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The Role of Reactive Oxygen Species in Autophagy Activation During Candida albicans Infection

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THE ROLE OF REACTIVE OXYGEN SPECIES IN AUTOPHAGY ACTIVATION DURING *CANDIDA ALBICANS* INFECTION

by

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Abstract

The immune system protects us against disease through a variety of mechanisms that result in pathogen elimination. Host innate immune cells can control infections by activating NADPH oxidase, which generates reactive oxygen species (ROS) to kill pathogens directly. A defective NADPH oxidase leads to chronic granulomatous disease (CGD), which causes recurrent infections within the host. Autophagy, a cellular recycling pathway, can also target pathogens for destruction and may be a pathway that is compromised within a CGD patient. Recent work suggests that autophagy can be activated by ROS *in vitro*. By utilizing transparent zebrafish, we are able to characterize the role of ROS and autophagy in innate immunity during fungal infection with *Candida albicans*. We administered DPI, α-tocopherol, and PMA to infected zebrafish to alter the levels of ROS then quantified the effects on autophagy using confocal microscopy. Our data suggests differences in autophagy activation *in vivo.* Additionally, we were able to enhance our understanding of loose phagosomes and provide a possible physiological difference between tight and loose phagosome morphologies. Further research is required in order to confirm new hypotheses regarding interactions between ROS and cell receptor signaling pathways that might activate autophagy and reasons for the distinct dichotomy of phagosomes observed within a live vertebrate host.

Dedication

I would like to dedicate my thesis to Mom and Dad who supported me when things weren't quite working out how I had planned and always encouraged me to do my very best.

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Introduction

The immune system provides protection from pathogens

The immune system is the body's line of defense that helps protect from and eliminate any pathogens through a variety of cellular and molecular mechanisms. There are two main branches of the immune system: innate immunity and adaptive immunity. Innate immunity is constantly active and protects against pathogens by physical barriers and cellular defenses. Phagocytic immune cells recognize microbes through pathogen-associated molecular patterns (PAMPs), which are antigens common to many pathogens (Janeway and Medzhitov, 2002). Adaptive immunity utilizes chemicals and immune cells that create a very specific response to and memory of pathogens (Kindt et al., 2007). Though both branches work together to remove microbial threats from the body, innate immunity plays a decisive role in many infections, such as candidiasis.

The innate immune system protects the body in a number of ways from pathogens. Physical barriers of the body are the first line of protection. These barriers include epithelial and mucosal membranes of the body (Janeway and Medzhitov, 2002). These physically block pathogens from entering the body and also secrete mucus and enzymes, such as lysozyme, that can neutralize the pathogen. Though the physical barriers prevent many pathogens from entering the body, disruptions in mucous membranes or skin provide pathogens with access routes. When entry occurs, cellular mechanisms of innate immunity take over.

A pathogen is quickly detected by cellular recognition and chemical signals released from local tissues. Chemokines and cytokines are major signaling molecules within the immune system. These are released during physical damage or infection and

recruit immune cells to the location of the attack (Kindt et al., 2007). The cells that are first to respond to the scene of infection are phagocytes.

There are three main types of phagocytes within the innate immune system: dendritic cells, neutrophils, and macrophages. Dendritic cells are embedded in the epithelial layers of the skin and are the first to respond to a pathogen because of their proximal location to most infections. Neutrophils and macrophages, also, are extremely important within the innate immune system. These white blood cells (WBCs) are able to detect pathogens by common PAMPs through a variety of Toll-like receptors (TLR) on their surfaces and engulf the intruder through phagocytosis (Akira et al., 2006). Once engulfed, pathogens are killed and degraded by different mechanisms in the phagocyte. One major killing pathway is fusion of the phagosome with lysosomes, which contain enzymes to break down the intruder. Another route of pathogen degradation utilizes reactive oxygen species (ROS) that are produced by a specialized enzyme called NADPH oxidase during an infection. Figure 1 shows the production of superoxide anion by NADPH oxidase. Subsequent reactions of superoxide anion with other enzymes in the phagocyte produce additional ROS. Also known as free radicals, ROS have the characteristic of having one or more unpaired electrons (Valko et al., 2007), which allows them to be extremely reactive with other substances in the cell; they can cause major damage to the pathogen as well as host cells. This method of pathogen elimination is known as respiratory burst, which phagocytes use to kill pathogens directly because of ROS toxicity. However, ROS may also have indirect roles of pathogen elimination that are in preliminary stages of exploration.

NADPH oxidase is important in innate immunity

NADPH oxidase, also known as *ph*agocyte *ox*idase (PHOX), plays an essential role in innate immunity and pathogen elimination. It is responsible for producing superoxide anion (O_2^-), which can react with a number of other oxidants creating ROS (Fig. 1, (Babior, 2004)). NADPH oxidase activation also causes changes to the redox potential (Rybicka et al, 2010) and pH within the phagosome (Jankowski et al., 2002), while also possibly activating proteases within the phagosome (Assari, 2006). Together these changes can help kill phagocytosed pathogens.

NADPH oxidase consists of multiple subunits that are separated into a membrane bound complex and a cytosolic complex. The membrane bound complex contains $gp91^{phox}$ and $p22^{phox}$ subunits, while the cytosolic complex contains $p47^{phox}$, $p40^{phox}$, and $p67^{phox}$ subunits (Assari, 2006; Babior, 1999). Phagocytosis of a pathogen causes phosphorylation of the cytosolic subunit $p47^{phox}$, facilitating translocation of the cytosolic complex to the membrane (Groemping et al, 2003; Clark et al., 1990). Interactions between the complexes at the membrane complete the activation cascade and NADPH oxidase becomes an active enzyme (DeLeo and Quinn, 1996; Babior, 1999), as shown in Figure 2 (Assari, 2006). Once active, the enzyme is able to use molecular oxygen to produce superoxide anion. NADPH oxidase activity can also be artificially activated by stimulation of protein kinase C (PKC), which phosphorylates $p47^{phox}$ (Fontayne et al., 2002). How phagocytosis is coupled with phosphorylation of $p47^{phox}$ to activate NADPH oxidase is still unknown; however, it may be the result of interactions with antibodies through Fcγ receptors (FcγR) or recognition of a pathogen by TLR signaling (Segal, 1996).

Defects within NADPH oxidase complex may arise from genetic abnormalities causing loss of function within the protein. The major subunits of NADPH oxidase that make up most of the genetic defects are $gp91^{phox}$ and $p47^{phox}$ (Dinauer, 2005). Because of these genetic defects, phagocytes cannot produce a sufficient amount of ROS to attack and destroy the pathogen. These mutations result in chronic granulomatous disease (CGD), which cause patients to be susceptible to more serious and recurrent infections (Babior, 1999; Heyworth et al., 2003). Understanding molecular mechanisms of how reduced amounts of ROS cause CGD patients to be more susceptible to disease will enhance treatment options and help recognize alternative ways in which the innate immune system fights infection. While ROS are understood to be important in killing pathogens directly, it is possible there are other important pathways that utilize ROS and ultimately require a functional NADPH oxidase, specifically autophagy activation.

Links between the innate immune system and autophagy

Autophagy is a general recycling pathway within all cells that has been shown to play an important role in controlling and eliminating intracellular pathogens (Levine and Deretic, 2007). Autophagy is usually initiated during periods of starvation, but has recently been shown to be active during an infection. There are different types of autophagy including microautophagy, chaperone-mediated autophagy, and macroautophagy (Klionsky, 2005; Mizushima et al., 2008). All types of autophagy recycle proteins and organelles of the cell; however, macroautophagy (known as autophagy throughout this thesis) is the major type of autophagy utilized during pathogen elimination (Schmid and Münz, 2007).

Autophagy is regulated through a number of different mechanisms. Inhibition of autophagy comes mainly from the *t*arget *o*f *r*apamycin (TOR) kinase, which blocks autophagy when ample nutrients are available (Levine and Kroemer, 2008). Activation of autophagy has been shown to occur through interferon-γ (IFN-γ), tumor necrosis factor (TNF), and TLR signaling (Virgin and Levine, 2009; Sanjuan et al., 2007), and possibly as a result of ROS generated from NADPH oxidase (Huang et al., 2009, Mitroulis et al., 2010). Once autophagy is activated, a cascade of events occurs where various signals and proteins accumulate near the phagosome. One important protein, light chain 3-I (LC3-I), becomes conjugated with phosphotidylethanolamine (PE) to become LC3-II (Ichimura et. al, 2000; Virgin and Levine, 2009), and is inserted into the autophagosomal membrane (Kabeya et al., 2000; Mizushima, 2004). When LC3 is fused with enhanced green fluorescent protein (EGFP), autophagy can be visualized because of the bright, green rings that are associated with the phagosomes surrounding the pathogen during confocal imaging. LC3-EGFP has been shown to be a useful method in identifying autophagic events (Mizushima, 2004). Using this method, we can quantify changes in autophagy activation by counting the number of LC3-EGFP phagosomal rings.

Recently, evidence supports that an active NADPH oxidase and presence of ROS is required for LC3 accumulation on zymosan, *Salmonella* (Huang et al., 2009), and *E. coli* phagosomes (Mitroulis et al., 2010) *in vitro*. This suggests that NADPH oxidase-generated ROS is an important activating signal of autophagy. Because these results are from *in vitro* experiments, it has lead us to investigate whether ROS do, in fact, have an indirect role in activating autophagy during an infection *in vivo*.

Along with ROS, TLR signaling has been shown to play a role in autophagy activation (Huang et al., 2009; Mitroulis et al., 2010). However, there has also been conflicting evidence that TLR activation causes LC3 to accumulate on the phagosome in an autophagy-independent way (Sanjuan et al., 2007). TLR signaling is an important aspect of the innate immune response and must be considered when exploring autophagy activation *in vivo.*

Limited research has been conducted in order to understand the dynamics and role of autophagy in the innate immune system. Understanding its connection in the context of other components of innate immunity is important and may lead to more effective or alternative treatments during an infection. We utilized the LC3 protein as a method of observing interactions between autophagy and NADPH oxidase, *in vivo,* during a *Candida albicans* infection.

Immunity to Candida albicans

C. albicans is a human commensal fungus that is kept under control by the innate immune system within healthy individuals. It is a dimorphic fungus, capable of living as yeast or hyphae (Mitchell, 1998). The innate immune system normally recognizes *C. albicans* through TLRs that detect specific components of its cell wall (Netea et al., 2008); this prevents proliferation of the fungi, but still permits commensal living within the gut.

