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Polyclonal anti-*Candida* antibody improves phagocytosis and overall outcome in zebrafish model of disseminated candidiasis

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Abstract

Fungal infections are a major cause of animal and plant morbidity and mortality worldwide. Effective biological therapeutics could complement current antifungal drugs, but understanding of their *in vivo* mechanisms has been hampered by technical barriers to intravital imaging of host-pathogen interactions. Here we characterize the fungal infection of zebrafish as a model to understand the mechanism-of-action for biological antifungal therapeutics through intravital imaging of these transparent animals. We find that non-specific human IgG enhances phagocytosis by zebrafish phagocytes *in vivo*. Polyclonal anti-*Candida* antibodies enhance containment of fungi *in vivo* and promote survival. Analysis suggests that early phagocytic containment is a strong prognostic indicator for overall survival. Although polyclonal anti-*Candida* antibodies protect against disease, this is not necessarily the case for individual monoclonal anti-*Candida* antibodies. Thus, the zebrafish appears to provide a useful model host for testing if a biological therapeutic promotes phagocytosis *in vivo* and enhances protection against candidemia.

1.1. Introduction

Fungal infections are a major cause of animal and plant morbidity and mortality worldwide (Brown et al., 2012). In healthy communities with good access to health care, opportunistic fungi such as *Candida* and *Aspergillus* account for the majority of lethal fungal infections of humans. These infections often result from iatrogenic immunocompromising events such as surgical procedures, allogeneic bone marrow transplantation, and use of in-dwelling catheters. Although several classes of antifungal drugs are approved for use in the United States, nearly one-third of all cases of disseminated candidiasis result in death (Enoch et al., 2006; Horn et al., 2009).

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Work over several decades has sought to develop effective vaccination protocols and biological therapeutics to complement the currently available chemotherapeutics. Recent work has led to the discovery of two different antibodies that can prevent infection in mouse models of candidiasis. The Cassone group identified an anti- β -glucan mouse IgG antibody with efficacy in preventing murine disseminated candidiasis and with *in vitro* killing activity (Torosantucci et al., 2005). The Cutler group identified and characterized an anti-mannan mouse IgM antibody with efficacy in a murine model (Han et al., 2001; Xin and Cutler, 2011; Xin et al., 2008). Nevertheless, the development of monoclonal antibodies for protection against candidemia has not yet realized its potential (Bromuro et al., 2002; Casadevall et al., 1998).

Therapeutic antibodies can act through a number of different mechanisms to block microbial pathogenesis, including direct neutralization, enhancement of complement deposition and/or activation, augmentation of opsonic uptake, activation of natural killer cells, and blockade of adhesion (Ramsland et al., 2015). While many of these activities can be and have been evaluated *in vitro* with in the presence of host cells, these *in vitro* evaluations do not fully recapitulate the host environment (Golay and Introna, 2012). Mechanistic *in vivo* mouse studies are limited in resolution, but genetic knockout experiments suggest that complement is a crucial component in the protective activity of both an IgM and an IgG monoclonal antibody specific for *Candida* (Han et al., 2001). Thus, the *in vivo* mechanism(s) of action of these antibodies remain obscure, in part because of the technical barriers to intravital imaging of host-pathogen interactions during fungal infection.

Zebrafish larvae represent an attractive model to discern mechanism, as their immune mechanisms are largely conserved with other vertebrates and it is possible to non-invasively image particle- and microbe-phagocyte interactions within an infected animal (Tobin et al., 2012). Opsonic recognition in fish occurs, as in mammals, through antibodies, complement and mannose-binding lectin pathways (Bengten and Wilson, 2015; Leiro et al., 1996; Leiro et al., 2008; Nakao et al., 2011; Zhang and Cui, 2014). In addition, scavenger receptors act as broad specificity non-opsonic receptors (Froystad et al., 1998). However, the roles and mechanisms of opsonic protection to infection remain only partially understood in the zebrafish and other teleosts.

Here we sought to characterize the use of a zebrafish model to evaluate mechanism-of-action for biological antifungal therapeutics. We assayed the ability of zebrafish to utilize mammalian antibodies for phagocytosis and the ability of anti-*Candida* antibodies to prevent lethal disseminated infection in these fish. We found that some mammalian IgG antibodies can promote phagocytosis of unopsonized beads in fish, and that polyclonal rabbit anti-*Candida* antibodies protect zebrafish against disseminated *C. albicans* disease. Intravital imaging revealed that these antibodies resulted in greater early phagocyte containment of *C. albicans* at the infection site. Non-invasive longitudinal studies further show that early containment is a strong prognostic indicator for protection against lethal disease. Although rabbit anti-*Candida* antibodies are effective in promoting phagocytosis and protecting against disease, the monoclonal mouse IgM anti-*Candida* antibody that demonstrated efficacy in a murine systemic infection model, neither protects against disseminated disease nor promotes phagocytosis. Thus, this zebrafish infection model appears to provide a useful

in vivo model for the ability of a biological therapeutic to promote phagocytosis *in vivo* and may be useful for assaying effectiveness in protection against candidiasis. Furthermore, the tight correlation between early phagocytosis and overall survival highlights how early containment could play an important role in innate immunity to *Candida*.

