Factors associated with Candida albicans dissemination via the intestinal mucosa

Emily Rochac Argueta

University at Albany, State University of New York, ecoles1387@gmail.com

The University at Albany community has made this article openly available. Please share how this access benefits you.

Follow this and additional works at: https://scholarsarchive.library.albany.edu/legacy-etd

Part of the Immunology of Infectious Disease Commons

Recommended Citation
https://scholarsarchive.library.albany.edu/legacy-etd/2153

This Master's Thesis is brought to you for free and open access by the The Graduate School at Scholars Archive. It has been accepted for inclusion in Legacy Theses & Dissertations (2009 - 2024) by an authorized administrator of Scholars Archive. Please see Terms of Use. For more information, please contact scholarsarchive@albany.edu.
Factors associated with *Candida albicans* dissemination via the intestinal mucosa

By

Emily Rochac Argueta

A Thesis

Submitted to the University at Albany, State University of New York

In Partial Fulfillment of

The Requirements for the Degree of

Master of Science

School of Public Health

Department of Biomedical Sciences

2018
ABSTRACT

A murine model that established GI colonization of Candida albicans and eventual dissemination was identified to be lacking by Koh and others. Koh’s model specifically employed a tailored combination of neutropenia, intestinal damage, and microbiome dysbiosis that would allow C. albicans to gain an opportunistic advantage. His model provided new opportunities to study the details of C. albicans pathogenesis with a focus on the innate immune mechanisms responsible for controlling C. albicans within the intestinal mucosa. The long-term goal of this research project was to specifically study the factors associated with Candida albicans dissemination via the intestinal mucosa. The model used treatment with N-acetylcysteine (NAc), Dextran Sodium Sulfate (DSS), and an antibiotic cocktail that would allow for C. albicans to have an advantage upon uptake.

Based upon the pathology of dissemination in humans, we sampled several tissues in mice such as stomach, kidneys, spleen, liver, lung, brain, heart, PP, feces, and blood. We learned from this research that DSS and antibiotic pretreatment were our most useful tools for establishing C. albicans colonization and invasive infection. Additionally, we were unable to replicate Koh’s gross standard of disseminated disease, i.e., weight loss of 20% or more from baseline, in any of our various approach models.

Future laboratory investigation will pursue constructing a multi-faceted approach that incorporates a variety of pretreatment exposures using lower concentrations of or without DSS; expanded sampling of blood and tissues outlined above; sequential sampling of PPs at times during and after intestinal exposure to C. albicans; and sampling of additional MALT tissue including mesenteric lymph nodes to determine the route and the time point at which the infection changes from candidemia to deep-seated candidiasis.
First, and foremost, I would like to thank my family. The most heartfelt thank you to my daughter Chloe, who joined me on this journey during the second week of the program; she has welcomed me home from the laboratory with open arms and a wide smile. One embrace from her, and everything else melts away. To my amazing husband and partner, who has always put me first and supported my journey, no matter what form that path has taken. To my father, who has been the ear and shoulder and to my mother who has always instilled upon me to never give up, I am grateful for such a supporting home. I want to especially thank my grandmother’s weekend phone calls and my grandfather’s quiet and stoic manner, which taught me to always face life’s obstacles with grace and, most importantly, all the facts.
ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Magdia De Jesus, whose constant scientific curiosity and unwavering energy at the bench has inspired me throughout my graduate school journey. I thank you for supporting my new role as a mother, while maintaining the highest of standards of me as a graduate student in your laboratory. Without your support and guidance, I would not have been able to achieve my goals in science and pursue my graduate degree. Furthermore, I wanted to recognize the significant role played by our laboratory technician, Heather Gallagher for her constant assistance, critical eye, and research skill. Another member of the De Jesus Laboratory is graduate student Steven Torres who has become an integral member of the lab and an enthusiastic and willing participant in my project.

Next, I would like to thank Dr. Nicholas J. Mantis, Mr. Richard Cole, Dr. Qing-Yu Zhang, and Dr. Sudha Chaturvedi for imparting their expertise as members of my thesis committee. I would especially like to thank Dr. Qing-Yu Zhang for her collaboration with our project and teaching me about processing and interpreting epithelial cells of the intestines. Also, I would like to thank Dr. Sudha Chaturvedi, Lynn Leach and the rest of her Mycology laboratory for running a significant number of assays for our project. Additionally, I wanted to acknowledge the fundamental role filled by Veterinary Pathologist Dr. Fernando Torres-Velez, who has assisted us in gross tissue analysis during necropsy and histologic evaluation which has allowed for a much more thorough evaluation of our research.

