Cytochrome C and Cell Death in the fungal pathogen *Aspergillus fumigatus*

Matt James [1], Ko-Wei Liu [1], Elisa Vesely [1], Tobias Hohl [2], Robert A. Cramer [1]

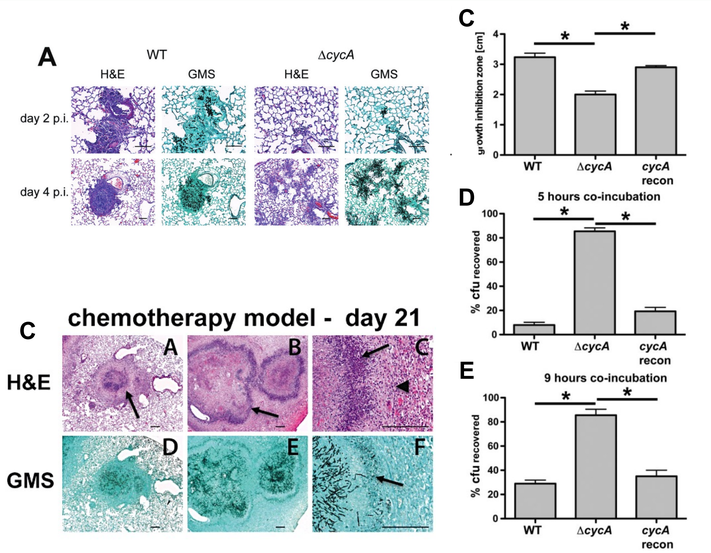
[1]Department of Microbiology and Immunology Geisel School of Medicine at Dartmouth

[2]Memorial Sloan Kettering Cancer Center

Background and hypothesis

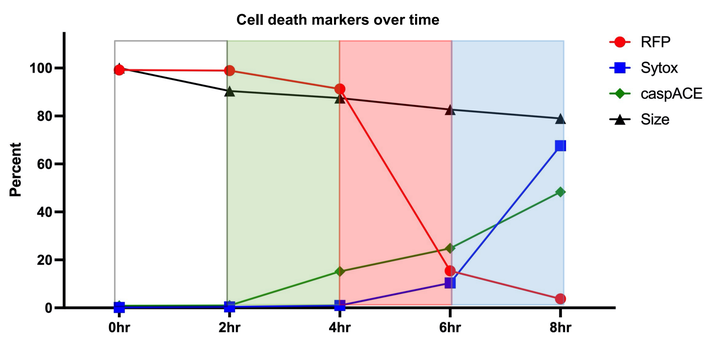
Aspergillus fumigatus is a ubiquitous environmental mold that can cause a life-threatening infection known as invasive aspergillosis (IA). IA is caused by defects in innate immune system function that result in failed clearance of inhaled conidia from the lung. While it is known that innate immune function, particularly NADPH oxidase activity, is responsible for clearance of these conidia from the lung, the fungal mechanisms by which these conidia die from leukocytes remains unknown. Here we investigate the role of *A. fumigatus* cytochrome c (*cycA*) in both hydrogen peroxide-induced and leukocyte-induced fungal cell death. Cytochrome c is a canonical cell death effector in higher order metazoa that functions in programmed and regulated forms of cell death and is conserved across eukaryotes. We hypothesize that deletion of *cycA* confers resistance to H_2O_2 and leukocyte – induced fungal cell death.