2. Materials and Methods

2.1. Zebrafish care and maintenance

Zebrafish were housed in recirculating systems (Aquatic Habitats, Apopka, FL) at the University of Maine Zebrafish Facility. All zebrafish care and husbandry were performed as previously described (Brothers et al., 2013). Zebrafish care protocols and experiments were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health under the University of Maine-approved Institutional Animal Care and Use Committee (IACUC) protocols A2012-11-03 and A2015-11-03. Larvae were grown at a density of 150 fish per dish in 150 mm Petri dishes containing 150 ml of E3 media (5 mM sodium chloride, 0.174 mM potassium chloride, 0.33 mM calcium chloride, 0.332 mM magnesium sulfate, 2 mM HEPES in nanopure water, pH=7). The transgenic zebrafish strain used was Tg(*mpx:EGFP*)^{j114}, which expresses enhanced green fluorescent protein (EGFP) in neutrophils (Renshaw et al., 2006). E3 media was supplemented with 0.3 mg/L methylene blue for the first 6 hours to prevent microbial growth. Larvae were transferred to E3 containing 0.02 mg/ml of 1-phenyl-2-thiourea (PTU) (Sigma-Aldrich, St. Louis, MO) to prevent pigmentation and incubated at 33°C.

2.2. Bead opsonization and preparation

Polystyrene beads at 1% w/v (SpheroTech, Lake Forest, IL, 2.5–4.5 µm diameter fluorescent beads, FP-3056–2) were opsonized with rabbit IgG, mouse IgG, human IgG, and human Fab (Jackson ImmunoResearch, West Grove, PA) at antibody concentrations of 250 µg/ml, by incubation in the dark for one hour at 37°C, then washed three times in phosphate-buffered saline (PBS) and resuspended in PBS to a final concentration of 1.5×10^7 beads/ml, using a hemacytometer to achieve the desired concentration.

2.3. Fungal strains, growth conditions, and antibody co-injection

Engineered red fluorescent *C. albicans* strain CAF2.1-dTom-NATr (CAF2-dTomato) (Gratacap et al., 2013) was grown on yeast-peptone-dextrose (YPD) agar (Difco, Livonia, MI; 20 g/L peptone, 10 g/L yeast extract, 20 g/L glucose, 2% agar). For infections, liquid cultures of *C. albicans* were grown overnight in YPD at 30°C on a roller drum (New Brunswick Scientific, Edison, NJ). The overnight-cultured *C. albicans* were washed twice with PBS and resuspended in 5% w/v polyvinylpyrrolidone (PVP) (Sigma-Aldrich, St. Louis, MO) to a final concentration of 1×10^7 cfu/ml. PVP ensures a consistent small-volume injection dose (Detrich et al., 2010). Separate aliquots of the resuspension were mixed with either rabbit anti-*Candida* IgG pAb (USBiological), mouse anti-*Candida* IgM mAb (Regeneron Pharmaceuticals, Inc., Tarrytown, NY) or mock opsonized with rabbit anti-GFP IgG pAb (USBiological, Salem, MA) at a final antibody concentration of 10 µg/ml. For co-injection experiments *C. albicans* were mixed with antibodies twenty minutes prior to

injection. Monoclonal mouse IgM anti-*Candida* antibody recognizing β -mannan (B6.1) was purified by Regeneron Pharmaceuticals, Inc. (Tarrytown, NY) using standard methods from hybridoma licenced through Ligocyte Pharmaceuticals, Inc.

2.4. Hindbrain injections and drug treatment

Zebrafish at the prim25 stage (at approximately 36 hours post fertilization; staged according to the method of Kimmel, et al. (Kimmel et al., 1995)), were manually dechorionated, and anesthetized in Tris-buffered tricaine methane sulfonate (Tricaine; 200 mg/ml; Western Chemicals, Inc., Frensdale, WA) prior to injection. For injections, approximately 5 nL of opsonized polystyrene bead suspension at 1.5×10^7 beads/ml in PVP, or 2 nL of *C. albicans* suspension at 1.0×10^7 cfu/ml was microinjected through the otic vesicle into the hindbrain ventricle to achieve a dose of approximately 15 beads or 15 fungal cells at the site of injection. Within one hour post-injection, larvae were screened using a Zeiss Axiobserver Z1 microscope equipped with Vivatome system (Carl Zeiss Microimaging, Thornwood, NJ) for selection of larvae containing 13–17 beads/fungal cells at the site of injection (SOI). Larvae were subsequently incubated at 33°C in E3 media containing PTU. In experiments to test the relationship between extracellular fungal number and mortality, larvae were preincubated for 1 hour pre-infection with either DMSO or diphenyleneiodonium (Enzo, Farmingdale NY) as described (Brothers et al., 2013).