C. albicans can cause mucosal infections or disseminated candidiasis. Mucosal infections are localized infections within a mucosal layer, such as the common yeast infection in women (Sobel et al., 1998). Disseminated candidiasis occurs when *C.*

albicans spreads throughout the body. This infection occurs in a high percentage of immunocompromised and hospitalized patients, who have weakened immune systems (Pfaller and Diekema, 2007). The dynamics of *in vivo* disseminated candidiasis has had limited research, providing us the opportunity to expand our understanding of how the innate immune system and autophagy work together in combating disseminated candidiasis.

Zebrafish as a model organism for candidiasis

Danio rerio, or zebrafish, has become an extremely useful tool in modeling infection. The zebrafish is an ideal model organism because it reproduces quickly, produces high amounts of offspring, and there are a variety of transgenic lines already established (reviewed by Tobin et al., 2012). They also have transparent larva, which is important when observing pathogen-host interactions and autophagy activation *in vivo.* They have been shown to exhibit characteristics of disseminated candidiasis (Brothers et al., 2011), which is essential in order to study the dynamics between innate immunity and autophagy during early stages of candidiasis.

During our research, we used a transgenic LC3-EGFP line of zebrafish (He, 2009). These fish have enhanced green fluorescent protein (EGFP) fused to LC3, the protein important when identifying autophagy and, because of their small size and transparency, we are able to image and visualize LC3-EGFP localization directly using confocal microscopy. Another important advantage of zebrafish is the ability to investigate the innate immune system on its own, since the adaptive immune system does not fully develop until 4-6 weeks post-fertilization (reviewed by Sullivan and Kim,

2008). By infecting the zebrafish at 36 hours post-fertilization (prim-25 stage; Figure 3), we took advantage of this delay in adaptive immunity and focused solely on innate immunity.

There are numerous advantages to using zebrafish over the mouse model and *in vitro* approaches with regards to confocal microscopy. During infection in mice, the animals must be sacrificed and slides prepared in order to view infected tissues. This limits the observational time points and may destroy valuable data when prepping the tissues. The zebrafish model avoids these constraints, as we are able to see directly into the fish without causing any damage. We are also able to observe transient processes and collect data about overall dynamics of the infection that may be overlooked in a mouse model. *In vitro* approaches can use confocal microscopy, however these experiments do not consider the interactions of multiple types of immune cells and the pathogen in a live host. These advantages, along with the recent establishment of disseminated candidiasis in zebrafish, makes the LC3-EGFP transgenic zebrafish a good candidate for characterizing autophagy activation using chemical treatments that alter ROS concentration.

Chemical treatments used to characterize the role of ROS in autophagy activation

A variety of chemicals are available that enable us to observe how NADPH oxidase-generated ROS play a role in autophagy activation *in vivo.* Treatments include diphenylethyliodium (DPI), which inhibits NADPH oxidase activity; α-tocopherol, which acts as an antioxidant to remove ROS; and phorbol myristate acetate (PMA), which stimulates NADPH oxidase activity (O'Donnell et al., 1993; Wang and Quinn, 1999;

McPhail et al., 1984). Distribution of LC3 during these treatments helped us elucidate whether or not ROS are involved in the activation of autophagy during an *in vivo* infection.

DPI acts to decrease NADPH oxidase activity by binding noncompetitively to the enzyme (O'Donnell et al., 1993). DPI has been used *in vitro* when studying autophagy, showing a significant decrease in the number of LC3+ phagosomal rings, which are indicative of autophagy (Huang et al., 2009). Evidence showing an effect of DPI treatment *in vitro* leads us to investigate this substance further in a live host. Also, by using this chemical inhibitor, we can potentially mimic CGD in zebrafish larvae to understand autophagy within patients suffering from this disease.

α-tocopherol, a type of vitamin E, is an antioxidant, which removes ROS and potentially inhibits autophagy. The major reason the general population uses antioxidants are to prevent the appearance of aging, which is commonly associated with tissue damage caused by ROS. However, the use of these chemicals may actually be interfering with the immune system's ability to fight infection. α-tocopherol acts extremely fast in a non-enzymatic reaction by transferring hydrogen to form tocopheroxyl radical. This radical is more stable than ROS, and does not cause tissue damage. The ability of α-tocopherol to be regenerated is an important function of the antioxidant allowing it to be reused multiple times to quench ROS (Wang and Quinn, 1999). It has also been shown to inhibit the respiratory burst of neutrophils by interacting with protein kinase C and inhibiting NADPH oxidase activity (Azzi et al., 2000). Autophagy research using α-tocopherol has shown decreased numbers of LC3+ phagosomal rings *in vitro* (Huang et al., 2009),

indicating a potential effect *in vivo* as well. Additionally, our research investigated how antioxidants may interfere with the immune system's ability to fight infections.

Phorbol myristate acetate (PMA) is known to artificially stimulate NADPH oxidase by acting upon protein kinase C (McPhail et al., 1984; Karlsson et al., 2000; Lundqvist et al., 1996; Wolfson et. al, 1985). Since PMA can stimulate NADPH oxidase intracellularly (Karlsson et al., 2000; Lundqvist et al., 1996), we can apply this treatment during infection with *C. albicans* because the fungus lives inside the phagocyte after it is engulfed. NADPH oxidase stimulation will allow us to fully assess how ROS may influence autophagy activation.

A summary of phagocytosis, ROS, and where they are thought to initiate autophagy activation is depicted in Figure 4. Included in this figure are the different treatments that we used to block or induce certain components of the activation pathway. With these different treatments and observations of LC3-EGFP distribution in the phagocyte, we can determine if ROS affect autophagy activation *in vivo* during an infection with *C. albicans*.

Materials and Methods

Zebrafish care

All zebrafish were maintained in recirculating systems (Aquatic Habitats, Apopka, FL) at the University of Maine Zebrafish Facility with water temperatures kept at 28°C. All zebrafish care protocols and experiments were conducted following NIH guidelines under Institutional Animal Care and Use Committee (IACUC) protocol A2009-11-01. Larvae were grown at a density of 100/dish in 10-cm petri dishes with 60

ml egg water. Egg water contained deionized water with 60 mg/liter Instant Ocean salts (Spectrum Brands, Mentor, OH). Fish strains used were LC3-EGFP (kindly provided by Daniel Klionsky, University of Michigan) and MPO-mCherry transgenic fish (plasmid provided by Anna Huttenlocher, University of Wisconsin-Madison; transformation conducted by Remi Gratacap, University of Maine). Microbial growth was prevented during the first 24 h of development by adding 0.00003% methylene blue. All zebrafish care and procedures were performed as previously described (Westerfield, 2007).

Fungal strains and growth conditions

Candida albicans CAF2-dTomato strain (kindly provided by Remi Gratacap, University of Maine) was used throughout these experiments. *C. albicans* was grown on yeast-peptone-dextrose (YPD) agar (Difco; 20g/liter peptone, 10 g/liter yeast extract, 20 g/liter glucose, 2% agar). Liquid cultures of *C. albicans* were grown overnight in YPD at 37°C for infections. Overnight cultures were washed in calcium- and magnesium-free phosphate-buffered saline (PBS; Lonza, Walkersville, MD) three times, counted on a hemocytometer, and adjusted to a final concentration of 1×10^7 yeast cells/ml.

Microinjection

Zebrafish at the prim-25 stage (36 hpf) were staged according to the method of Kimmel et al. Larvae were manually dechorionated and anesthetized in Tris-buffered tricaine methane sulfonate (tricaine; 200 μg/ml) (Western Chemicals, Inc., Frendale, WA). To infect, 5 to 10 nL of *C. albicans* suspension at 1×10^7/ml in PBS was microinjected through the otic vesicle into the hindbrain ventricle (Figure 3). Our goal

was to achieve a dose of approximately 10 CFU. Larvae were immediately screened after infection using a Zeiss Axiobserver Z1 microscope equipped with a Vivatome system (Carl Zeiss Microimaging, Thornwood, NJ) in order to limit the range of the initial infection to 10-25 yeast cells.

Fluorescence microscopy

An Olympus IZ-81 inverted microscope with an FV-1000 laser scanning confocal system was used for confocal imaging (Olympus). An objective lens with a power of x40/0.75 NA was used. Fish were anesthetized as previously described with tricaine and further immobilized in a mixture of 0.5% low-melting-point agarose (Lonza, Walkerville, MD) in egg water including tricaine (200 μg/ml). Images are overlays of fluorescence image panels (red-green) or overlays of differential interference contrast (DIC) and fluorescence images. Optical filters were used to detect dTomato and enhanced green fluorescence protein (EGFP) with excitation/emission at 554/581nm and 488nm/510nm, respectively.

Treatments and incubations

Zebrafish were treated with diphenylethyliodium chloride (DPI; 100 μM; Enzo, Farmingdale, NY) in 0.825% DMSO (v/v), α-tocopherol (100 μM, Sigma-Aldrich, St. Louis, MO) in 0.1% DMSO (v/v), phorbol 12-myristate 13-acetate (PMA, 0.304 μM; Sigma-Aldrich, St. Louis, MO) in egg water. DPI and α-tocopherol treated fish were immersed immediately after injection and incubated for 4 h at 28°C. PMA treated fish were immersed for 30 min at 3.5 hpi, immediately before confocal imaging. Shortened

exposure of PMA was due to extreme chemical sensitivity and reduced zebrafish survival during extended exposure.

Respiratory burst assay

The respiratory burst assay was conducted using dihydrodichlorofluorescein-diacetate (H_2DCF-DA) as previously described (Hermann and Kim, 2005) with some minor changes. Twelve larvae per treatment were induced with PMA (Sigma-Aldrich, St. Louis, MO), while twelve larvae were not induced for each treatment (control DMSO, DPI, or α-tocopherol). A Synergy2 plate reader (Biotek, Winooski, VT) was used to measure fluorescence every hour for a total of 8 h and nine readings (one reading at 0 h). Excitation and emission wavelengths were 485 nm and 528 nm, respectively. Differences in the ratios (induced/uninduced) of control and DPI or α-tocopherol treated fish were examined using bootstrapped confidence intervals obtained from 1,000 replicates, using the Pop-Tools add-in for Microsoft Excel. The degree of significance was determined by observing whether 95%, 99%, and 99.9% confidence intervals overlapped.

Results

Development of a classification scheme for phagosome morphology and LC3-EGFP distribution was necessary for quantitative analyses.