The role for *cycA* in pathogenesis



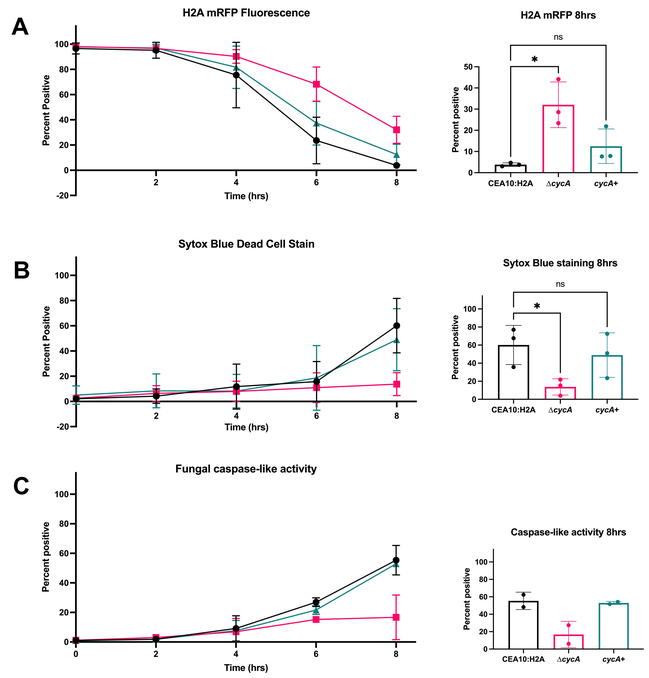
Deletion of *cycA* alters pathogenesis and confers resistance to reactive oxygen species and leukocyte killing. **A)** H&E and GMS staining reveals that despite a growth defect, the Δ*cycA* strain can achieve in vivo levels of growth like that of WT in immunocompromised mice. **B)** The Δ*cycA* strain can persist in immunocompromised mice of up to 21 days. **C)** Deletion of *cycA* confers resistance to menadione. **D, E)** Loss of *cycA* confers resistance to ex vivo macrophage killing. Grahl et al, 2012.

A. fumigatus displays multiple cell death markers



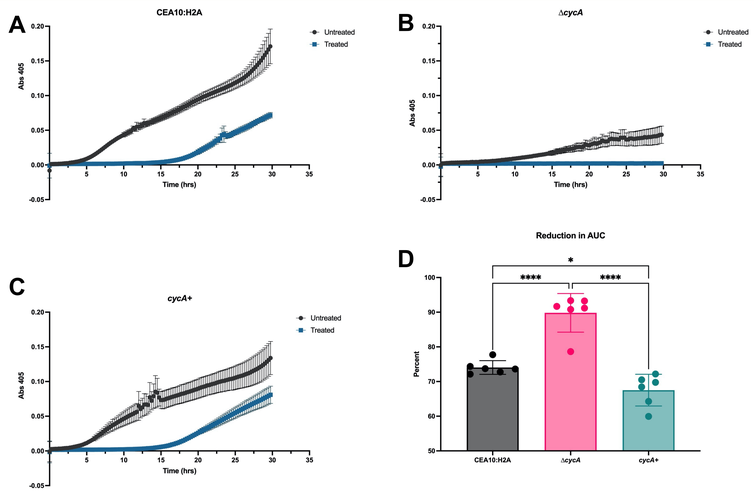
A. fumigatus displays multiple cell death markers over time after treatment with 10mM H_2O_2. Swollen conidia were treated with 10mM H_2O_2 for 8hrs. At two– hour intervals conidia were stained with Sytox blue dead cell stain to determine viability and CaspACE to determine caspase-like activity. Size is determined by mean fluorescence intensity (MFI) of FSC-A and displayed as a percent of untreated. Fluorescence is relative to untreated conidia.

Deletion of *cycA* alters markers of cell death under short-term ROS exposure



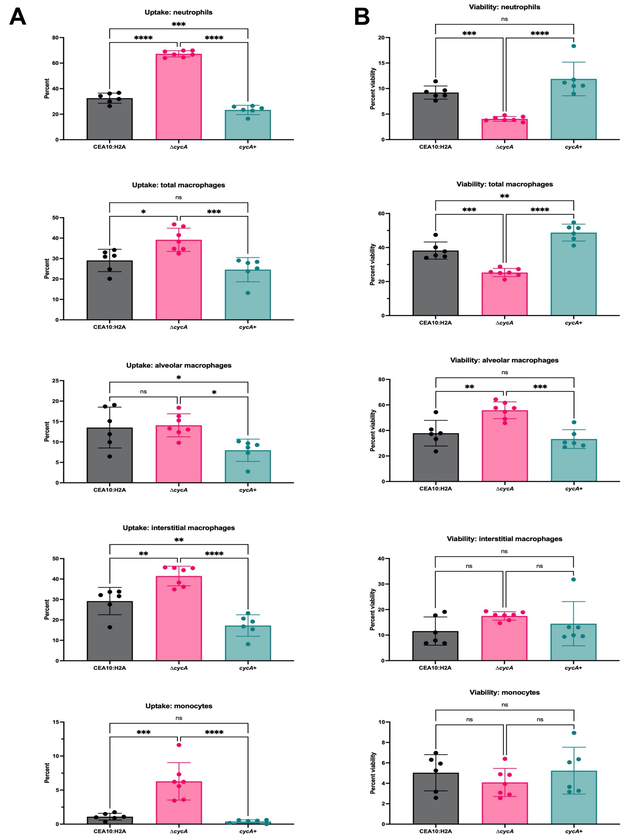
Deletion of *cycA* alters multiple cell death markers after treatment with 10mM H_2O_2. H2A:mRFP – labeled conidia were swollen and then treated with 10mM H_2O_2 for 8hrs. **A)** The Δ*cycA* displays a retention of mRFP fluorescence as compared to WT and recon strains, indicating reduced DNA fragmentation. **B)** The Δ*cycA* strain displays reduced sytox blue staining, indicating an increase in viability at 8hrs of treatment. **C)** The Δ*cycA* strain displays reduced caspase-like activity as indicated by staining with CaspACE, a FITC-VAD-fmk probe. * p<0.02 by Friedman test with Dunn's post test.