2.5. Fluorescence microscopy

At four hours post injection (HPI) larvae were anesthetized in Tricaine and immobilized in 0.5% low-melting-point agarose (Lonza, Walkersville, MD) in E3 media containing Tricaine, and placed in a 96-well glass-bottom imaging dish (Greiner Bio-One, Monroe, NC; 655892). Confocal images were acquired using an Olympus IX-81 inverted microscope with an FV-1000 laser scanning confocal system (Olympus, Waltham, MA). The EGFP and dTomato fluorescent proteins were detected by laser/optical filters at 40x magnification (NA 0.9) for excitation/emission at 488/505–525 nm (SDM 560) and 543/560–620 nm (SDM 640), respectively. Larvae were imaged by confocal microscopy in 24-well coverslip-bottom plates (Mattek, Ashland, MA) in a small volume of egg water (deionized water with 60 mg/L Instant Ocean salts, Spectrum Brands, Mentor, OH) plus anaesthetic (200 μ g/ml MS-222). Filter sets were at 543/610 and 488/510 excitation/emission wavelengths for dTomato and EGFP, respectively, and a 40x/0.75 N.A. UPlanFL N objective were used to collect Z-stacks of 31–59 slices at an interslice distance of 2 μ m. Z-stack images of the hindbrain ventricle were scored for the following at the site of infection: 1) Number of intracellular *Candida*, 2) Number of extracellular *Candida*, 3) Number of neutrophils recruited, 4) Number of neutrophils that had phagocytosed *Candida*, and 5) Number of macrophage-like cells that had phagocytosed *Candida*. Images were collected and processed using Fluoview (Olympus, Waltham, MA) and Photoshop (Adobe Systems, Inc., San Jose, CA), and subsequently analyzed with Fluoview to quantify bead/phagocyte interaction at the site of injection. After imaging, larvae were euthanized by Tricaine overdose.

2.6 Statistics

Parametric unpaired T-tests or non-parametric Mann-Whitney tests were conducted on data (Graphpad Prism 6, Graphpad Software, Inc., La Jolla, CA) to analyze the statistical

significance of differences between the opsonized and mock-opsonized treatment groups, depending on whether the data were normally distributed. For multiple-comparisons, one-way ANOVA was used with post-test analysis (Graphpad Prism). For imaging experiments in which the number of fish examined per cohort in an individual experiment was small due to technical limitations, data was analyzed by Fisher's Exact Test with two or three groups and appropriate post-test analysis using Graphpad Prism.

3. Results

3.1. Human IgG promotes macrophage phagocytosis of microspheres in the zebrafish hindbrain.

The zebrafish larva offers a transparent vertebrate model with significant similarities to the human immune system, and holds promise as a tool for pre-clinical evaluation of therapeutics (Sullivan and Kim, 2008; Tobin et al., 2012). Because there is a significant clinical need for new anti-fungal therapies, we sought to use this model to characterize the mechanism of action for potentially therapeutic antibodies. We first tested whether mammalian antibodies can stimulate phagocytosis in zebrafish. We coated polystyrene microspheres with IgG antibodies of human, rabbit, or mouse origin, using human Fab as a control, and injected them into the hindbrain ventricle as previously described (Fig. 1A; Brothers et al., 2011). We used *mpx:EGFP* transgenic zebrafish that have green neutrophils so we could characterize the phagocyte types involved in response to microspheres (Brothers et al., 2011; Renshaw et al., 2006). Previous work has showed that EGFP-negative phagocytes are macrophages, so we will refer to EGFP-negative cells that have engulfed fungi as macrophages (Ellett et al., 2011; Gray et al., 2011). We ensured a consistent inoculum across groups by screening within the first 30 minutes post-injection and imaged representative fish four hours post-injection (Fig. 1B). Quantification of immune cell interactions with coated microspheres revealed that only coating with human IgG resulted in significantly enhanced phagocytosis of microspheres in comparison to the Fab-coated control (Fig. 1C). Furthermore, the percentage of antibody-coated microspheres both in contact with and contained within a phagocyte was significantly higher in the human IgG-opsonized group as compared to the Fab control group (Fig. 1D). Additionally, we categorized the microsphere-phagocyte interaction by immune cell type, and observed that opsonized microspheres are primarily phagocytosed by non-neutrophil mononuclear cells, likely macrophages (Fig. 1E). Representative images demonstrate that inocula were similar but there was greater phagocytosis of beads coated with human IgG (Fig. 1F–I). These experiments demonstrate that opsonization of polystyrene microspheres with human IgG enhances zebrafish immune cell phagocytosis, specifically by macrophages. These initial results suggested that opsonization of microbes with polyclonal antibodies might also enhance immune cell activity and phagocytosis in this *in vivo* infection model.

