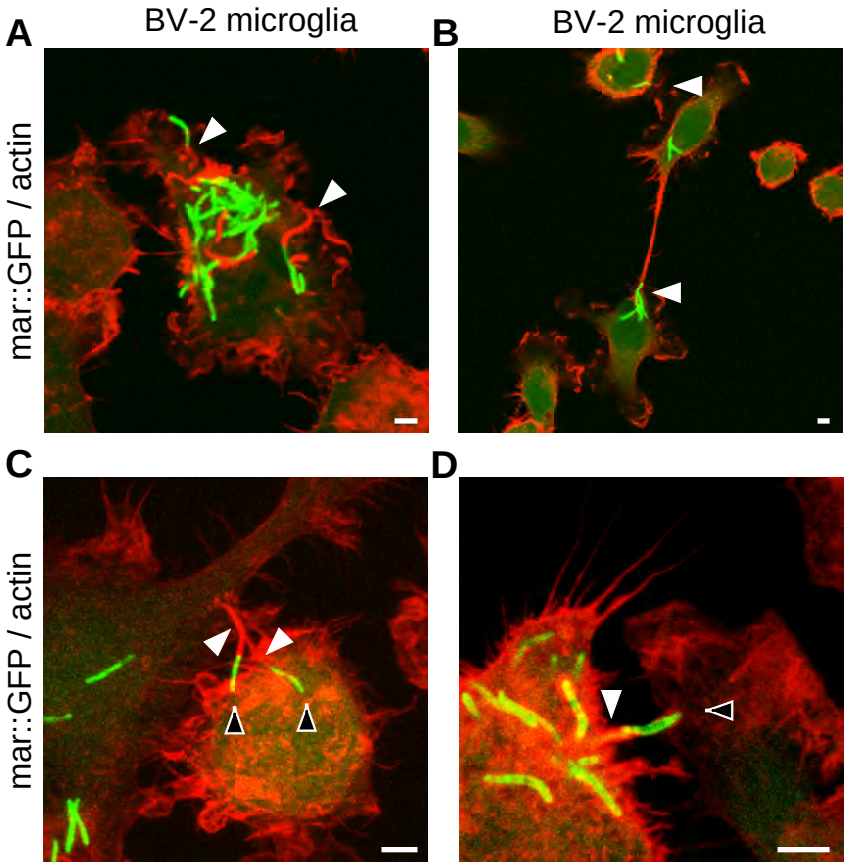

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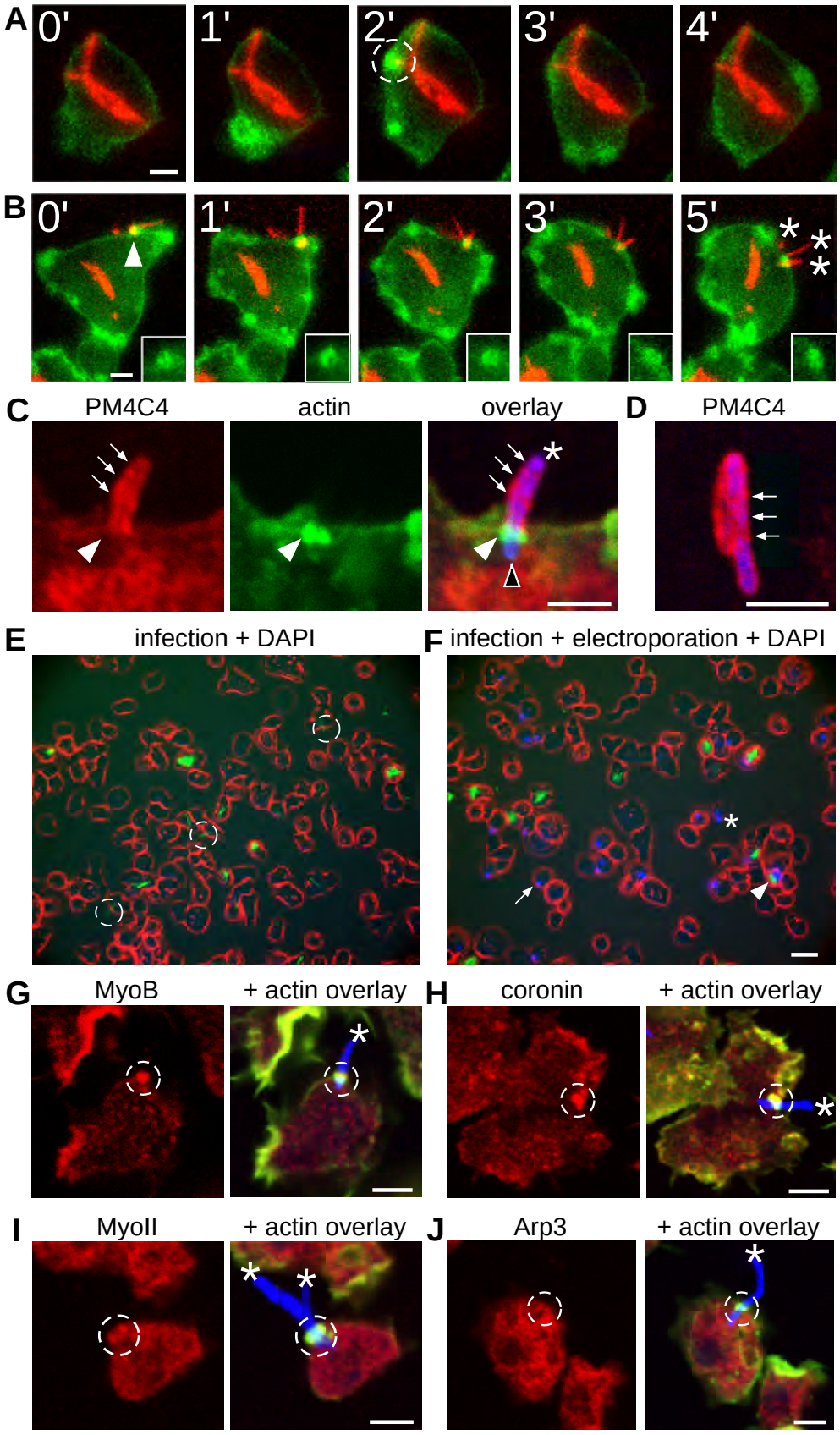