C. albicans can be contained within tight or loose phagosomes in human neutrophils and mouse macrophages (Marquis et al., 1991; Fernandez-Arenas et al., 2009); however, the mechanisms of exactly how these morphologies arise are still unknown. There has been work showing that damage to the cell wall of *C. albicans* and killing of the parasite *Leishmania donovani* occur more often within loose phagosomes

(Marquis, 1991; Gueirard et al, 2008). Investigating potential physiological differences between these types of phagosomes are important in understanding pathogen elimination within host immune cells. Additionally, it has been shown that NADPH oxidase activity is associated with loose phagosomes (Reeves et al, 2002). Similar to these observations, we found two types of morphologies, tight and loose, in the phagosome population *in vivo*, enabling us to assess potential differences between these two morphologies. Furthermore, LC3 accumulation has also been shown to be dependent on NADPH oxidase production of ROS *in vitro* (Huang et al., 2009; Mitroulis et al., 2010). To characterize how LC3-EGFP localization varies as a function of NADPH oxidase activity, ROS, and phagosome morphology *in vivo,* it was necessary to create a classification scheme.

Figure 5 depicts the different types of phagosomes and LC3-EGFP distributions seen throughout the experiments. These categories were seen during most treatments and represent over 1000 phagosomes observed. Tight phagosomes (Fig 5A) and loose phagosomes (Fig. 5B) were associated with weak cytoplasmic LC3-EGFP, strong cytoplasmic LC3-EGFP, and LC3-EGFP phagosomal rings.

There was a clear dichotomy of phagosome morphology during the infections *in vivo*. Tight phagosomes displayed no visible space between the phagosomal membrane and fungal cell wall during analysis of differential interference contrast (DIC) or fluorescent images. Loose phagosomes exhibited a phagosomal lumen larger than the enclosed fungi, which could be seen in DIC images. Furthermore, there was a strong distinction and separation between the phagosomal membrane and fungal cell wall with

DIC and fluorescent imaging, where dTomato and EGFP were noticeably separated. Both fluorescence and DIC images were used to confirm phagosome morphology.

To understand the relationship between ROS and autophagy clearly, it was necessary to describe and separate different LC3-EGFP distributions. Preliminary experiments showed cytoplasmic and phagosomal LC3-EGFP distributions. From these experiments, categories of strong cytoplasmic LC3-EGFP (s. cyt), weak cytoplasmic LC3-EGFP (w. cyt), and LC3-EGFP phagosomal rings (phag. rings) were created (Fig. 5). The strength of cytoplasmic LC3-EGFP fluorescence was analyzed in relation to other images of the same experiment to differentiate between strong and weak. Phagosomal rings were defined as having stronger expression of LC3-EGFP directly associated with the phagosomal membrane surrounding *C. albicans* than the cytoplasmic LC3-EGFP expression. In some phagocytes containing phagosomal rings, there was cytoplasmic LC3-EGFP visible, but in others the only appearance of LC3-EGFP was in the ring. Once phagosome morphology and distribution of LC3-EGFP categories were established, we were able to sort our images and conduct quantitative analyses to compare control and treated zebrafish.

ROS are not necessary for activating autophagy but absence may influence phagocytosis and proliferation of C. albicans *within phagocytes.*

ROS have been shown to be an essential activator of autophagy. Treatment with α-tocopherol has previously been used *in vitro* and was found to reduce the number of LC3+ phagosomal rings (Huang et al., 2009). After infection, we treated zebrafish with α-tocopherol to characterize the activation of autophagy *in vivo*. We also examined the

relationship between LC3-EGFP localization and phagosome morphology. Data obtained from these experiments demonstrate that most phagosomes were tight, with the majority of those having cytoplasmic LC3-EGFP (Fig. 6A). There seemed to be a decreased frequency of loose LC3-EGFP phagosomal rings (Fig. 6B), but this was not found to be statistically significant ($p=0.066$).

We also looked at the percentage of tight phagosomes with LC3-EGFP rings compared to the percentage of loose phagosomes with LC3-EGFP rings (Fig. 7). Analyzing the frequency of LC3-EGFP rings within the phagosome populations provides a possible physiological distinction between these morphologies. Raw numbers of LC3-EGFP rings were similar between tight and loose populations; however, relative percentages of each are significantly different (Fig. 7A).

Comparing the percentages of tight and loose LC3-EGFP phagosomal rings within either the α-tocopherol or the control treatment suggests that this bias towards loose phagosomes having LC3-EGFP phagosomal rings is independent of treatment, and therefore independent of ROS ($p\leq0.0001$ by Fisher's Exact Test; Fig. 7B). Control tight and loose phagosome populations are broken down into the percentages of LC3-EGFP rings or no LC3-EGFP rings, highlighting the bias of LC3-EGFP to accumulate on loose phagosomes (Fig. 7C). These results present one physiological difference between tight and loose phagosomes.

During analysis of LC3-EGFP distribution, phagocytes and phagosomes were observed to have various amounts of *C. albicans*, causing us to question if the absence of ROS affects the amount of fungi within phagocytes or phagosomes. Previous data have shown that macrophages commonly contain three or more fungi (≥3) while neutrophils

only have one or two (<3; Brothers et al., 2011). Because the zebrafish do not have a marker for the different cell types, we tried to separate neutrophils and macrophages based on the amount of *C. albicans*. Eventually, we decided this was not an ideal method of distinguishing phagocyte type; however, interesting data were still apparent.

A phagocyte with ≥3 *C. albicans* was determined by counting all the fungi, regardless of the number of phagosomes (Fig. 8A). Phagosomes with ≥3 *C. albicans* were determined by analyzing LC3-EGFP of the phagocyte around the fungus to elucidate how many fungal cells the phagosome encompassed (Fig. 8B). Phagocytes with ≥3 *C. albicans* increased significantly in α-tocopherol treated fish compared to control fish ($p \leq 0.001$ by Fisher's Exact Test; Fig. 8C). Additionally, phagosomes with ≥3 *C. albicans* increased during α-tocopherol treatment; however, it was not determined to be significant (Fig. 8D). These results suggest that phagocytes have a decreased ability to kill the fungi directly. Together, our findings imply that ROS do not have an effect on autophagy activation, but may alter the host's ability to control *C. albicans* infection.

PMA-enhanced respiratory burst does not significantly increase autophagy

According to *in vitro* data, autophagy activation increases when NADPH oxidase is stimulated by PMA (Mitroulis et al., 2010). We tested if NADPH oxidase stimulation, *in vivo*, also produced a higher rate of autophagy. Because of sensitivity to the chemical, zebrafish were exposed to PMA for only 30 min, after a 3.5-h incubation period to allow *C. albicans* to establish an infection. We hoped that exposure at the end of incubation would still allow us to observe the full effects of increased ROS during imaging. Two types of transgenic zebrafish were utilized in these experiments, our normal LC3-EGFP

cross and LC3-EGFP:MPO-mCherry to determine if our findings were sensitive to zebrafish strain background. These fish had no qualitative differences among them, and showed similar results (individual data not shown). This suggests that this phenotype is not strain background-dependent. The graphs therefore show PMA data combined from both types of zebrafish (Fig. 9). We found similar characteristics to α-tocopherol results, where the majority of phagosomes were tight with cytoplasmic LC3-EGFP (Fig. 9A). There was an increase within the total LC3-EGFP phagosomal ring population; however, it was not significant by Fisher's Exact Test (Fig. 9B). These data suggest that, *in vivo*, NADPH oxidase stimulation does not have the same effect on autophagy activation as previously found *in vitro*.

NADPH oxidase inhibition does not significantly decrease autophagy

α-tocopherol treatments showed that ROS do not reduce autophagy activation significantly, however, this treatment removed ROS from the phagocyte after it had already been produced, potentially allowing activation to occur within the short lifespan of ROS. In order to understand the relationship between ROS and autophagy activation fully, production of ROS must be blocked, which can be accomplished by inhibiting NADPH oxidase activity using DPI. DPI treatment has been shown to significantly decrease autophagy *in vitro* (Huang et al., 2009). We expected that DPI might elicit stronger changes in LC3-EGFP localization than seen with α-tocopherol, but this was not observed.

We found tight phagosomes with cytoplasmic LC3-EGFP dominating the phagosome population (Fig. 10A). DPI treated zebrafish showed no significant difference

in amount of LC3-EGFP phagosomal rings compared to control zebrafish (Fig. 10B). There was an increase in percent of phagocytes (Fig. 10C) and phagosomes (Fig. 10D) with ≥3 *C. albicans*, similar to α-tocopherol. Phagocytes, however, were not significantly different between DPI and control zebrafish, rather the percent of phagosomes was significantly greater between treatment and control ($p \leq 0.05$ by Fisher's Exact Test). These results, again, suggest that ROS removal does not affect autophagy activation as extensively *in vivo*, but may affect the phagocyte's ability to control the fungal infection.

The DPI experiments yielded similar populations of tight and loose LC3-EGFP phagosomal rings as α-tocopherol treatment (Fig. 11). Raw numbers between these tight and loose populations were comparable, but the percentages of each respective population showed a significant difference (Fig. 11A). Biased accumulation of LC3-EGFP on loose phagosomes, seen previously in α-tocopherol treatments, was visible within DPI treatment and control as well (Fig. 11B). This tendency of loose phagosomes to have LC3-EGFP phagosomal rings was apparent even without any chemical addition (Fig. 11C). This suggests something, that we are unaware of, is driving LC3-EGFP to accumulate more frequently on loose phagosomes.

Respiratory burst assay confirms α-tocopherol's antioxidant activity, while contradicting expected action of DPI

DPI and α-tocopherol had similar results with each analysis conducted, however it was not clear why DPI did not have more of a dampening effect on autophagy activation, since DPI consistently reduces autophagy events significantly *in vitro* (Huang et al., 2009). In the essence of time, we found DPI and α-tocopherol concentrations from

sources that showed significant decreases in the number of LC3+ phagosomal rings (Huang et al., 2009; Mitroulis et al., 2010), but that also allowed for survival of the zebrafish (Yoo et al., 2006; Niethammer et al, 2009; Cordero et al., 2009). In order to understand what is occurring *in vivo*, it was necessary to confirm the actions of the chemicals in zebrafish by conducting a respiratory burst assay (RBA). After wild-type AB zebrafish were injected at the prim-25 stage with PBS and incubated for 4 h at 28°C in α-tocopherol, DPI, or their controls at the same concentrations previously used in our experiments, respiratory burst was induced by phorbol myristate acetate (PMA). The amount of fluorescence by oxidized dihydrodichlorofluorescein-diacetate (H_2DCF-DA) was measured at 3 h post-PMA addition (7 h post-PBS injection). Monte Carlo analysis with 1,000 replicates was used to find the average ratio of induced to uninduced fluorescence (Fig. 12). Respiratory burst was significantly dampened in α-tocopherol treated zebrafish compared to control treated zebrafish ($p<0.001$; Fig. 12A). In contrast, DPI treated zebrafish did not have a dampened respiratory burst compared to control zebrafish (Fig. 12B) These results indicate that α-tocopherol was quenching ROS as expected, but DPI was not blocking ROS production efficiently. This information is important in order to interpret our previous results and what they indicate within the context of autophagy activation and infection control in the immune system.