3.2. Co-injection of polyclonal anti-*Candida* antibodies enhances early phagocytosis of *C. albicans* in zebrafish.

To test the ability of microbe-specific antibodies to enhance early phagocytosis in the zebrafish, we co-injected rabbit polyclonal anti-*Candida* or mock anti-GFP antibodies with CAF2-dTomato *C. albicans* in the hindbrain ventricle of *mpx:EGFP* transgenic zebrafish and

measured phagocytosis. We first determined a concentration of polyclonal antibody that provided for specific recognition, comparing binding to yeast by utilizing a fluorescent secondary antibody and quantifying fluorescence by flow cytometry. We found that 10 µg/ml was the highest concentration of antibody that provided for maximal opsonization while eliminating non-specific binding by the mock anti-GFP antibodies (Suppl Fig 1). We then co-injected *anti-Candida* or anti-GFP antibodies at this concentration with CAF2-dTomato fungal cells and measured phagocytosis. Within the first 30 minutes post-injection, fish were imaged, fungal cells were counted, and only fish injected with the proper inoculum (15 $\pm$ 5 cells) were used. Fish were then imaged at 4 hpi at high resolution by confocal microscopy and the following parameters were scored in the hindbrain ventricle: intracellular fungi, extracellular fungi, neutrophil recruitment, neutrophil-phagocytosed fungi, or fungi phagocytosed by macrophage-like cells.

We found that early interaction between *C. albicans* and phagocytes is altered by co-injection of *Candida*-specific antibodies. Preliminary counts established that fungal inocula were comparable between groups (Fig. 2A). However, there was a significantly higher percentage of total fungal cells that were intracellular when they were co-administered with the *Candida*-specific antibodies. Parsing the location of intracellular *C. albicans* relative to phagocyte type revealed that, with antibody co-administration, macrophages but not neutrophils phagocytosed both a higher proportion of fungi (Fig. 2B) and a higher average number of fungi per infected fish (Fig. 2C). The total number of extracellular fungal cells was significantly lower with co-injection of *Candida*-specific antibody (mean $\pm$ SEM; 8.1 $\pm$ 2.9 vs. 16.3 $\pm$ 2.7; anti-*Candida* vs. anti-GFP). Thus, the higher percentage of intracellular fungi is attributable primarily to a significantly lower number of extracellular fungal cells rather than a higher number of intracellular fungal cells. This suggests that intracellular fungi were killed or prevented from replicating over the first four hours of infection.

On the host side, there was overall low recruitment of neutrophils in all animals and consequently low phagocytosis by neutrophils (mean $\pm$ SEM; 4.0 $\pm$ 1.26 vs. 2.4 $\pm$ 0.74 neutrophils per fish; anti-*Candida* vs. anti-GFP). The relatively minor importance of neutrophils in phagocytosis may also be due to the nature of the infection site, as neutrophils tend to be relatively limited in bacterial phagocytosis in fluid-filled spaces (Colucci-Guyon et al., 2011). There was no difference in the number of macrophage-like cells at the infection site that had phagocytosed *C. albicans*, despite the fact that more fungal cells resided within macrophage-like cells with addition of the anti-*Candida* antibody (mean $\pm$ SEM; 3.75 $\pm$ 0.46 vs. 3.42 $\pm$ 0.76 macrophage-like cells with internalized fungi per fish; anti-*Candida* vs. anti-GFP). Taken together, these results suggest that the polyclonal anti-*C. albicans* antibody enhances early phagocytic efficiency at the injection site.

3.3. Early phagocytosis efficiency is highly predictive for survival from hindbrain ventricle infection of *C. albicans*.

Increased early *C. albicans* containment at the injection site has previously been shown to be associated with survival, and conversely poor early engulfment is related to enhanced mortality (Brothers et al., 2013). However, the predictive value of non-invasive imaging has

not been examined in detail. To more closely examine the relationship between failed early phagocytosis and mortality, relative to other measures of immune response, we measured a number of fungal and host variables over the first four hours of infection through longitudinal time-course confocal imaging. Infections and scoring were performed essentially as described above, although no antibody co-injection was used and fish were instead treated with either vehicle or the NADPH oxidase inhibitor diphenyleneiodonium (DPI), as previously described (Brothers et al., 2013). This inhibitor was used to reduce early phagocyte recruitment and enhance susceptibility to infection, increasing the range of host responses with varying phagocytosis efficiency thereby providing a better test set for exploring the relationship between fungal containment and survival. To analyze this data, we first plotted the relationship between the number of extracellular fungi at 4 hpi and the ultimate fate of the fish in the first 24 hpi. We found a strong and statistically significant difference in the number of extracellular fungal cells in fish that survived versus died (Fig. 3A). To identify a choice-point between survival and death, we then plotted the data cumulatively (Fig. 3B). For surviving fish, the plot indicates the percentage of surviving fish with X or more extracellular fungi. Thus, 0% of surviving fish have greater than 20 extracellular fungi. For dead fish, the plot indicates the percentage of dead fish with X or fewer extracellular fungi. Thus, 0% of dead fish have fewer than 5 extracellular fungi. This analysis demonstrated that the overwhelming majority of fish that died (91%) had more than 11 extracellular fungi, while all had at least 7 extracellular fungi at 4 hpi (Fig. 3B). Conversely, the overwhelming majority of fish that survived (85%) had 12 or fewer extracellular fungi at 4 hpi. Previous work had used an arbitrary level of 5 or greater extracellular fungi to stratify those fish with effective versus ineffective early phagocytosis (Brothers et al., 2013). This more detailed analysis suggests that the break-point between survival and mortality is closer to 10–12 extracellular fungi at 4 hpi.

