Supplementary Material for: **Conserved mechanisms of *Mycobacterium marinum* pathogenesis within the environmental amoeba, *Acanthamoeba castellanii***

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Contents:

The Supplementary Material includes Materials and Methods, References, and 3 Figures with legends.

Materials and Methods

Bacterial Strains and Growth Conditions

Mycobacterium marinum strains were grown in Middlebrook 7H9 (Sigma Aldrich, St. Louis, MO) with 0.1% Tween-80 (Fisher Scientific, Pittsburgh, PA) as described previously (2). The plasmid expressing DsRed (3) was maintained in *M. marinum* using 20μg/ml Kanamycin (IBI Scientific, Peosta, IA). The *M. marinum* M strain and the M strain bearing a deletion in the RD1 region (ΔRD1) was the kind gift of Dr. Eric J. Brown.

Protozoa Strains and Growth Conditions

Acanthamoeba castellanii (Douglas) Page was obtained from the American Type Culture Collection (ATCC #30234) and was grown and maintained axenically in PYG (peptone yeast glucose-712 medium, ATCC, Manassas, VA) at room temperature. Amoebae were grown to confluence in 9 inch diameter petri plates at room temperature (22-25°C), resuspended in fresh PYG-712 media and counted using a hemocytometer.

M. marinum infections of A. castellanii

M. marinum was grown in 7H9 media as described above at 30°C to late log phase (OD_{600} 0.8 to 1.0), washed three times and resuspended in sterile PBS. The bacteria were syringe filtered to remove clumps and used to infect amoebae either on glass cover slips, 24-well culture dish or in a 9 inch diameter treated petri dish (Nunc, Rochester, NY). Mycobacteria were added to multiplicity of infection (MOI) of one, five or 10 and incubated with *A. castellanii* for 30 minutes at room temperature. Following this incubation, the media containing the extracellular bacteria was aspirated and fresh PYG-712 media was used to wash the amoebae monolayer three times to remove extracellular bacteria. We count the addition of fresh media as time = 0. When appropriate, extracellular *M. marinum* were killed by gentamicin treatment (150μg/ml,

InvivoGen, San Diego, CA) in PYG-712 for two hours (Figure 1A), allowing study of only those organisms that infected amoeba. Following this incubation the media was removed and replaced with fresh PYG-712 broth.

For determination of *M. marinum* colony forming units, infected *A. castellanii* were mechanically lysed using a syringe (lysis was confirmed via microscopy) and the *M. marinum* removed by centrifugation. The resulting bacterial pellet was washed three times in sterile PBS, and diluted and plated for colony forming units (CFUs) on 7H10 (Sigma Aldrich) agar. The bacteria were counted following 10-14 days of growth at 30°C. When conducting the experiments in Figures 1B and 1C, *M. marinum* were washed three times in PYG-712 containing 50 μg/ ml gentamicin and visualized using a 20x objective on a Zeiss AxioObserver Inverted Microscope. Each infection was imaged at five different fields per time point using phase contrast and Rhodamine fluorescence filters. The exposure time of red channel images was standardized across each time course experiment. Overlay images were generated using ImageJ (1). The percentage of amoeba infected was calculated based on counting >100 amoeba in five different microscopic fields. These data were then used for statistical analysis. Amoebae with greater than one colocalizing DsRed expressing bacterium were counted as infected.

Actin tail formation

A. castellanii ($5x10^5$ per well) were seeded on glass cover slips (Fisher Scientific) in a 12-well plate and allowed to adhere for one hour. Monolayers were infected as above at an MOI of one with *M. marinum* expressing DsRed (5). At 22 hours post infection, the cover slips were removed, washed in PBS and the amoebae were permeabilized and fixed using cold Methanol. Actin tails were visualized using Alexa 488 Phalloidin (Life Technologies, Grand Island, NY).

Images were acquired with an Evolution QEi CCD (Media Cybernetics, Bethesda, MD) on a Nikon Eclipse TE300 (60X) using IPLab software (Scanalytics, Ontario, NY).

LDH release assay

M. marinum infections of *A. castellanii* were performed as above. The assay was performed at 72 hours post infection using the CytoTox96 kit (Promega, Madison, WI), which measures Lactate Dehydrogenase release according to the manufacturer's instructions. We report raw optical densities in Figure 1C.