Lastly, I would like to thank Dr. Janice Pata, Dr. Valerie Bolivar, Anthony Torres and the Department of Biomedical Sciences of SUNY Albany School of Public Health, for providing constant support and resources.
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Page:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
</tr>
<tr>
<td>List of Abbreviations</td>
</tr>
<tr>
<td>List of Tables</td>
</tr>
<tr>
<td>List of Figures</td>
</tr>
<tr>
<td>Chapter 1 Introduction</td>
</tr>
<tr>
<td>Chapter 2 Materials and Methods</td>
</tr>
<tr>
<td>Chapter 3 Results</td>
</tr>
<tr>
<td>Chapter 4 Discussion</td>
</tr>
<tr>
<td>References Cited</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

Ab- Antibodies
Abx- Antibiotics
ASCA- Anti-Saccharomyces cerevisiae antibodies
CFU- Colony forming unit
CLR- C-type Lectin Receptor
DAPI- 4’,6-diamidino-2-phenyldole
DC- Dendritic Cell
DSS- Dextran Sodium Sulfate
DT- Diphtheria toxin
FAE- Follicle-associated epithelium
GALT- Gut-associated Lymphoid Tissue
HeLa cells- human epithelial cells
Ig- Immunoglobulin
IL- Interleukin
IP- Intraperitoneal injection
FAE- Follicle-associated epithelium
M cell- Microfold cell
mAb- Monoclonal antibody

MALDI-TOF- Matrix-assisted laser desorption/ionization time of flight

MLN- Mesenteric lymph node

NAc- N-acetylcysteine

PP- Peyer’s patch

PCN-G- Penicillin-G

PCR- Polymerase chain reaction

SED- Sub-epithelial dome
LIST OF TABLES

Table:                                                                                   Page:

1. Detailed outline of the six approaches that were used to optimize *C. albicans* dissemination in a murine model 20

2. Localization of *C. albicans* to tissue-specific sites 35

3. *Candida* species Multiplex Real-Time PCR Assay Primers and Probes 38
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The microanatomy of a Peyer’s patch allows for selective sampling immune sensing that is specific to the gut.</td>
<td>18</td>
</tr>
<tr>
<td>2. Organ and tissue sites associated with pathogenesis of invasive candidiasis in humans.</td>
<td>19</td>
</tr>
<tr>
<td>3A. Colony-forming unit in collected tissues.</td>
<td>36</td>
</tr>
<tr>
<td>3B. Colony-forming unit in collected tissues.</td>
<td>37</td>
</tr>
<tr>
<td>4. Approach 4: Histopathologic evaluation of invasion of <em>C. albicans</em> (stained with PAS) into the lumen of the stomach.</td>
<td>39</td>
</tr>
<tr>
<td>5. Initial and final weights for mice receiving heat-killed and live <em>C. albicans</em> are not a predictor for dissemination.</td>
<td>40</td>
</tr>
<tr>
<td>6. Proposed model of live and heat-killed <em>C. albicans</em> distribution in a human based upon a mouse-model</td>
<td>41</td>
</tr>
<tr>
<td>7. Future directions that improve upon the current murine model of candidiasis.</td>
<td>42</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

The human digestive system is essential for the processing of food and its conversion into usable energy for metabolism and growth.\(^1\) This process is aided by commensal bacteria in the gut collectively known as the microbiome.\(^1\) Low pH, various enzymes, mucous, and commensal bacteria that comprise the microbiome all contribute to the innate primary defenses against pathogens that humans ingest. Beneath the GI tract’s large mucosal surface area, lies the submucosal immune system which is a key second line of defense. Several enteric organisms such as *E. coli*, *Shigella*, *Poliovirus*, or *Candida albicans* have the capacity to invade the body by overcoming adaptive or innate mucosal immune system defenses (Figure 1).

For example, enterohemorrhagic *E. coli*’s (EHEC) pathogenic strain O157:H7 differs from the commensal strain in part due to the multitude of sugars catabolized including galactose, hexuronates, mannose, and ribose which provide the carbon sources for causing colonization defects.\(^2\) It is speculated that these sugars are available to pathogenic strains as they are not consumed by the commensal intestinal microbiota and the outcompeting for these resources allows for EHEC to outcompete and colonize the intestines.\(^2,3\) The primary method by which *E. coli* is introduced into the gut where it colonizes, is via the oral route, and the presence of iron in drinking water increases the number of *E. coli* bacteria and cultivability.\(^3\)

*Shigella dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei* are the four serogroups that are the causative agents of shigellosis, another common and sometimes fatal intestinal bacterial infection that is responsible for at least 80 million infections and 700,000 deaths worldwide due to the unrelenting diarrhea it causes.\(^4–6\) Our bodies naturally produce bile as an integral component to digestion, but bile is also relevant to our immunity as it is able to destroy a number of harmful bacteria.\(^4\) Unfortunately for the host, bile is incapable of protecting against *E. coli*, *Salmonella*,
*Vibrio*, or *Shigella*. *Shigella* is capable of not only evading the antibiotic effects of bile, but also utilizing hepatic-derived alkaline fluid to elicit changes in its gene expression allowing for increased resistance to bile and to also upregulate its virulence gene expression. As an effective *Shigella* vaccine is currently unavailable, antibiotics are the only way to combat this infection, which is also gaining antibiotic resistance. Antibiotic resistance is common in intestinal infections partly due to the pathogen’s interactions with bile salts prompting a more infectious organism and increased expression of virulence genes such as *ospE1* and *ospE2* that promote outer membrane localization so these proteins may function as adhesins and thereby adhere to and invade epithelial cells.