To examine the contribution of other early immune and fungal factors to infection outcome, we hierarchically clustered all of the data collected. However, incorporating this additional data resulted in a less precise divide for stratification of fish at 4 hpi (Fig. 3C). Instead, clustering based only on the number of intracellular and extracellular fungi provided a crisp divide between fish destined to survive and die (Fig. 3D). Clustering these data using the same parameters but limiting the data to that of individual time points (1, 2, 3, or 4 hpi) revealed that the data at 4 hpi is equivalent to using all of the data between 1 and 4 hpi for clustering. In this case, we achieved a sensitivity (true positive rate) of 87% combined with a specificity (true negative rate) of 90%. Taken together, these data demonstrate the power of using phagocytosis efficiency as an early non-invasive test that reliably predicts survival to 24 hpi. The predictive value of early phagocytosis efficiency for survival in this fungal infection model suggests that enhancing early phagocytosis could improve outcomes.

3.4. Co-injection with polyclonal antibodies enhances host survival in a dose-dependent manner

Because we found that co-injection with a polyclonal anti-*Candida* antibody increases early phagocytosis (Fig. 2), we hypothesized that this antibody preparation would also protect fish from overall mortality. We measured survival in fish injected and imaged as described above. We found that co-injection with this antibody at the concentration that promotes

phagocytosis and early containment (as shown in Fig. 2A) led to significant protection from mortality (Fig. 4A). The survival curve of larvae infected with opsonized *Candida* was significantly different to that of mock-opsonized *Candida* infected larvae ($p = 0.0017$; Fig. 4B). Larvae that were infected with opsonized *Candida* had a higher end point survival of 70.3% compared to that of larvae infected with mock-opsonized *Candida* of 34.3% (Fig. 4B). Further testing with lower doses of antibody revealed a dose-dependent protection from long-term mortality for the anti-*Candida* as compared to the non-specific antibody (Fig. 4B–C).

3.5. A mannan-directed monoclonal antibody does not improve infection outcome in disseminated candidiasis in zebrafish

We attempted to extend these studies to determine if a previously characterized anti-*Candida* monoclonal antibody would be effective in enhancing early phagocytosis and/or overall survival in the context of this zebrafish larval infection model. We used an IgM anti-*Candida* antibody previously described to react with mannan epitopes on the fungal cell surface and protect mice from disseminated candidiasis (Han and Cutler, 1995). *C. albicans* was co-injected with control anti-GFP or monoclonal anti-*Candida* IgM, and inocula were validated to ensure equivalent doses (Suppl. Fig. S2A). Larvae were scored for survival for four days post-infection, but there was no protection provided by this antibody in the larval fish model (Suppl. Fig. S2B). Taken together, these experiments suggest that this single monoclonal antibody may not be effective in enhancing survival in this zebrafish model of *C. albicans* infection, but do not exclude the possibility that other monoclonal antibodies may be effective.

4. Discussion

Humoral immunity is a crucial component in adaptive immunity and successful vaccination. Although antibodies play a number of well-recognized protective roles, technical obstacles limit our ability to characterize how they actually promote protection in the context of a whole animal infection model. The zebrafish larval model offers the advantages of a small and transparent vertebrate host with an immune system that is similar to man (Carradice and Lieschke, 2008; Meeker and Trede, 2008; Tobin et al., 2012; Torraca et al., 2014). Here, we examined the possibility for using these animals to characterize the ability of antibodies to enhance phagocytosis of *Candida albicans* and thereby promote resistance to disease. We found that polyclonal anti-*Candida* antibodies can promote containment and survival in this model, whereas the single monoclonal tested was not effective in promoting survival.