References:

1. **Abramoff, M. D., Magalhaes, P.J., Ram, S.J.** 2004. Image Processing with ImageJ Biophotonics International, **11:**36-42.
2. **Champion, P. A., M. M. Champion, P. Manzanillo, and J. S. Cox.** 2009. ESX-1 secreted virulence factors are recognized by multiple cytosolic AAA ATPases in pathogenic mycobacteria. Mol Microbiol **73:**950-62.
3. **Cosma, C. L., O. Humbert, and L. Ramakrishnan.** 2004. Superinfecting mycobacteria home to established tuberculous granulomas. Nat Immunol **5:**828-35.

Figure S1

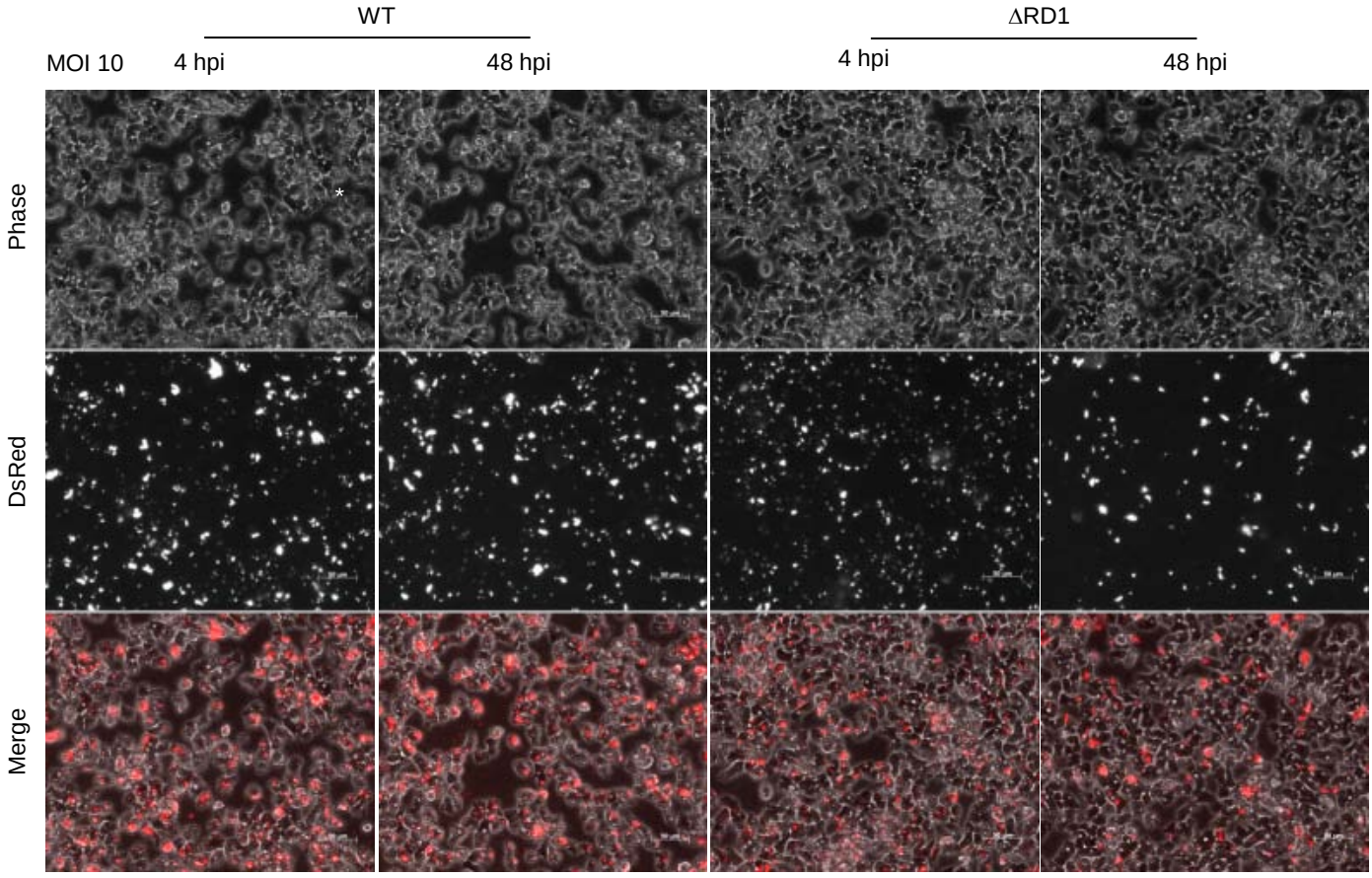

Figure S1: *M. marinum* lacking the ESX-1 secretion system are cleared from *A. castellanii*. A. Infection by *M. marinum* results in a stable population of infected amoebae. In contrast, the population of infected amoebae decreased over time in the absence of the ESX-1 system. *A. castellanii were* infected with *M. marinum* and ΔRD1 *M. marinum* expressing DsRed at MOI 10. Monolayers were imaged using 20x objective on a Zeiss AxioObserver Microscope.