Discussion

We demonstrated that it is possible to explore the relationship of autophagy and its role in the immune system within a live host. Utilizing the zebrafish, we were able to characterize autophagy activation *in vivo* through non-invasive imaging and describe a

potential physiological difference between tight and loose phagosomes. Additionally, we were able to explore how changing the amount of ROS can affect the ability of the immune system to control infection.

Autophagy activation is not dependent on ROS in vivo

The main focus of this research pertains to the relationship between NADPH oxidase-generated ROS and autophagy activation. We hypothesized that autophagy activation would be comparable to *in vitro* studies when exposed to chemical treatments that alter the amount of ROS within a live host. (Huang, 2009; Mitroulis, 2010).

Our research showed that the amount of ROS does not affect autophagy, which differs from *in vitro* data. Because we determined DPI did not inhibit NADPH oxidase efficiently (Fig. 12B), we cannot use the DPI data for conclusive evidence. However, α-tocopherol and PMA treatments, which were shown to reduce and increase the amount of ROS respectively during the respiratory burst assay, had no significant differences in the number of LC3-EGFP phagosomal rings, and therefore no significant differences in autophagy activation, when compared to controls. Previous research *in vitro* has shown that autophagy is dependent upon the presence of ROS, and detoxifying these reactive molecules with α-tocopherol results in decreased rates of autophagy (Huang, 2009). Additionally, PMA-stimulated NADPH oxidase induces respiratory burst and has previously shown to increase autophagy (Mitroulis, 2010). These differences from previous research launched our investigation into potential defects in the immune system of the inbred LC3-EGFP zebrafish strain. However, we found our LC3-EGFP:MPO-mCherry crosses mirrored the LC3-EGFP results indicating there were no obvious

genetic defects within the immune system of the LC3-EGFP transgenic fish, leading us to believe there are actual differences in autophagy activation *in vivo.*

Differences in methodology may also be a reason our data do not reflect previous research. The zebrafish model allows for multiple interactions from a variety of cells and soluble signaling factors that may not be replicated in cell culture experiments, as well as the physical location within a live host compared to a petri dish. These experimental differences may be the culprit of the discrepancies.

Figure 13 proposes that ROS and cell surface receptors, such as FcγR or TLR signaling, work together to fully activate autophagy *in vivo*. Further experiments, like using morpholinos to knockdown cell surface receptor genes, would help determine if these interactions are indeed necessary to activate autophagy.

Loose phagosomes may elicit a stronger immune response causing autophagy activation to be morphologically biased

Another aspect of this research was to address potential physiological differences between tight and loose phagosomes. We observed a dichotomy of morphologies in the phagosomes surrounding the fungi, similar to previous observations (Marquis, 1991; Fernandez-Arenas, 2009). We wanted to understand if there was any relationship between phagosome morphology and autophagy.

We found a high frequency of loose phagosomes was associated with LC3-EGFP phagosomal rings, which is indicative of autophagy activation (Fig. 7). Previous *in vitro* research has not had a formal discussion of a potential link between loose phagosomes

and autophagy, but representative images of these studies are consistent with this idea (Sanjuan et al. 2007).

It has been suggested that an active NADPH oxidase, and therefore an increase in ROS, is associated with loose phagosomes (Reeves et al., 2002). With this information, and the *in vitro* model of autophagy activation (Huang, 2009; Mitroulis, 2010), this bias of autophagy to loose phagosomes would be easily explained. However, as we have previously described, autophagy activation does not depend on ROS *in vivo*, providing a reason to believe another mechanism, specifically associated with loose phagosomes, plays a role in activating autophagy. One mechanism could be that a maturation process of tight phagosomes into loose phagosomes occurs, potentially creating a stronger immune response within the loose phagosomes. This maturation hypothesis is indicated in Figure 13, which also shows, by the relative weights of the arrows, the frequency of LC3-EGFP associated with loose and tight phagosomes.

Our findings of autophagy occurring preferentially within loose phagosomes may be an additional reason for the increased capacity of loose phagosomes to kill pathogens. Loose phagosomes have been shown to be more capable of killing *C. albicans* and *Leishmania donovani* (Marquis 1991; Gueirard et al, 2008). Though we didn't witness any fungi degradation between 4-6 hpi, killing may occur at later time points. It would be interesting to extend the imaging period to understand more about pathogen killing in loose phagosomes or LC3-EGFP phagosomal rings. Ultimately, we were successful in observing a physiological difference between the morphologies, however additional experiments must be conducted in order to confirm our hypotheses.

Antioxidants may affect immune function negatively because of the role of ROS in pathogen containment within phagocytes

α-tocopherol and DPI treatments seemed to have an effect on the number of phagocytes and phagosomes containing ≥3 *C. albicans.* However, because DPI was not blocking ROS production in the context of PMA-induced respiratory burst, we focused our attention only on the effects of α-tocopherol. There was an increase in phagocytes and phagosomes containing multiple fungi during α-tocopherol treatment; however, only the increase in phagocytes was statistically significant (Fig. 8C). Reduced neutrophil migration and phagocytosis has been observed in NADPH oxidase knockdown and DPI-treated zebrafish (Brothers and Wheeler, unpublished). This may account for what is happening during our α-tocopherol treatment; more macrophages, which commonly phagocytose three or more *C. albicans*, must migrate to the site of infection and phagocytose fungi in order to compensate for depleted neutrophil activity. We were unable to confirm this hypothesis in our experiments because we do not have a marker differentiating neutrophils and macrophages in the LC3-EGFP zebrafish line.

Another hypothesis relates to the hypothesis that ROS are capable of directly damaging the pathogen (Segal, 2005). It is possible that phagocytosis occurs normally; however, once inside the phagocyte, reduced amounts of ROS may diminish the ability of the phagocytes to destroy the fungi, which are subsequently able to divide more readily. Though we did not confirm these theories experimentally, they could be addressed by observing migration of neutrophils and macrophages using transgenic zebrafish that differentiate between the two, and by assessing the fungal burden during α-tocopherol treatment and controls.

Our research showed that autophagy activation was not inhibited when the antioxidant α-tocopherol was administered, but more research will need to be conducted to fully understand activation *in vivo*. Autophagy was activated more frequently in loose phagosomes, regardless of ROS presence, illustrating differences between tight and loose phagosomes that have yet to be fully understood. Furthermore, we found that antioxidants may have a negative impact on the ability of the immune system to directly attack pathogens. Additional investigations must be conducted to confirm our new hypotheses about ROS, autophagy activation, and the role of autophagy in controlling fungal infection. Ultimately, we have shown that it is possible to address these questions pertaining to autophagy within a live, vertebrate host.

Figures

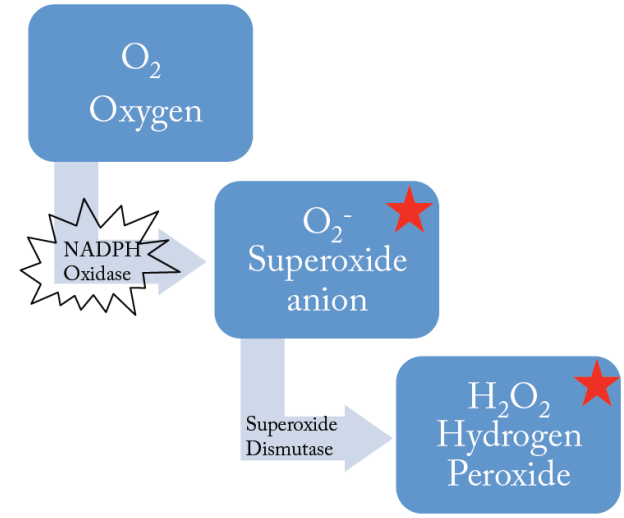


Figure 1: Reactive oxygen species (ROS) production pathway. Oxygen and NADPH oxidase react to produce superoxide anion, which reacts with other enzymes to form additional ROS.

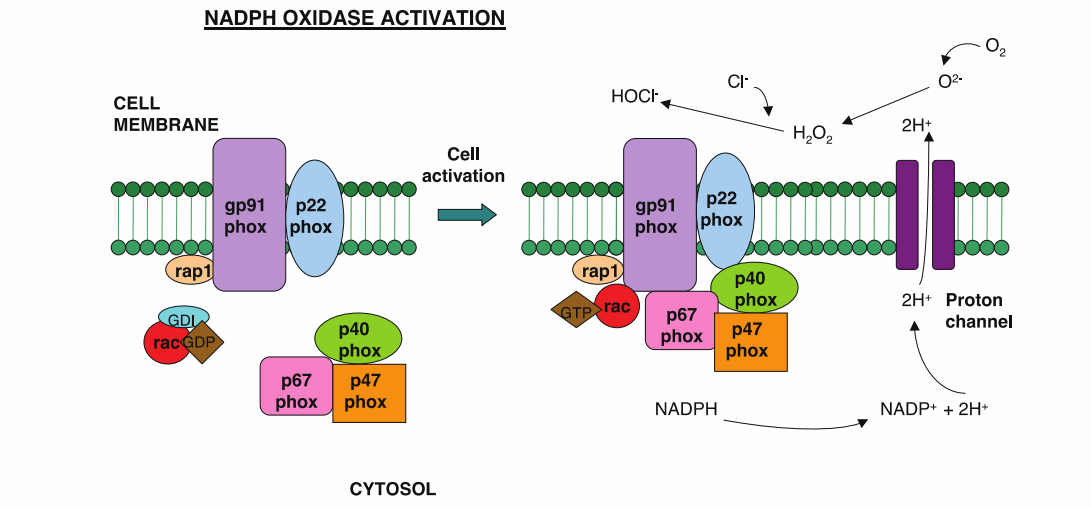


Figure 2: Translocation of NADPH oxidase. Cytosolic subunits translocate to the membrane, which activates the enzyme and leads to production of superoxide anion (Assari, 2006).