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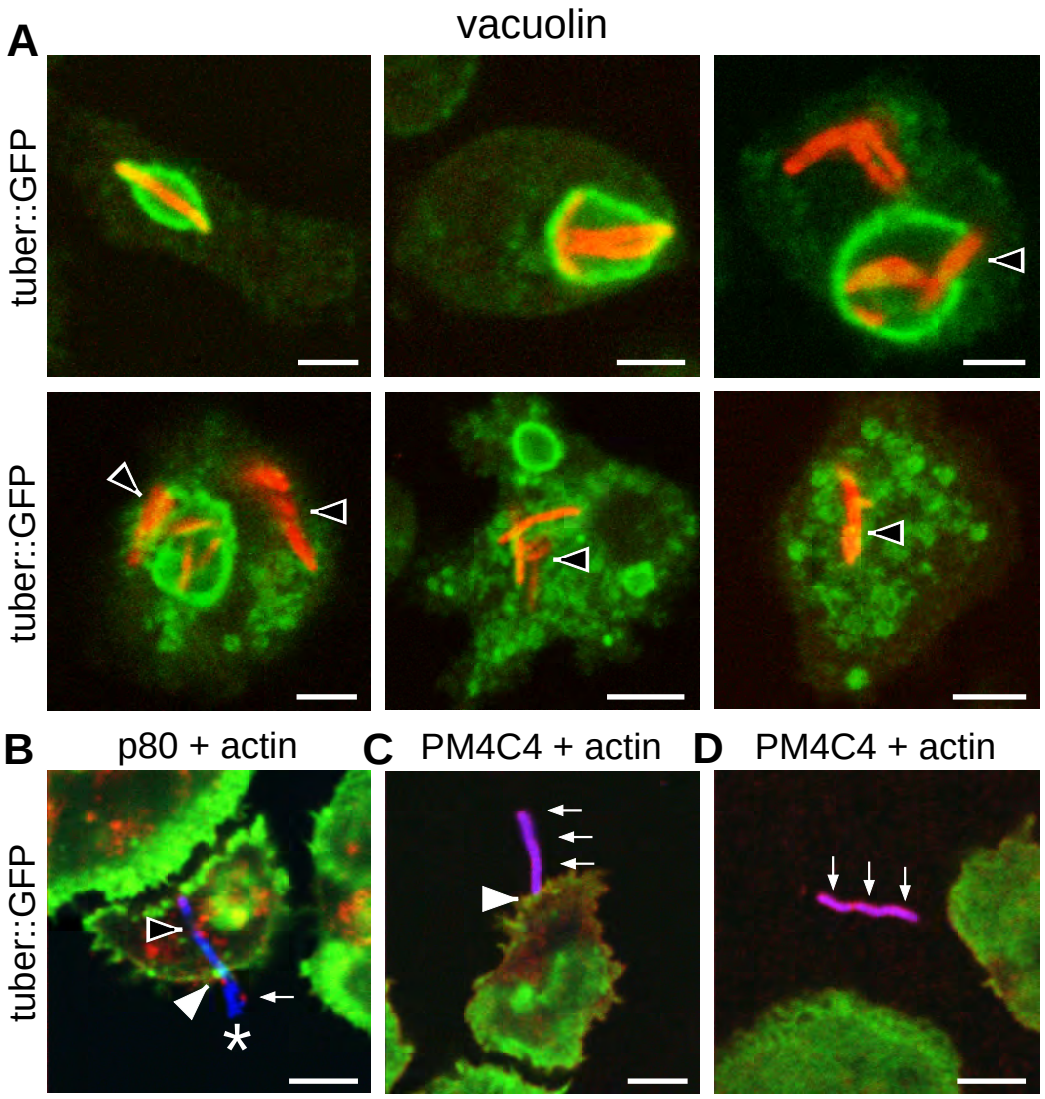