Figure S2

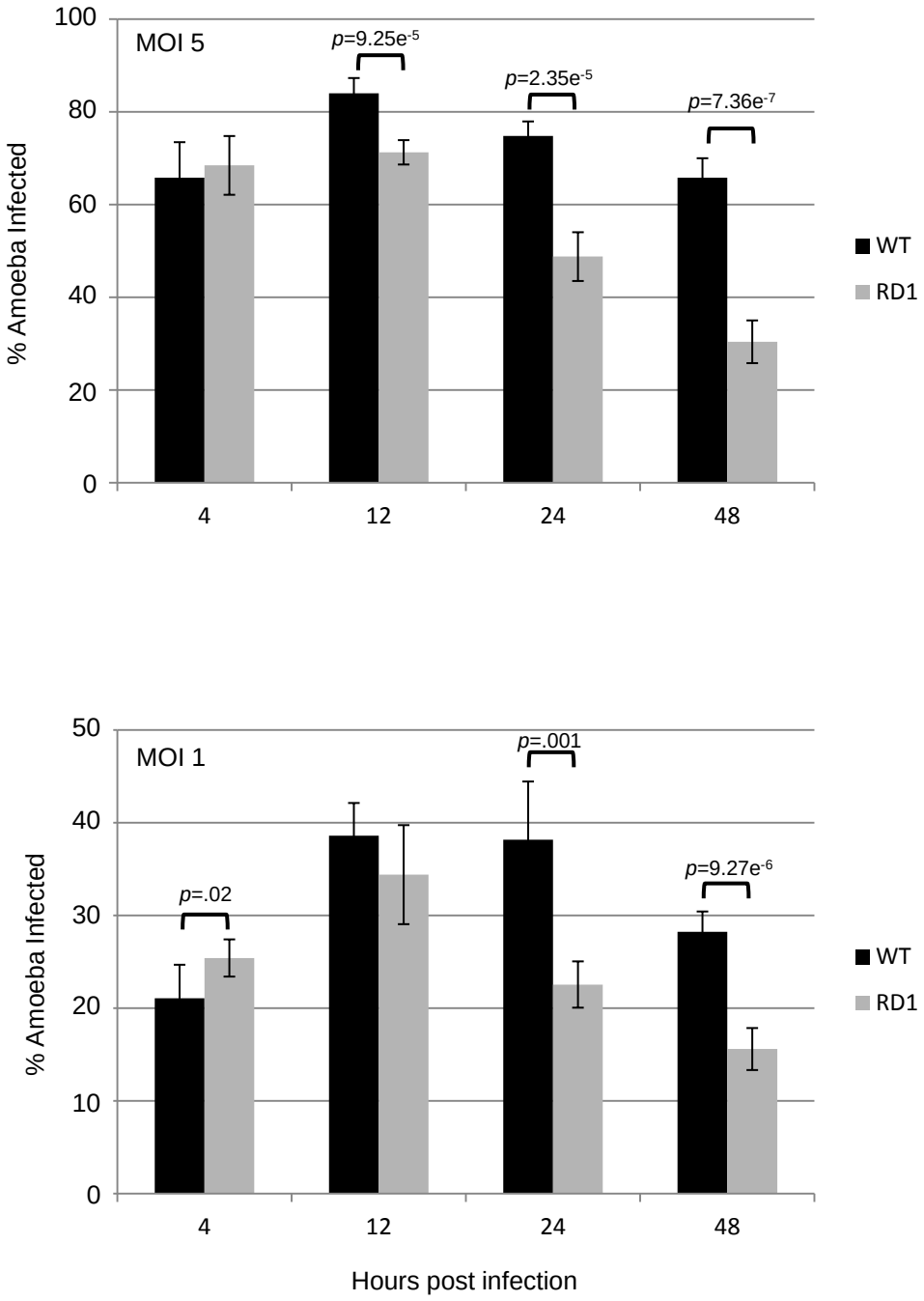

Figure S2: *M. marinum* lacking the ESX-1 secretion system are cleared from *A. castellanii*. A. Infection by *M. marinum* results in a stable population of infected amoebae. In contrast, the population of infected amoebae decreased over time in the absence of the ESX-1 system. *A. castellanii* as in Figure S2. Error bars denote standard deviation.The percentage of infected amoebae was established after counting >100 cells in each of five different fields at the indicated time points. Asterisks represent statistically significant differences based on an unpaired tailed Student's T Test. P values < 0.05 were considered significant, and are listed above.