We first demonstrated that opsonization of polystyrene microspheres with human IgG enhances zebrafish immune cell activity and phagocytosis, specifically by macrophages. This may result from phagocytosis through scavenger receptors, complement receptors, and/or antibody receptors. Scavenger receptor-mediated phagocytosis has been demonstrated for protein-coated beads in rainbow trout (Froystad et al., 1998). Complement- and antibody-mediated phagocytosis by macrophages has also been demonstrated in teleosts (Leiro et al., 1996; Leiro et al., 2008). Intriguingly, opsonization with human serum also enhances phagocytosis of beads by salmon macrophages in a process dependent on

complement deposition (Johnson and Smith, 1984). Although an Fc gamma receptor homolog has not yet been found in zebrafish, expansions of related receptors may allow for a more broad-specificity phagocytosis of antibody-opsonized targets (Akula et al., 2014). In the context of an artificial polystyrene substrate, rabbit and mouse IgGs may not interact as efficiently as human IgGs with zebrafish receptors on phagocytes or elicit sufficient complement deposition to increase uptake. Given the evolutionary conservation of polysaccharide pattern recognition receptors, it is possible that coating microspheres with fungal cell wall polysaccharides as well as IgG will enhance their recognition and phagocytosis through a combination of opsonic and non-opsonic receptors that more closely mimics a fungal pathogen (Netea et al., 2015). It will also likely be informative to test if morpholino knock-down of complement pathway components affects phagocytosis of different IgG-coated microspheres.

We found that mammalian antibodies enhance immune cell activity and phagocytosis in this *in vivo* infection model, similar to the IgG-coated microspheres. Closer analysis made possible using intravital imaging in the zebrafish revealed that, in the presence of anti-*Candida* polyclonal antibodies, macrophages but not neutrophils phagocytose a higher proportion of fungi. Taken together, these results demonstrate that the polyclonal anti-*C. albicans* antibody tested in these studies can enhance early phagocytic efficiency at the injection site. It is notable that antibody-enhanced phagocytosis of fungi has not been demonstrated before *in vivo* to our knowledge, and this highlights the utility of the zebrafish model to measure specific immune responses in the complex *in vivo* environment. Efficient recognition and ingestion of particles requires crosstalk among phagocytic receptors (Freeman and Grinstein, 2014). Thus, enhanced phagocytosis of *Candida* in the presence of anti-*Candida* antibodies may result from recognition by a combination of scavenger receptors, complement receptors, and/or antibody receptors. Similar to the mammalian situation, fish complement binds well to yeast (Ichiki et al., 2012). It is possible that opsonization with polyclonal antibodies enhances this complement deposition and phagocytosis.

Previous work had used an arbitrary level of extracellular fungi to stratify those fish with effective versus ineffective early phagocytosis (Brothers et al., 2013). The more detailed studies presented here suggest that the break-point between survival and mortality is closer to 10–12 extracellular fungi at 4 hpi. Taken together, these data demonstrate the power of using phagocytosis efficiency as an early non-invasive test that can be used to predict survival at 24 hpi. This may be especially important in the hindbrain ventricle model of infection, in which phagocytosis blocks hyphal germination of *C. albicans* and thereby prevents lethal invasive growth (Brothers et al., 2013). In addition, phagocytosis provides a critical break on infection progression in other zebrafish infection models in which it has been monitored non-invasively *in vivo* (Bojarczuk et al., 2016; Clay et al., 2007). The importance of early phagocyte responses is thus clear when it is possible to measure these responses non-invasively and longitudinally follow the consequences of differential efficiency in early pathogen containment.

As a consequence of enhanced early containment and blockade of hyphal growth, the survival of larvae infected with opsonized *Candida* is significantly enhanced compared to

that of mock-opsonized *Candida* infected larvae. Antibody-enhanced containment of fungi may be generally relevant to vaccine-induced protection against pathogens in other fish, as others have shown that vaccine-induced antibodies mediate complement deposition that enhances phagocytosis of ciliates and microsporidian fungi in turbot (Leiro et al., 1996; Leiro et al., 2008). In addition to enhanced phagocytosis, complement deposition can also activate fish immune cells (Rotllant et al., 2004) and mammalian adaptive immune responses (Li et al., 2008; Peng et al., 2008). Similar pathways may play an analogous role in antibody-mediated protection in humans.

The single anti- β -mannan specific mouse IgM monoclonal antibody tested is ineffective in enhancing survival in this zebrafish model of *C. albicans* infection. Monoclonal anti-*Candida* antibodies are generally not very effective in the mouse candidemia model, and some are even anti-protective (Bromuro et al., 2002; Casadevall et al., 1998; Polonelli and Cassone, 1999). It is not known why some are more protective than others. The IgM we chose has been well characterized to be protective in the mouse model by targeting a mannan epitope (Han and Cutler, 1995). The lack of efficacy in this zebrafish model may be because this IgM provokes a species-or mammalian-specific response through its constant region. Mouse IgMs may not as efficiently interact with the zebrafish complement system, a known mechanism by which the anti- β -mannan IgM provides protection in mice (Han et al., 2001). Furthermore, it is not known if this IgM antibody stimulates phagocytosis *in vivo*, which may be a required mechanism-of-action for enhanced protection in this infection model where the fungi are delivered through injection into the hindbrain ventricle.