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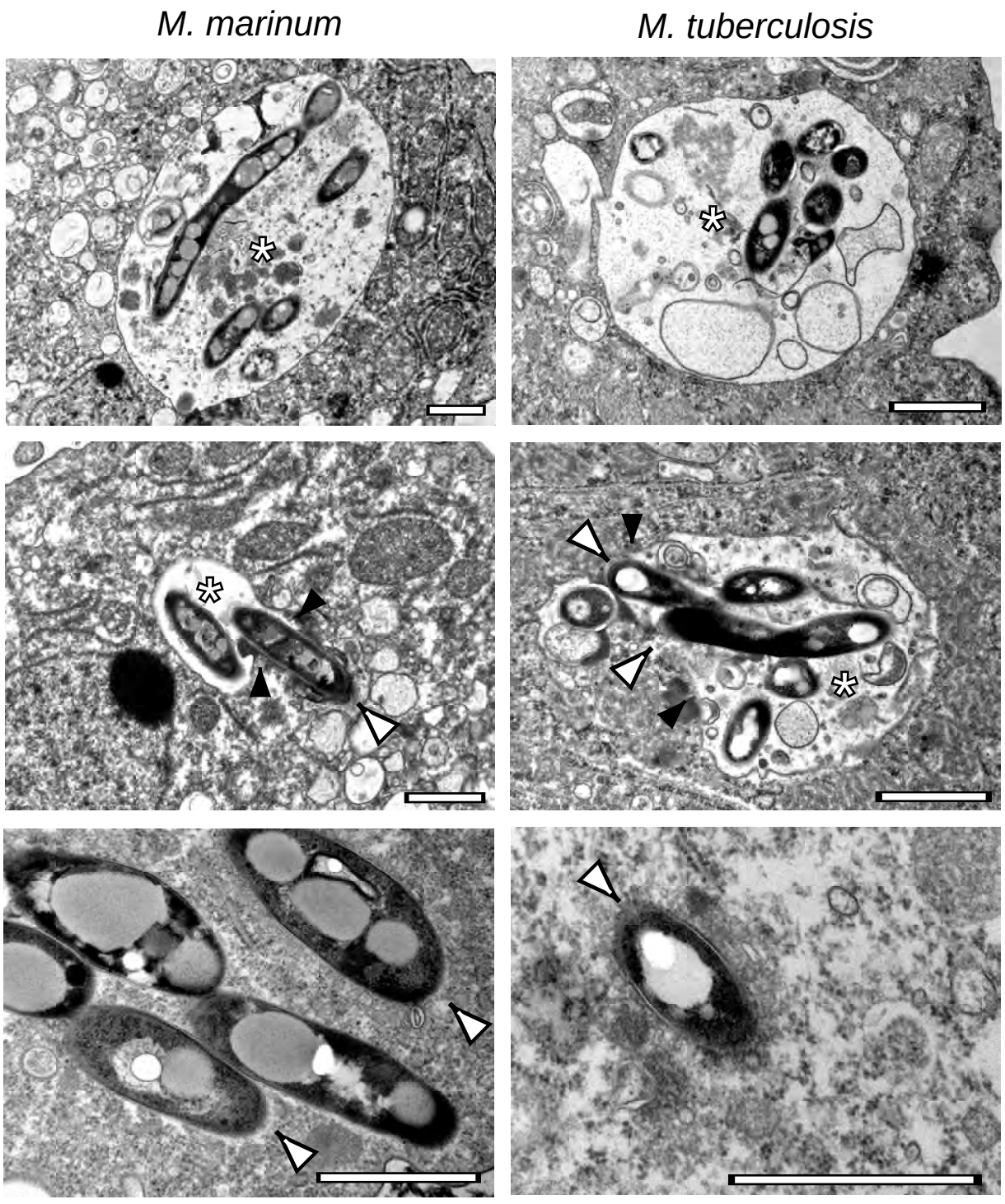

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