Macrophage activation by IFN- γ triggers restriction of phagosomal copper from intracellular pathogens

Qian Shen, Matthew J. Beucler, Stephanie C. Ray, and Chad A. Rappeleye
Department of Microbiology, Ohio State University, Columbus, OH 43210, USA

Objectives: This study investigates how macrophages alter copper availability to control intracellular pathogens and the mechanisms by which *Histoplasma capsulatum* combats copper restriction.

Methods: A forward genetic screen of *H. capsulatum* identified loci required for proliferation within macrophages. Quantitative RT-PCR (qRT-PCR) was used to measure the transcriptional response of *Histoplasma* yeasts to high or low copper. To estimate intraphagosomal copper concentrations in macrophages, a *gfp* transcriptional reporter was used to measure the activity of the copper-responsive *CTR3* promoter. *Histoplasma* yeasts lacking Ctr3 were tested for virulence using a murine model of pulmonary histoplasmosis.

Results: The loss of the ability of *Histoplasma* yeast to replicate in macrophages was linked to disruption of the *CTR3* locus, which encodes a fungal copper transporter. Loss of Ctr3 function impairs yeast growth in limiting copper consistent with Ctr3 functioning as a copper-specific importer. Transcription of the *CTR3* gene is induced both by differentiation of *H. capsulatum* into pathogenic yeasts and by low available copper. Infection of macrophages with a strain having a transcriptional fusion to the *CTR3* promoter demonstrated that phagosomes of non-activated macrophages have moderate copper levels that are not antimicrobial to *H. capsulatum*. However, IFN- γ activation of phagocytes causes phagosomal copper restriction both in culture and in vivo. Accordingly, in a respiratory model of histoplasmosis, Ctr3-deficient yeasts are fully virulent during innate immunity but are attenuated after the onset of adaptive immunity.

Conclusion: Activation of macrophages during cell-mediated immunity, switches the phagosome from a copper-replete to a copper-depleted environment, forcing *H. capsulatum* reliance on Ctr3 for copper acquisition.

Figure 3. Activation of macrophages decreases phagosomal copper availability. (A) Representative images of *H. capsulatum* yeasts with the *CTR3* promoter-*gfp* fusion grown in vitro with different copper concentrations. (B) *CTR3* promoter activity in BMDMs with and without IFN- γ activation as indicated by the fluorescence of the P_{CTR3} -*gfp* transcriptional reporter fusion.

Figure 4. *H. capsulatum* virulence in vivo requires Ctr3 during the adaptive immune response. The relative fitness (competitive index) of Ctr3-deficient (*ctr3*) compared to Ctr3-producing (*CTR3*) yeasts was determined by co-infecting mice intranasally with equal amounts of *CTR3* and *ctr3* yeasts. Relative fungal burdens were determined for yeasts co-cultured in vitro and in the lungs at 6, 9, 12, 15, 18, and 21 days post-infection. Asterisks indicate significant (* $P < 0.05$, ** $P < 0.01$) differences compared to the *ctr3* : *CTR3* ratio of the inoculum.

Figure 1. Ctr3 enables *H. capsulatum* growth in limited copper. Dose response curves for Ctr3-expressing (*CTR3*, black), Ctr3-deficient (*ctr3*, red), and the complemented (*ctr3/CTR3*, blue) strains grown in liquid culture with the copper-specific chelator BCS.

Figure 2. Activation of macrophages impairs proliferation of Ctr3-deficient yeasts. Bone marrow derived macrophages (BMDMs) were activated with IFN- γ and/or TNF- α , and infected with Ctr3-expressing (*CTR3*, black), Ctr3-deficient (*ctr3*, red) and complemented (*ctr3/CTR3*, blue) *H. capsulatum* yeasts. Intracellular yeasts were enumerated (CFU) after 48 hours. Asterisks indicate significant differences (* $P < 0.05$) in the intracellular proliferation of yeasts.

Figure 5. IFN- γ treatment restricts copper availability in vivo. BMDMs or mice were infected with *H. capsulatum* yeast with *TEF1* (P_{TEF1}) or *CTR3* (P_{CTR3}) promoter-*gfp* fusions. *CTR3* promoter activity yeasts following infection was determined by measurement of GFP-fluorescence by microscopy and normalized to the average *TEF1* promoter activity. Data shows the distribution of *CTR3* promoter activity and horizontal bars represent the mean *CTR3* promoter activity. Asterisks indicate significant differences (** $P < 0.01$, *** $P < 0.001$).