Here we have described cross-species enhancement of fungal phagocytosis by co-injection of polyclonal rabbit anti-*Candida* antibodies in the zebrafish larva. The enhanced fungal containment improves survival of infection, likely by the previously described mechanism of blocking invasive hyphal growth (Brothers et al., 2013). We also found that coating with human IgG can enhance phagocytosis of polystyrene beads, in line with previously described effects of human serum for phagocytosis by fish macrophages (Johnson and Smith, 1984). Our results with the polyclonal anti-*Candida* antibody demonstrate the potential power of the zebrafish model for assaying the cellular effects of potential biological therapeutics *in vivo*. As zebrafish are not a natural host for *C. albicans*, this model holds more promise in elucidation of well-conserved immune mechanisms and has limited utility for discovery of human-or mammalian-specific immune responses. While more data will be necessary to test its broader utility as a screening model, this first set of results suggest the zebrafish larva is worth pursuing as a new pre-clinical model for testing the efficacy of opsonizing reagents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- > Opsonization of beads with human IgG stimulates zebrafish phagocytosis.
- > Polyclonal anti-*Candida* antibody opsonization stimulates fungal containment *in vivo*.
- > Efficient early fungal engulfment is prognostic for infection survival.
- > Polyclonal anti-*Candida* antibody treatment enhances overall survival of candidemia.

Fig. 1. Opsonizing polystyrene beads with human IgG increases bead-phagocyte interaction at SOI.

Prim25 stage *mpx:EGFP* transgenic zebrafish larvae (staged as described by Kimmel et al., 1995) with EGFP-expressing neutrophils and non-fluorescent macrophages (Renshaw et al., 2006) were injected into the hindbrain with polystyrene beads opsonized with human IgG, rabbit IgG, mouse IgG, and human IgG Fab fragment. (A) Microinjection of a 5 nL volume into the hindbrain ventricle through the otic vesicle was performed as described (Brothers and Wheeler, 2012), and inoculum was verified by screening larvae immediately post-injection. (B) Inoculum size within one hour post-injection is consistent among groups. (C-I)

Images of the hindbrain ventricle were acquired via confocal microscopy at 4 hpi. Bead and phagocyte numbers were quantified based on 2 μ m Z-stacks using a 40x/0.75 N.A. UPlanFL N objective. (C, D) A two-tailed Fisher's exact test indicates a significant difference in the percentage of beads observed inside a phagocyte (C, $p < .0001$) and in contact with a phagocyte and intracellular (D, $p < 0.05$) between human IgG and human Fab opsonized groups. (E) Opsonized polystyrene beads are phagocytosed primarily by GFP-negative macrophages. Representative images reveal more bead-phagocyte interaction in fish injected with human IgG opsonized beads (F) as compared to beads opsonized with rabbit IgG (G), mouse IgG (H) and human Fab fragment (I). Images were acquired at 1024×600 pixels, and the number of slices for panels F, G, H, and I are 58, 53, 45, and 59, respectively. Movies including all of the slices are included as Supplemental Data Files M1E, M1F, M1G and M1H. Data are pooled from seven independent experiments. Although technical constraints limited cohort sizes in each experiment, reproducible differences were found from experiment to experiment. Number of fish scored; Human IgG n=18; Rabbit IgG n=17; Mouse IgG n=14; Human Fab fragment n=26. Scalebar = 50 μ m.

Fig. 2. Opsonization with anti-*Candida* polyclonal antibody enhances phagocytosis *in vivo*. (A-E) Zebrafish of the *mpx:EGFP* line at the prim25 stage were infected in the hindbrain with 10–15 cells of CAF2-dTomato *C. albicans*, with screening within 30 minutes for confirming dose. *C. albicans* was co-injected with either anti-*Candida* or anti-GFP PAb at a final concentration of 10 μ g/ml. Images were acquired via confocal microscopy at 4 hpi. Fungal and phagocyte numbers were quantified based on 2 μ m Z-stacks using a 40x/0.75 N.A. UPlanFL N objective. (A) Initial inoculum of *C. albicans* cells in hindbrain ventricle (within 30 minutes of inoculation). (B) Average percent of total *C. albicans* cells in zebrafish

hindbrain ventricle that are either inside neutrophils or macrophages or extracellular at 4 hpi. Significant difference between treatment groups in the percentages of total *Candida* phagocytosed by macrophages ($p < 0.05$; t-test), total phagocytosed by neutrophils ($p < 0.05$; Mann-Whitney), and total percent extracellular ($p < 0.05$; Mann-Whitney). (C) Average number of *C. albicans* inside neutrophils or macrophages or extracellular at 4 hpi. The only significant difference between treatment groups was in the number of extracellular *Candida* ($p < .01$; Mann-Whitney). (D-E) Representative images of the hindbrain ventricle in zebrafish larvae infected with *C. albicans* opsonized with (D) anti-GFP or (E) anti-*Candida* PAb at 36 hpf (scalebar = 50 μm). Images were acquired at 1024×600 pixels, with 35 slices for panel D and 52 slices for panel E. Movies including all of the slices are included as Supplemental Data Files M2D and M2E. Data are combined from three independent experiments, $n=18$ larvae per treatment group, whiskers = minimum and maximums. Although technical constraints limited cohort sizes in each experiment, reproducible differences were found from experiment to experiment.