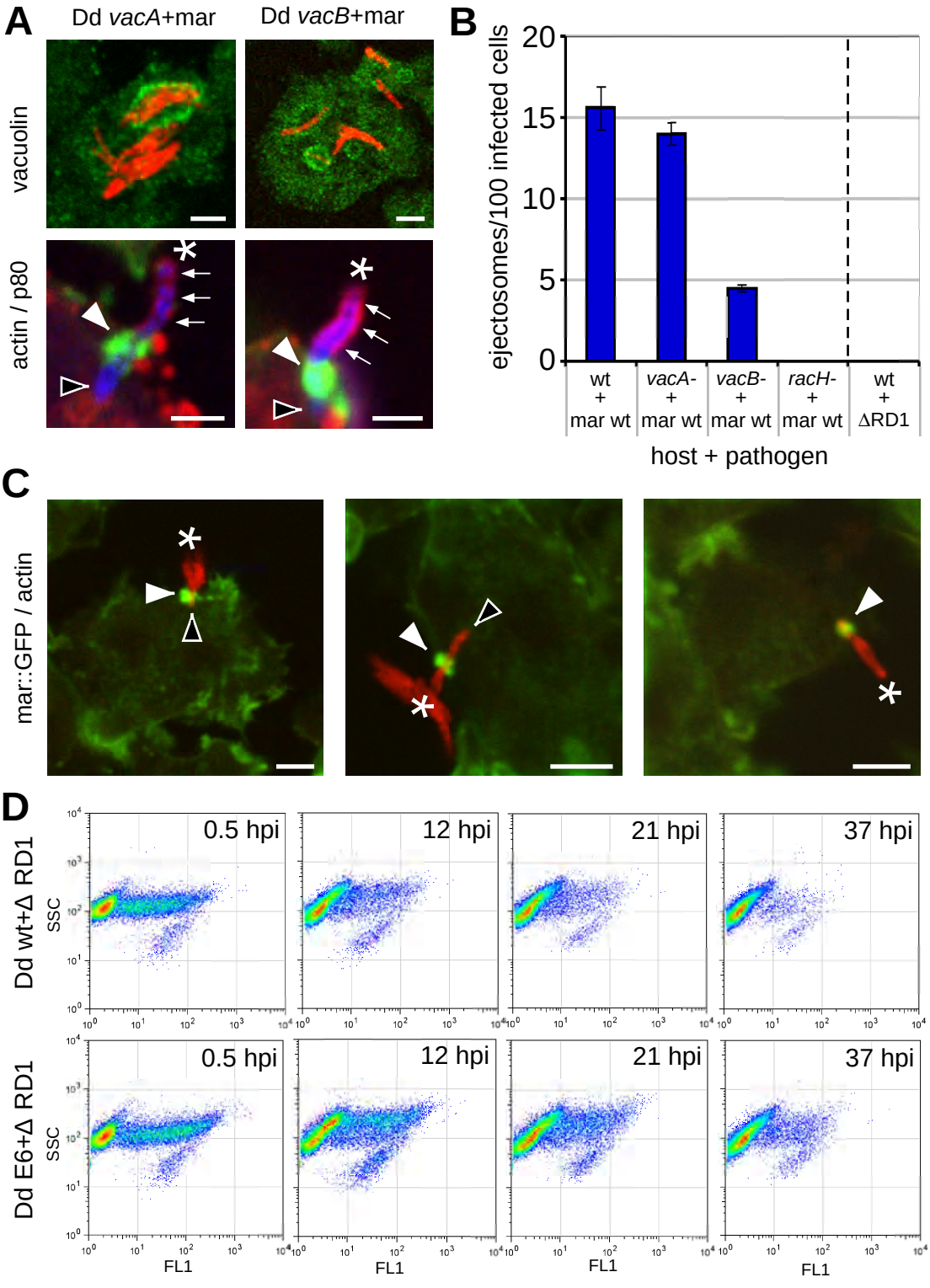

Figure S9
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