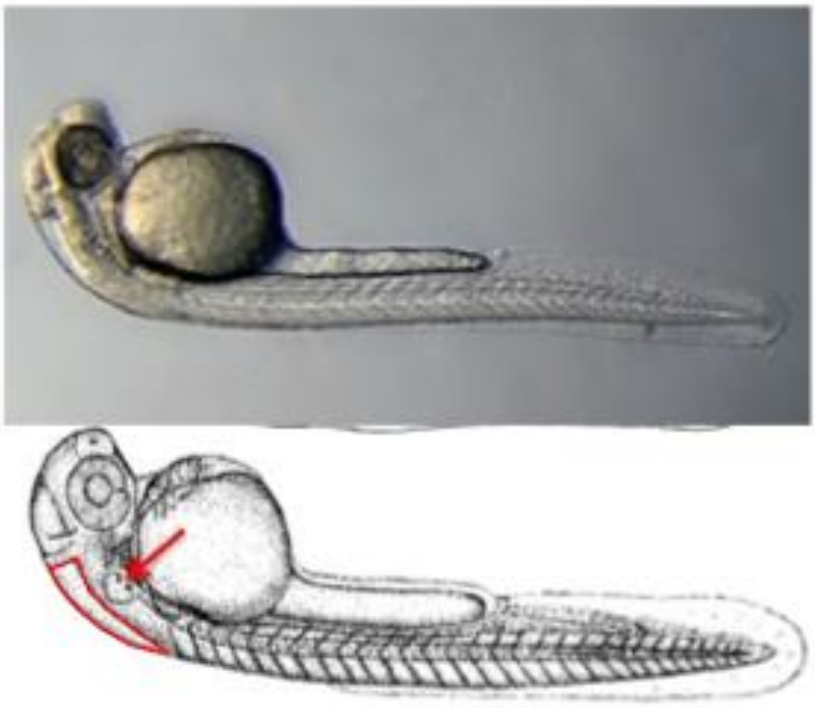


Figure 3: Infection of the hindbrain ventricle in larval zebrafish. Zebrafish at the prim-25 stage (~36 hpf) were infected in the hindbrain ventricle, outlined in red, through the otic vesicle, indicated by the red arrow, with 10-25 *Candida albicans* yeast, and immediately screened using a Zeiss Axiobserver Z1 microscope to confirm ideal infection. After injection, fish were incubated for 4 h at 28°C and imaged between 4-6 hpi using confocal microscopy. Images were analyzed using Fluoview Software to classify phagosome morphology, LC3-EGFP distribution, and to count the number of *C. albicans* within phagocytes and phagosomes.

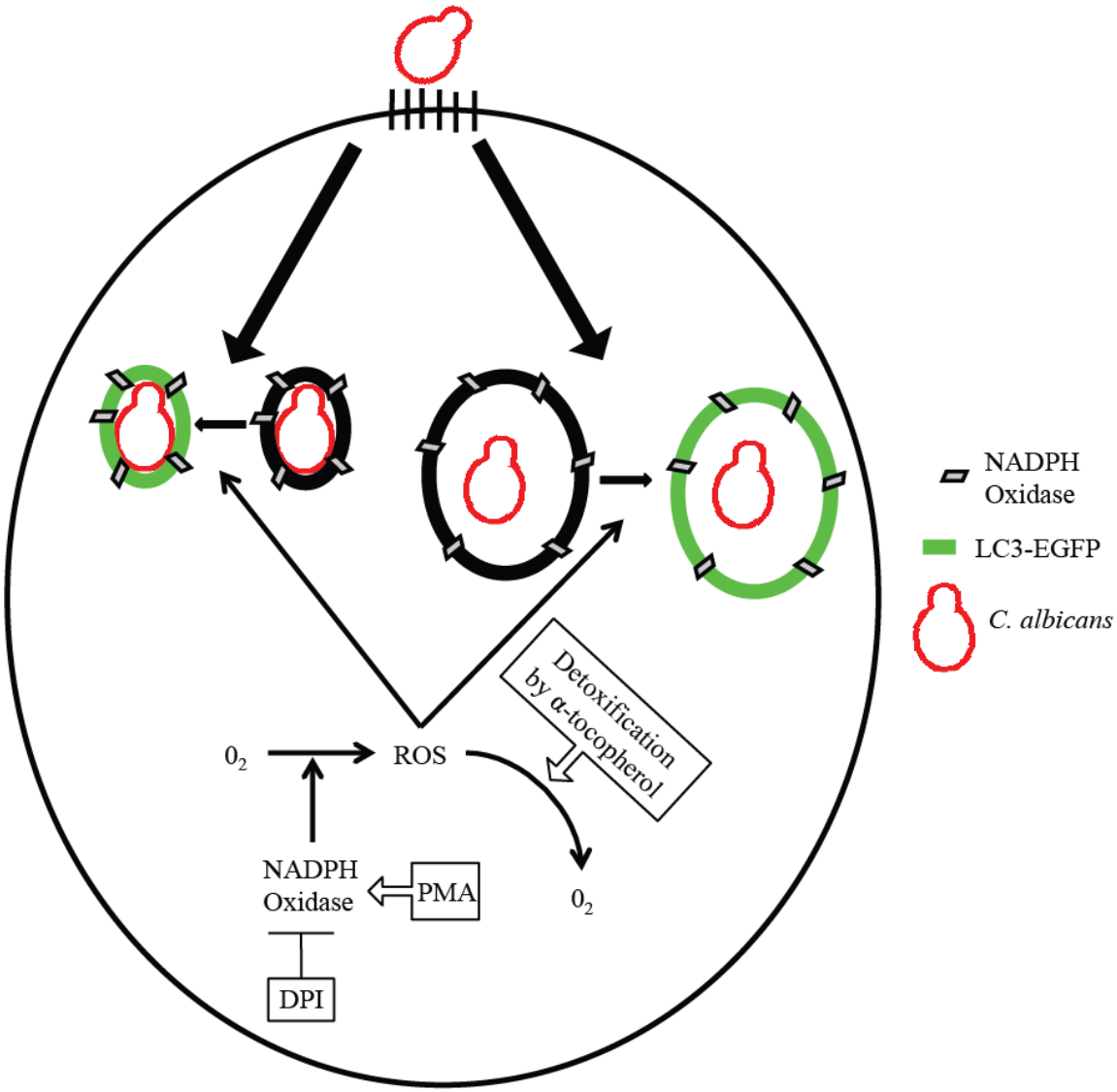


Figure 4: Hypothesized autophagy pathway with chemical treatments. Upon recognition and phagocytosis of *C. albicans*, NADPH oxidase translocates to the phagosomal membrane and reacts with molecular oxygen to produce ROS. *In vitro*, activation of autophagy by ROS leads to localization of LC3-EGFP on the phagosome containing the pathogen, forming phagosomal rings that can be visualized by confocal microscopy. Changes in the frequency of LC3-EGFP phagosomal ring appearance are indicative of changes in autophagy activation. DPI, which inhibits NADPH oxidase from producing ROS, and α-tocopherol, which acts as an antioxidant and removes ROS, treatment and was expected to reduce the frequency of LC3-EGFP phagosomal rings. PMA treatment stimulates NADPH oxidase activity, increasing ROS production, and was expected to increase the frequency of LC3-EGFP phagosomal rings. The changes in concentration of ROS will help us characterize autophagy activation *in vivo*.

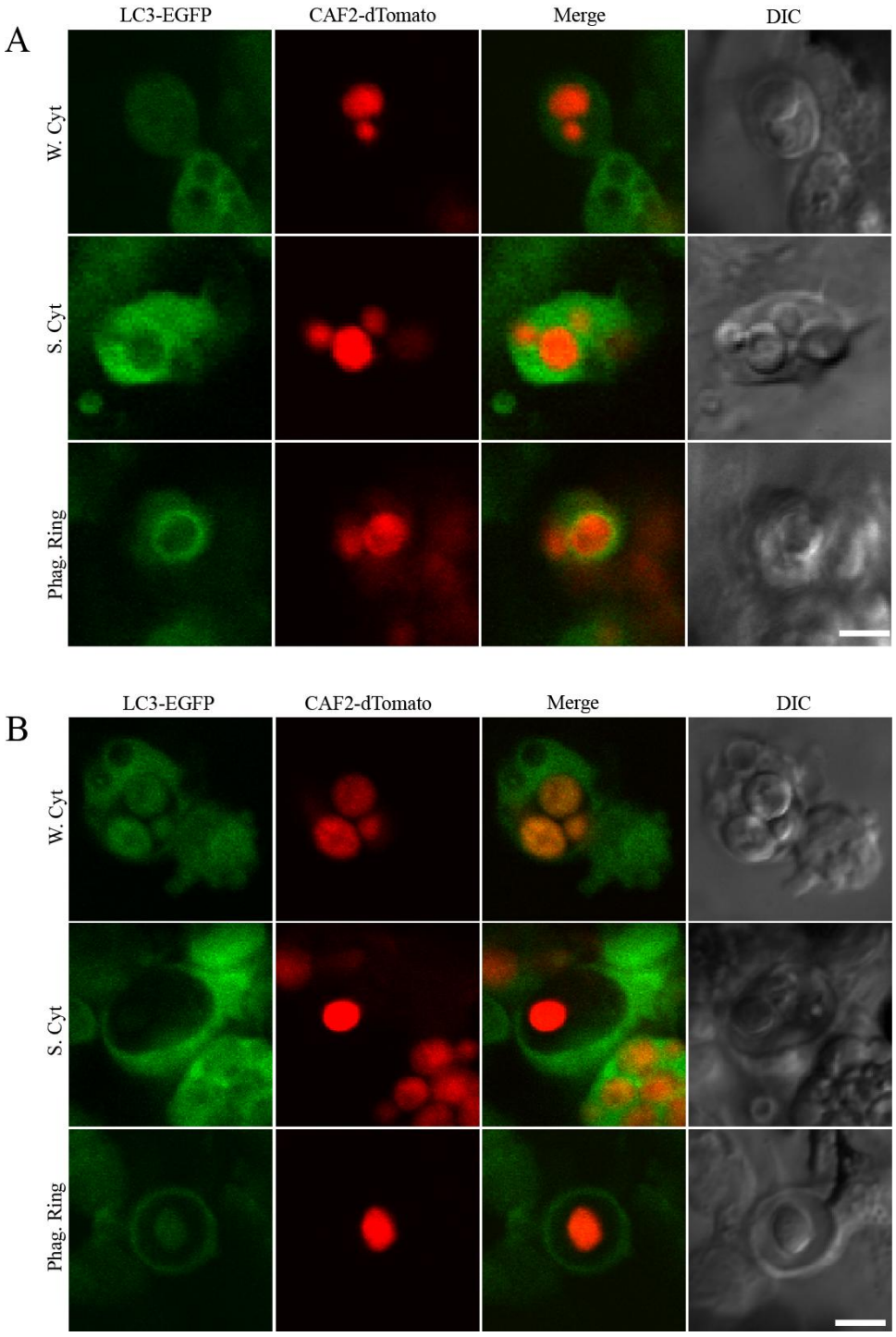


Figure 5: Classification scheme of phagosome morphology and LC3-EGFP localization. LC3-EGFP zebrafish were infected as described in Figure 3 with CAF2-dTomato *C. albicans*. Phagosome morphology was characterized as either tight (A) or loose (B) by analysis of DIC and fluorescent images. Both morphologies showed LC3-EGFP localization as weak cytoplasmic (w. cyt), strong cytoplasmic (s. cyt) or as phagosomal rings (phag. ring). Images are representative of over 1000 tight phagosomes and over 50 loose phagosomes that were the result of 16 independent experiments. Scale bars = 5 μm.