Fig. 3. Inefficient early phagocytosis is a strong predictor of mortality in the zebrafish hindbrain infection model.

(A-D) Zebrafish of the *mpx:EGFP* line at the prim25 stage were infected in the hindbrain with 10–15 CAF2-dTomato *C. albicans*, with screening within 30 minutes for confirming dose. Fish were longitudinally imaged by confocal microscopy from 1–4 hpi and images were scored for fungal and host phenotype: fungal cells were scored as either intracellular/extracellular and yeast/filament; phagocytes in the hindbrain with ingested fungal cells and/or EGFP fluorescence were also counted. Fish were then removed from low-melt agarose with tricaine and kept until 24 hpi, when they were scored for survival. (A) Surviving and dead fish were scored for the number of extracellular fungi they had had at 4 hpi. Box-plots are shown; T-test was used for statistics, *** $p < 0.001$. (B) Cumulative distributions of the number of extracellular fungi at 4 hpi for surviving (green) and dead (gray) fish are plotted. For surviving fish, the plot indicates the percentage of surviving fish with X or more extracellular fungi. Thus, 0% of surviving fish have greater than 20 extracellular fungi. For dead fish, the plot indicates the percentage of dead fish with X or fewer extracellular fungi. Thus, 0% of dead fish have fewer than 5 extracellular fungi. (C) Quantitative phenotypes at 1–4 hpi for individual fish were hierarchically clustered. Green bar above indicates cluster with greater survival and black bar above indicates cluster with greater mortality. Black asterisks indicate dead fish within survival cluster and green asterisks indicate survivors within death cluster. Red indicates high number and green

indicates low number. (D) Only intracellular and extracellular fungal cell number was used in hierarchical clustering. Red indicates high number and green low number. Gray indicates lack of data. Black and green bars and asterisks are as in panel C. The discriminatory power of clustering for predicting mortality using data for each time point or the combined dataset (1–4 hpi) is shown in the table below. Sensitivity is calculated as $(\# \text{ true positives})/(\# \text{ true positives} + \# \text{ false negatives})$. Specificity is calculated as $(\# \text{ true negatives})/(\# \text{ true negatives} + \# \text{ false positives})$.

Fig. 4. Dose-responsive antibody-mediated protection from disseminated candidiasis. (A-C) Zebrafish of the *mpx:EGFP* line at the prim25 stage were infected in the hindbrain with 10–15 cells of CAF2-dTomato *C. albicans*, with screening within 30 minutes for confirming dose. Images were acquired via confocal microscopy at 4 hpi. Fungal and phagocyte numbers were quantified based on 2 μ m Z-stacks using a 40x/0.75 N.A. UPlanFL N objective. (A) Average Survival Kaplan-Meier curves of larvae infected with *C. albicans*, co-injected with either anti-*Candida* or anti-GFP pAb at 10 μ g/ml. Initial inoculum was consistent and is shown in Fig. 2A. After screening, fish were observed until 96 hpi for

survival. Results pooled from two independent experiments. Difference between anti-*Candida* and anti-GFP is significant ($p < 0.01$ by Log-Rank; $n=23$ anti-*Candida*, 21 anti-GFP, 7 uninfected) (B-C) Dose-response test (2 trials combined; number of zebrafish per treatment group = 32, 11, 18, 22, 17, 29, 45 and 53 respectively for uninfected, 0 $\mu\text{g/ml}$, 1 $\mu\text{g/ml}$ anti-GFP, 1 $\mu\text{g/ml}$ anti-*Candida*, 5 $\mu\text{g/ml}$ anti-GFP, and 5 $\mu\text{g/ml}$ anti-*Candida*, 10 $\mu\text{g/ml}$ anti-GFP, and 10 $\mu\text{g/ml}$ anti-*Candida*; note that 10 $\mu\text{g/ml}$ data from two trials in 4A was comparable and was therefore pooled with 2 experiments performed with all doses). *C. albicans* was co-injected with either anti-*Candida* or anti-GFP pAb at 1–10 $\mu\text{g/ml}$. (B) Initial inoculum of *C. albicans* Caf2-dTomato cells in zebrafish larvae hindbrain ventricle. No significant difference between inoculum in each treatment group; $p > 0.05$ by ANOVA. (C) Survival Kaplan-Meier curves of zebrafish larvae infected with *C. albicans*, anti-*Candida* IgG or anti-GFP IgG at 0, 1, 5 and 10 $\mu\text{g/ml}$ over 96 h.p.i. Survival curves of the combined data from duplicate trials (for 0, 1, and 5 $\mu\text{g/ml}$) or four trials (10 $\mu\text{g/ml}$). Significant differences between anti-*Candida* and mock at 10 $\mu\text{g/ml}$ ($p < 0.01$) and between anti-*Candida* and un-opsonized at 1 $\mu\text{g/ml}$ ($p < 0.05$, Log-Rank of Kaplan-Meier).