Figure S3

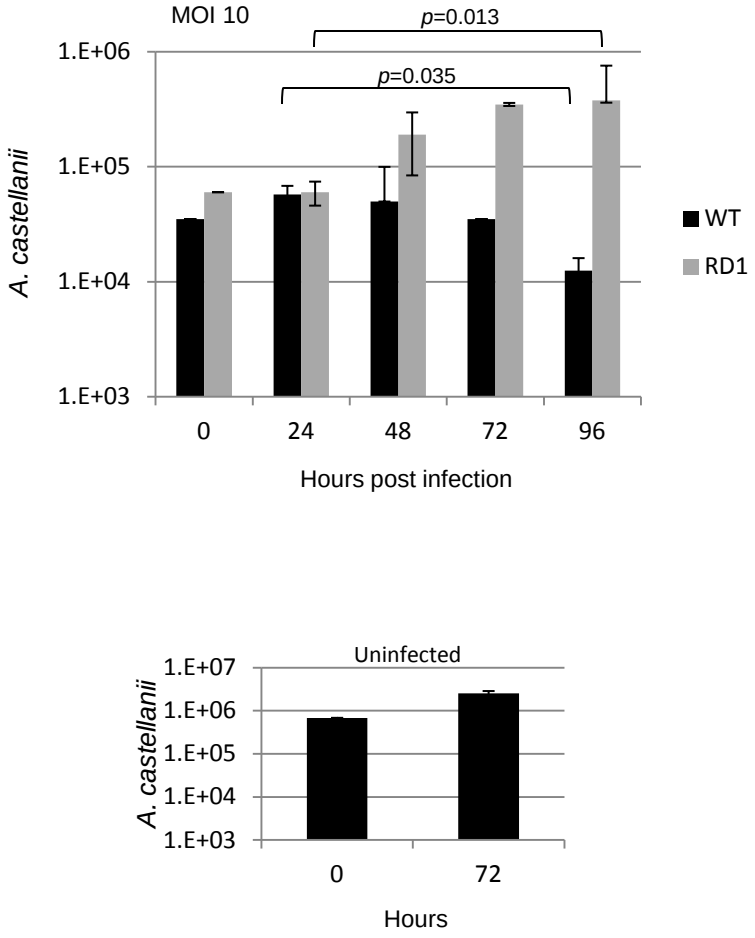

Figure S3: C. Infection of *A. castellanii* results in a relatively constant number of amoebae over time, while infection by *M. marinum* lacking ESX-1 leads to amoebae growth. At each time point following infection, the number of amoebae were counted. Data shown corresponds to Figure 1C. Statistically significant differences based on an unpaired tailed Student's T Test, P values < 0.05, are listed above. Lower panel represents growth of uninfected amoeba between 0 and 72 hours, which represents ~3.7 fold increase in growth.