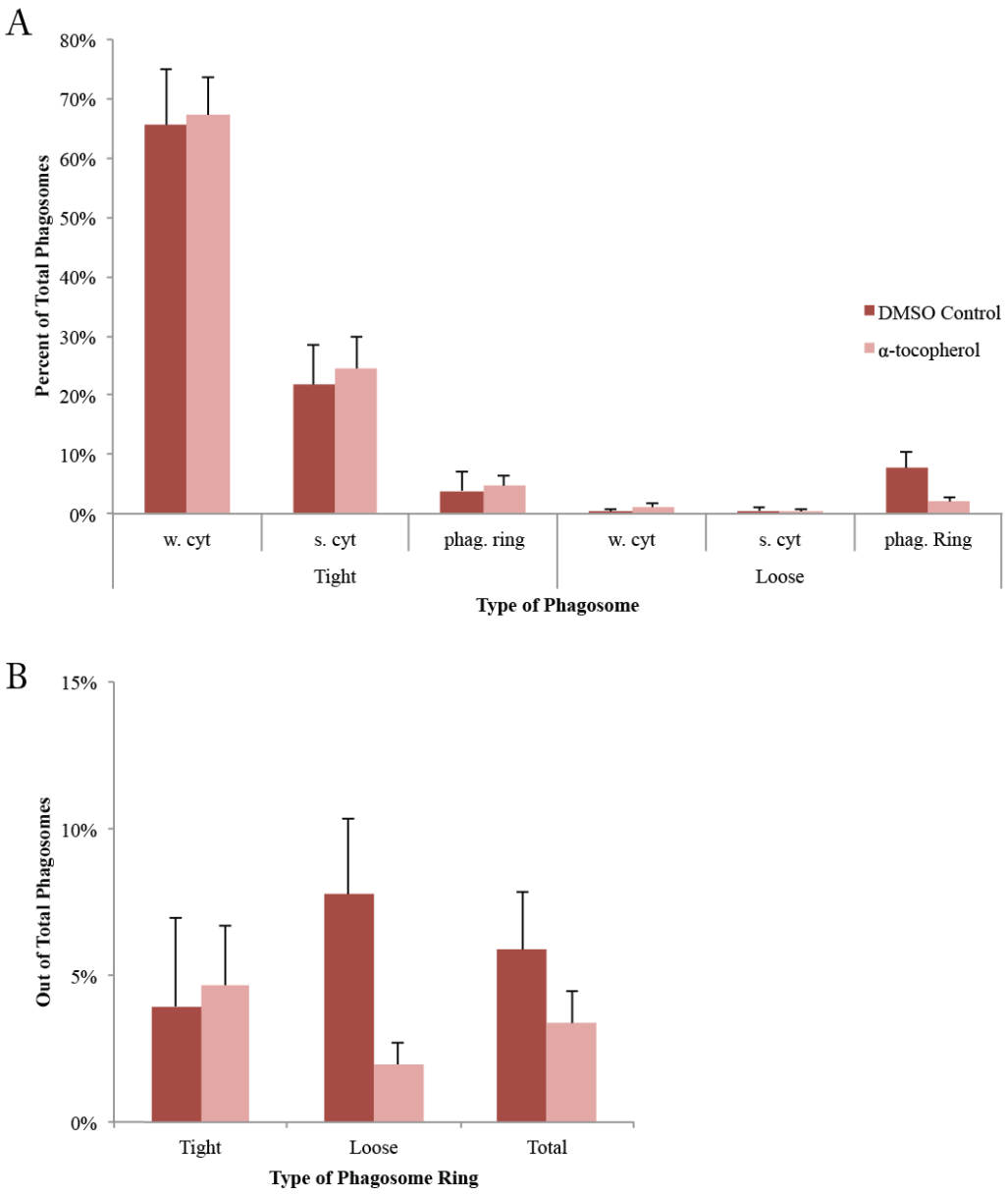
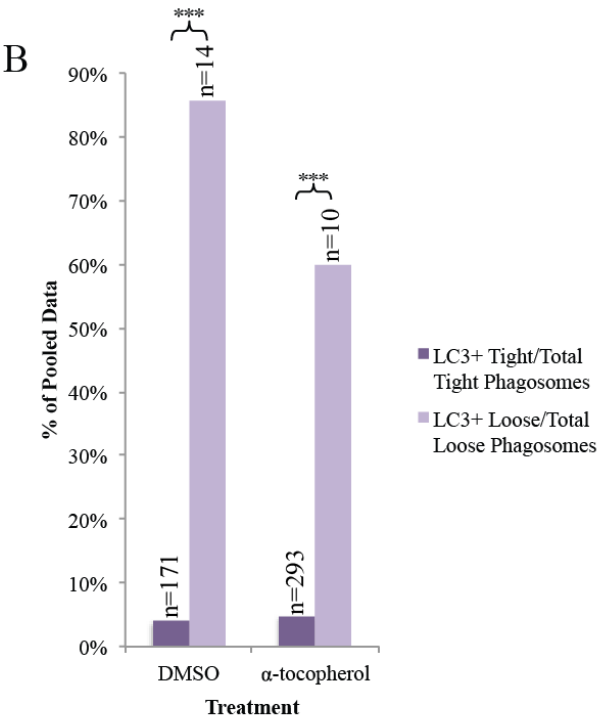


Figure 6: Removal of ROS by α-tocopherol does not affect LC3-EGFP distribution significantly. LC3-EGFP zebrafish were infected as described in Figure 3 with CAF2-dTomato *C. albicans* and treated with α-tocopherol (100 μM) and DMSO (0.1%) or control DMSO (0.1%) during incubation. Phagosomes were categorized accordingly. The graphs show the mean percentage of each category out of total phagosomes from five independent experiments, with bars representing standard error. (A) Total distribution of phagosome population during α-tocopherol treatment compared to control. (B) LC3-EGFP phagosomal ring distribution during α-tocopherol treatment compared to control. Tight, loose and combined total of LC3-EGFP phagosomal rings are shown. Tight LC3-EGFP phagosomal rings and total LC3-EGFP phagosomal rings show little difference between control and α-tocopherol treatment groups. Percent of loose phagosomes with LC3-EGFP phagosomal rings did decrease during α-tocopherol treatment, but not significantly ($p=0.066$ by T-test).

A

	Treatment	Total Tight Phagosomes	Total Loose Phagosomes	LC3+ Tight Phagosomes	LC3+ Loose Phagosomes
Raw Numbers	DMSO	171	14	7	12
	α-tocopherol	293	10	14	6
Percentage out of Total Type	DMSO	–	–	4.1%	85.7%
	α-tocopherol	–	–	4.8%	60.0%



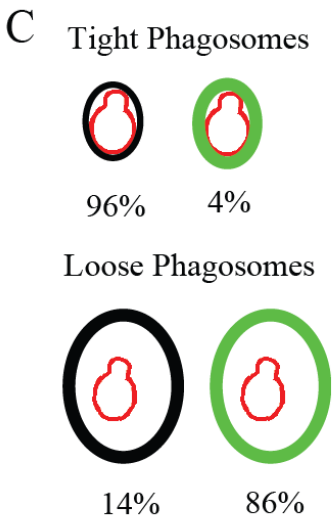


Figure 7: LC3-EGFP localizes more frequently on loose phagosomes than tight phagosomes. LC3-EGFP zebrafish were infected as described in Figure 3 with CAF2-dTomato *C. albicans* and treated with α-tocopherol (100 μM) and DMSO (0.1%) or control DMSO (0.1%) during incubation. Data are pooled from five independent experiments. (A) Raw data and percentages of tight and loose LC3-EGFP phagosomal rings. (B) Percentage of tight phagosomes with LC3-EGFP rings compared to percentage of loose phagosomes with LC3-EGFP rings within each treatment. n represents the number of total tight or total loose phagosomes quantified during α-tocopherol or control treatment. *** $p \leq 0.0001$ by Fisher's Exact Test. (C) Percentages of control tight and loose phagosomes with LC3-EGFP rings and without LC3-EGFP rings are shown beneath pictorial representations of each to illustrate the biased nature of LC3-EGFP accumulation on loose phagosomes in the absence of any treatment.