Deletion of *cycA* reduces long term viability after acute ROS exposure



Deletion of *cycA* results in diminished long-term viability after acute exposure to H_2O_2. Swollen conidia were exposed to 10mM H_2O_2 for 2.5hrs then washed with fresh media. Treated conidia were re-counted and inoculated into new cultures to monitor growth. Post-treatment growth was determined by reading Abs405 every 15mins for 30hrs. **A-C)** Growth kinetics of untreated cultures vs cultures treated with 10mM H_2O_2. **D)** Reduction in area under the curve (AUC) of treated cultures compared to untreated. The Δ*cycA* strain displays no observable growth 30hrs after treatment with close to 100% reduction in AUC as compared to the CEA10:H2A background and complement strains which display observable growth around 15hrs and only 65%-75% reduction in AUC. <p = 0.0001 as determined by one-way ANOVA with Tukey's post-test.

cycA contributes to cell survival in a sterilizing immunity model



In vivo FLARE experiment to determine the effect of cytochrome c deletion on innate immune killing of *A. fumigatus*. H2A:mRFP conidia were stained with alexa-fluor 633 to monitor conidia in relationship to immune cells. Immune competent C57BL/6 mice were inoculated with with 3e7 stained conidia and sacrificed 36hrs post inoculation. Cells were harvested from murine lungs and and stained for respective leukocyte subsets. Loss of *cycA* resulted in increased uptake **A)** across all leukocyte subsets, except for aveolar macrophages. Moreover, loss of *cycA* resulted in lower viability in neutrophil and total macrophage subsets, as determined by H2A:mRFP fluorescence **B)**. Interestingly the Δ*cycA* strain displayed higher viability in the aMac subset. <p = 0.0001 as determined by one-way ANOVA with Tukey's post test.

Summary & Conclusion

We observed that a Δ*cycA* strain displays altered cell death phenotypes including reduced histone fragmentation, reduced caspase-like activity, and reduced sytox blue staining after 6hr and 8hr exposure to 10mM H_2O_2. However, using a germination assay to monitor growth 30hrs after acute 2.5hr exposure to 10mM H_2O_2, we observed that loss of *cycA* results in no observable growth after treatment, suggesting a loss of viability as compared to the WT and complement strains. Examining in vivo leukocyte killing by FLARE technology, we observe that loss of *cycA* results in lower in viability in leukocyte subsets including neutrophils and total macrophages as compared to WT and complement strains. However, the Δ*cycA* strain displayed higher viability in specifically the alveolar macrophage subset as compared to other leukocyte subsets. Taken together, these data suggest cytochrome c presence in *A. fumigatus* contributes to cell survival under death inducing conditions and future studies will seek to define the underlying mechanisms.

Acknowledgements

This work was supported by funding from the NIH/NIAID grants R01AI139632 (RAC). RAC holds an Investigators in the Pathogenesis of Infectious Diseases Award from the Burroughs Wellcome Fund. This research is also supported by the Cystic Fibrosis Foundation (CF RDP – Stanton) as well as DartCF.