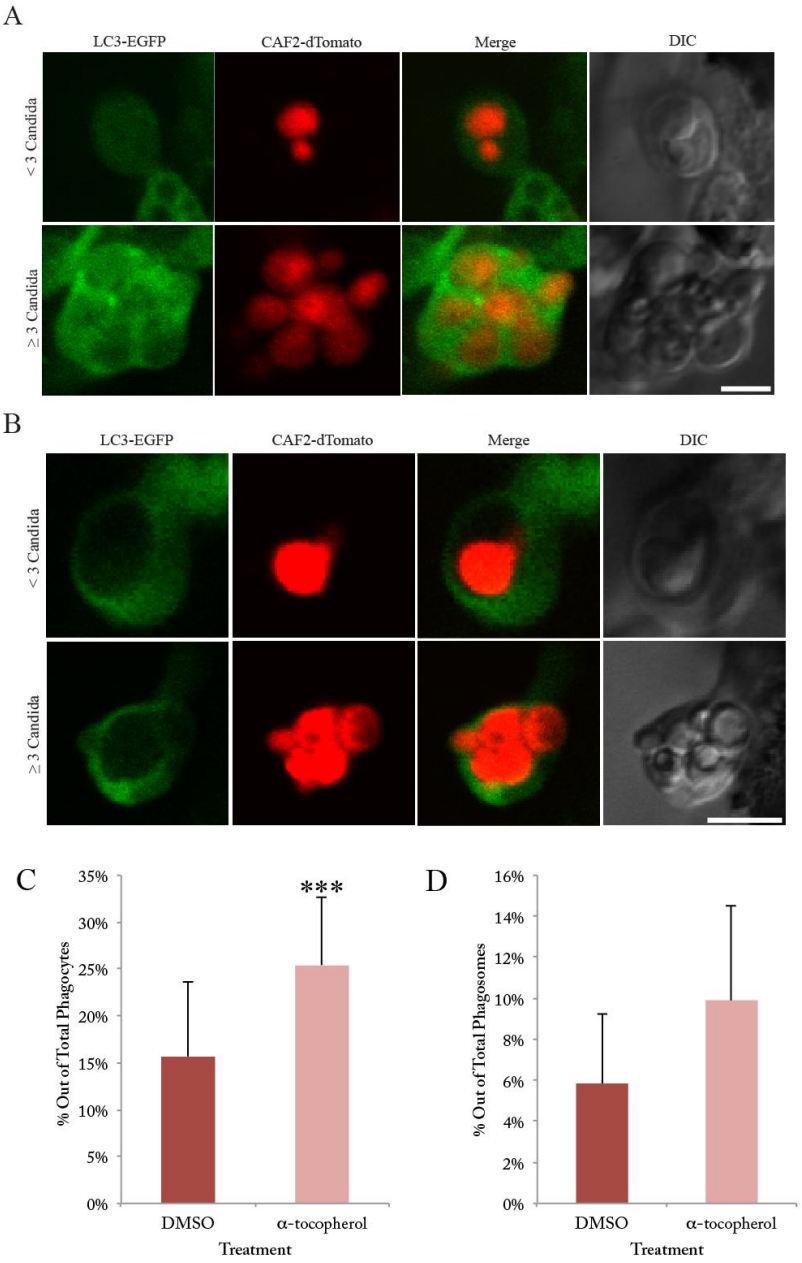


Figure 8: Removal of ROS by α-tocopherol may impact phagocyte ability to control *C. albicans.* LC3-EGFP zebrafish were infected as described in Figure 3 with CAF2-dTomato *C. albicans* and treated with α-tocopherol (100 μM) and DMSO (0.1%) or control DMSO (0.1%) during incubation. The number of *C. albicans* fungi within phagocytes and phagosomes was quantified. Images show phagocytes (A) and phagosomes (B) that contained ≥3 *C. albicans* or <3 *C. albicans.* Images are representative of over 600 phagocytes and 1000 phagosomes from 12 independent experiments. Scale bars = 5μm. (C) Phagocytes with ≥3 *C. albicans.* Percentage of phagocytes increased significantly in α-tocopherol treated zebrafish. ***$p \leq 0.0001$ by Fisher's Exact Test. (D) Phagosomes with ≥3 *C. albicans.* Percentage of phagosomes increased during α-tocopherol treatment, however not significantly. Graphs show mean percentages from 5 independent experiments, with bars representing standard error.

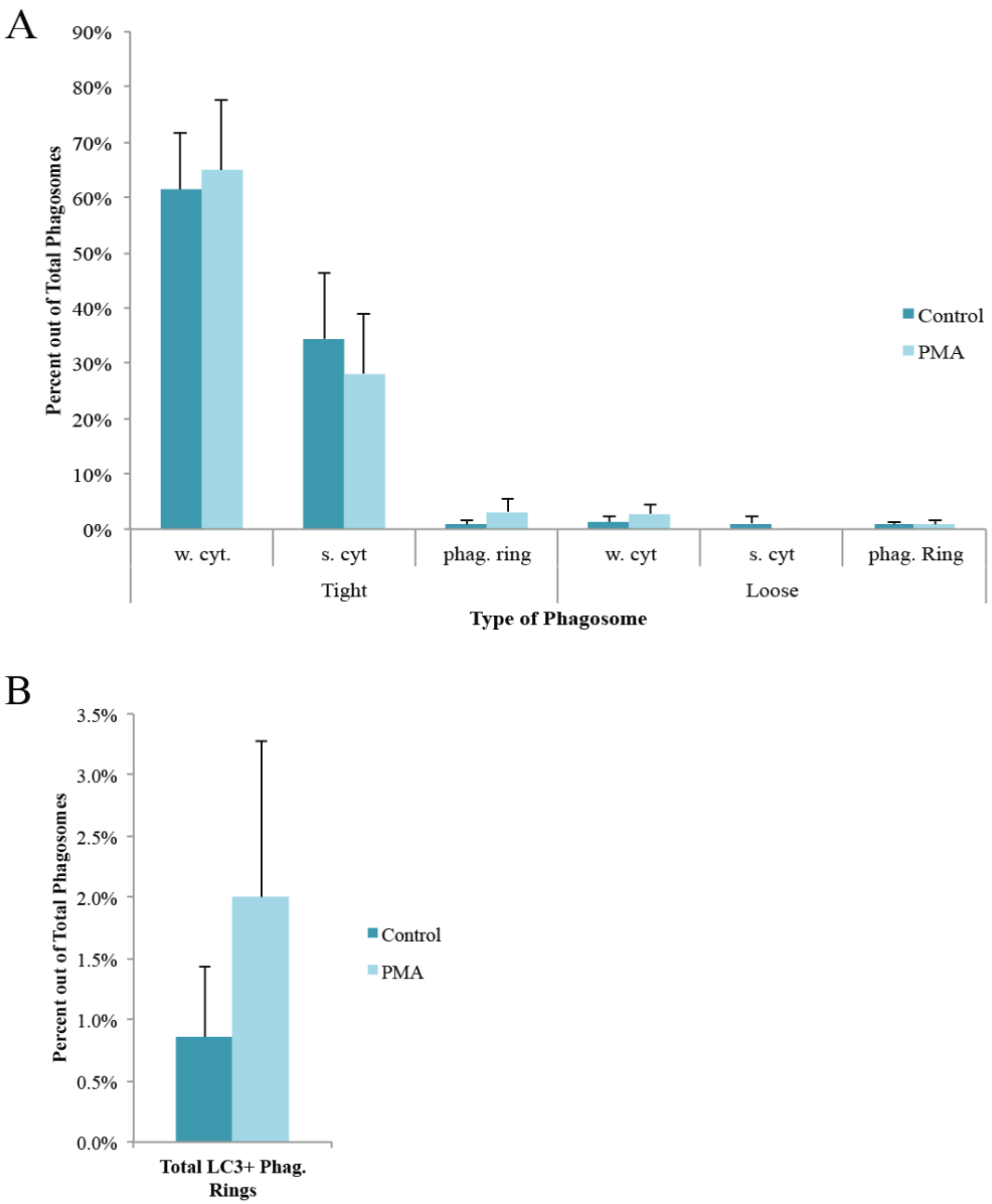


Figure 9: LC3-EGFP distribution is not significantly affected during NADPH oxidase stimulation. LC3-EGFP and LC3-EGFP:MPO-mCherry zebrafish were infected as described in Figure 3 with CAF2-dTomato *C. albicans*. Fish were incubated for 3.5 hpi at 28°C in egg water then submersed in PMA (0.304 µM) and egg water or control egg water for 30 min prior to imaging. Fish were imaged, analyzed and phagosomes were categorized accordingly. The graphs show the mean percentages of combined data from both types of zebrafish from 4 independent experiments, with bars representing standard error. (A) Total distribution of phagosome population during PMA treatment compared to control. (B) Total percentage of LC3-EGFP phagosomal rings during PMA treatment compared to control. Increased amounts of LC3-EGFP phagosomal rings were seen, however they were not found to be statistically significant.

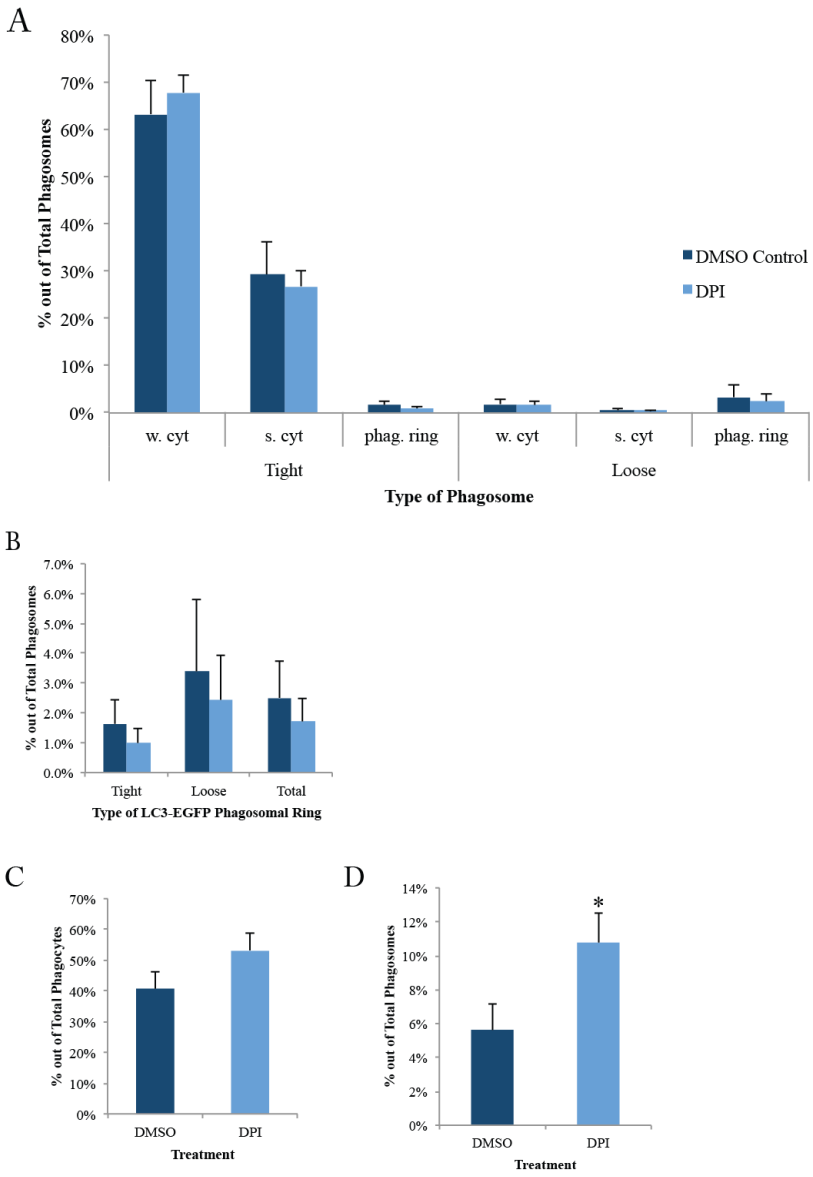
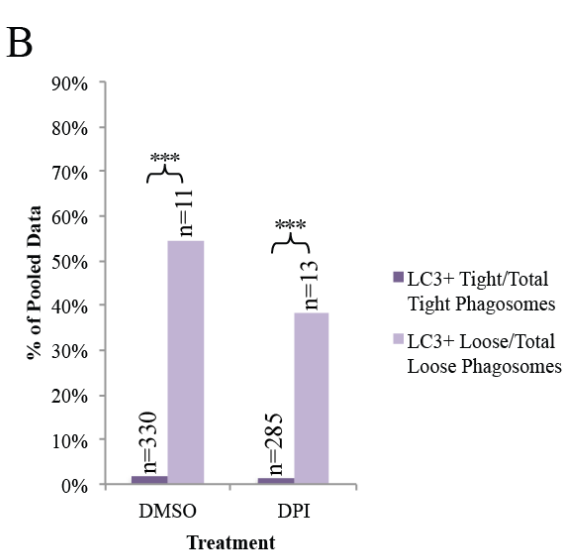


Figure 10: NADPH oxidase inhibition does not affect LC3-EGFP distribution or phagocytosis, but may impact infection control. LC3-EGFP zebrafish were infected as described in Figure 3 with CAF2-dTomato *C. albicans* and treated with DPI (100 μM) and DMSO (0.825%) or control DMSO (0.825%) during incubation. Phagosome morphology, LC3-EGFP distribution, and number of *C. albicans* were categorized accordingly. The graphs show the mean percentages of seven independent experiments, with bars representing standard error. (A) Total distribution of phagosome population during DPI treatment compared to control. (B) LC3-EGFP phagosomal ring distribution during DPI treatment compared to control. Tight, loose and combined total LC3-EGFP phagosomal rings are shown with little difference in number of LC3-EGFP rings between control and DPI treated zebrafish. (C) Phagocytes with ≥3 *C. albicans.* Percentage of phagocytes increase in DPI treated zebrafish, however not significantly. (D) Phagosomes with ≥3 *C. albicans.* Percentage of phagosomes increased significantly during DPI treatment. *$p \leq 0.05$ by Fisher's Exact Test.

A

	Treatment	Total Tight Phagosomes	Total Loose Phagosomes	LC3+ Tight Phagosomes	LC3+ Loose Phagosomes
Raw Numbers	DMSO	330	11	6	6
	DPI	285	13	4	5
Percentage out of Total Type	DMSO	–	–	1.8%	54.5%
	DPI	–	–	1.4%	38.5%



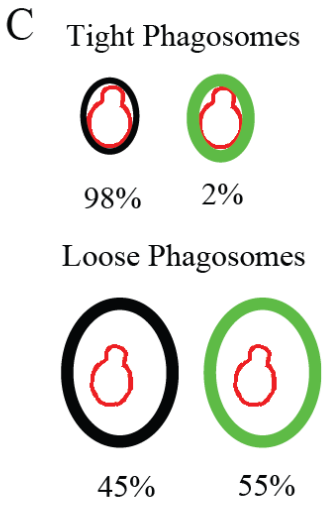


Figure 11: LC3-EGFP localizes more frequently on loose phagosomes than tight phagosomes. LC3-EGFP zebrafish were infected as described in Figure 3 with CAF2-dTomato *C. albicans* and treated with DPI (100 μM) and DMSO (0.825%) or control DMSO (0.825%) during incubation. Data is pooled from seven independent experiments. (A) Raw data and percentages of tight and loose LC3-EGFP phagosomal rings. (B) Percentage of tight phagosomes with LC3-EGFP rings compared to percentage of loose phagosomes with LC3-EGFP rings during DPI or control treatment. n represents the number of total tight or loose phagosomes quantified during each treatment. *** $p \leq 0.0001$ by Fisher's Exact Test. (C) Percentages of control populations are shown beneath diagrams depicting tight and loose phagosomes with LC3-EGFP rings and without LC3-EGFP rings to illustrate the biased nature of LC3-EGFP accumulation on loose phagosomes even in the absence of any treatment.

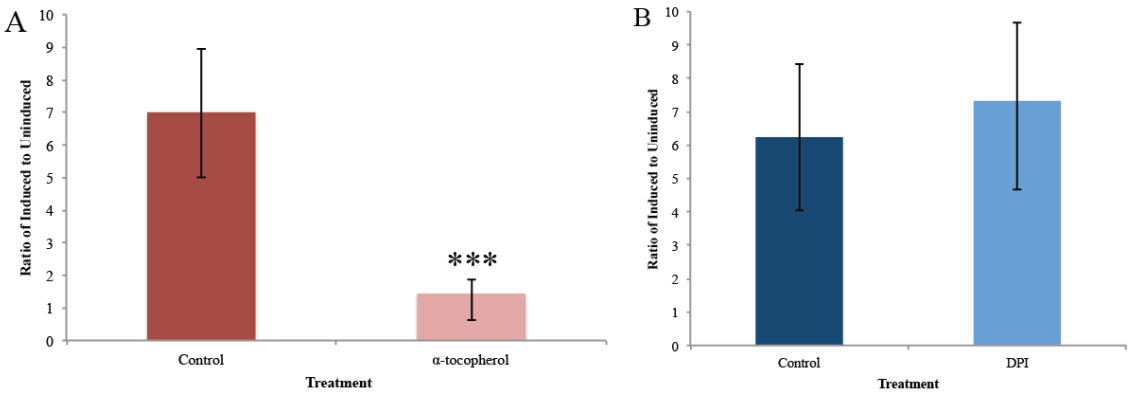


Figure 12: α-tocopherol and DPI differ in respiratory burst dampening. Wild-type AB zebrafish were injected with PBS into the hindbrain ventricle through the otic vesicle at the prim-25 stage and immediately submersed in (A) α-tocopherol (100 μM) and DMSO (0.1%) or control DMSO (0.1%) or (B) DPI (100 μM) and DMSO (0.825%) or control DMSO (0.825%) and incubated at 28°C for 4 h. After 4 h, respiratory burst was induced by PMA addition in the presence of H_2DCF-DA and was assayed. Monte Carlo analysis with 1,000 replicates was used to find the average ratio of fluorescence between induced and uninduced fish at 3 h post-PMA addition (7 h post-PBS injection). Graphs are representatives of three independent trials, with bars showing 95% confidence intervals. (A) α-tocopherol treatment shows significant dampening of respiratory burst ($p<0.001$) compared to the controls, indicating it is removing NADPH oxidase-generated ROS as expected. (B) DPI treatment does not show a dampened respiratory burst compared to the controls, indicating it is not blocking ROS production by NADPH oxidase as efficiently as expected.

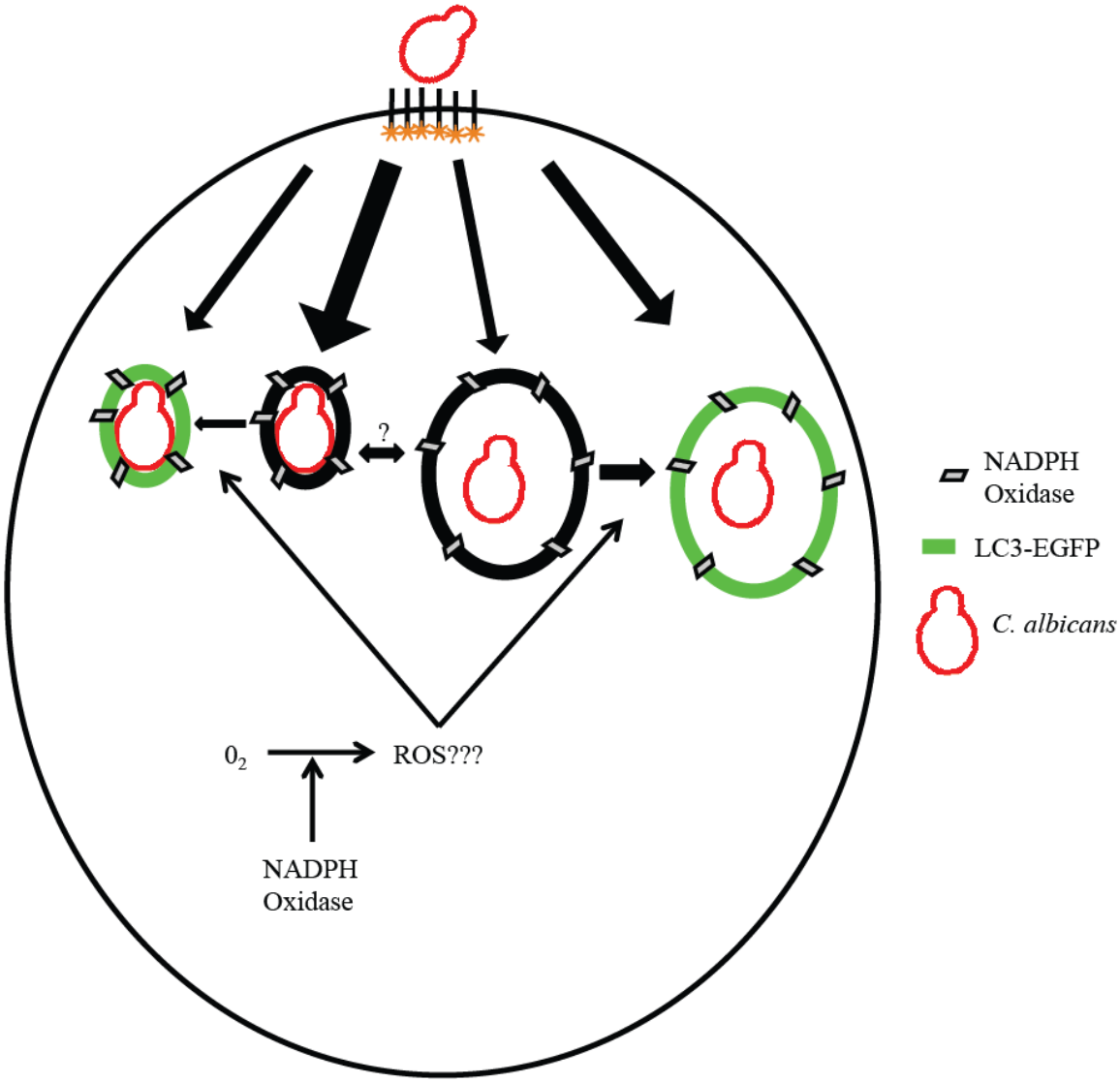


Figure 13: Summarizing our experimental findings and new hypotheses. Two phagosomes morphologies were observed, with the majority of them being tight cytoplasmic LC3-EGFP. When loose phagosomes are present, there is a high frequency of LC3-EGFP rings, which seems to be independent of ROS. Our findings question the role of ROS in autophagy activation *in vivo*. Additionally, there may be a maturation process of tight phagosomes into loose, which might be the reason autophagy is biased toward loose phagosomes. We hypothesize that ROS work in conjunction with cell surface receptors (TLRs, FcγRs) to complete the activation pathway, but necessitates additional investigation. Weights of the arrows in the diagram represent relative frequencies of each type of phagosome and LC3-EGFP rings according to our data.

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After graduation, Ashley will be attending Pacific University College of Optometry in Forest Grove, Oregon as a member of the Class of 2016, in order to become an optometrist.