Poliovirus is an enterovirus and the causative agent for paralytic poliomyelitis. Over 90% of infections are asymptomatic or manifest as a nonspecific fever, but in less than 1% the virus produces tissue damage to the central nervous system (CNS) resulting in flaccid paralysis. Apoptosis results from the activity of the Poliovirus receptor CD155 and binding of various ligands such as tumor necrosis factor. The Poliovirus mutant CD155 gene also seems to delay PV-induced lysis during persistent infection.

From roughly 611,000 known fungal species, only roughly 1% are human pathogens. *C. albicans* is one of these pathogenic species. *C. albicans*, although a commensal, also has the ability to be pathogenic in humans. *C. albicans* is a pleo- or polymorphic, meaning it may exist as budding fungal organism, that resides within the normal microflora of the human gastrointestinal tract, oral mucosa, vagina, or skin; as a pseudomycelium of elongated cells; or in its hyphal form more characteristic of its invasive state. This type of hyphal growth is considered anisotropic, a non-uniform cellular volume expansion along a polarized axis, a strategy employed for colonization and nutrient acquisition to compensate for its lack of motility. While innocuous in healthy
individuals, this 10-12 micron commensal organism may become opportunistic in immunocompromised patients and lead to lethal infection. C. albicans is capable of growing either as an ovoid-shaped budding unicellular yeast, or as a more invasive multicellular elongated hyphal form, with environmental cues such as pH responsible for causing these changes. C. albicans has a very high pathogenic potential due to its ability to self-regulate and adapt at to the host microenvironment by downregulating or upregulating its actions to sustain a state of commensalism or incite host tissue damage while still evading immune surveillance.

Various characteristics of C. albicans contribute to its virulence potential such as adhesion-mediating molecules potentiating host invasion, the secretion of hydrolases, the transition from yeast to hyphal form, contact sensing and thigmotropism, biofilm formation, phenotype switching, and various fitness strategies. Hyphal growth is responsible for hyphal-specific transcriptional processes leading to virulence-factor upregulation, such as increased adherence, biofilm formation and cellular invasion. Cell-wall expansion occurs at the hyphal tip as stimulated by a constant delivery of exocytic vesicles bringing building blocks from the cytoplasm and localizing the scaffolding proteins to the tip such as GTPases of the Ras-and Rho-family of enzymes. There are two mechanisms of action allowing hyphal C. albicans to invade epithelial and endothelial cells: active penetration and induced endocytosis. Active penetration occurs sans the host’s cellular machinery, whilst induced endocytosis takes advantage of recruiting the host cytoskeletal components actin and cortactin and recent research supports that successful invasion into HeLa (human epithelial) cells is independent of hyphal length but instead is more related to the ability of C. albicans to adapt to the oxidative stressors induced by the host immune system. Adhesion to epithelial surfaces is paramount to the initiating steps of a disseminated infection, which requires access to the bloodstream and mucosal tissue. Adhesins are specific proteins that serve to regulate
adherence between *C. albicans* cells and other microorganisms; some examples are the agglutinin-like sequence (ALS) proteins that encode glycosylphosphatidylinositol (GPI)-linked cell surface glycoproteins.\(^9\)

*C. albicans* invades non-professional phagocytic host cells by one of two possible strategies: induced endocytosis and active penetration.\(^{12}\) The host cell is stimulated by the presence of an the invasive organism and as a result, produces pseudopodia-like actin microfilament structures to endocytose the fungus.\(^{12}\) The catalyst of this action is the interaction between the Als3 fungal adhesin and either a human E-cadherin or epidermal growth factor receptor 2 (Her2) present on epithelial cells.\(^{12}\) Once actin rearrangement has commenced and fungal cells internalized, *C. albicans* subsequently exploits the clathrin-dependent endocytosis to invade human epithelial cells.\(^{12}\) Active penetration does not require the host’s cellular machinery and instead is reliant upon the physical pressure (turgor) applied by the hyphal tip penetrating the surface in combination with secreted extracellular hydrolases.\(^{11,12}\) Ssa1 and Ssa2 heat shock proteins are part of the HSP70 (heat shock protein 70) family proteins and contribute to the ability of *C. albicans* adhere to, invade, and damage epithelial and endothelial cells.\(^{13}\) Ssa1 specifically is expressed on the hyphal surface and is responsible for binding host epithelial cells, acting to incite endocytosis and exploit the host’s defense mechanisms, bringing it into the cells for further fungal invasion and subsequent dissemination.\(^{13}\) It is hypothesized that Ssa1 and Als3 bind to the same endo and epithelial cell surface proteins as either individual proteins or as a complex of proteins.\(^{13}\) A second theory is that Ssa1 serves as a chaperone required for folding and trafficking of Als3, but this is less likely as proper folding has been observed in the absence of Ssa1.\(^{13}\) In vitro studies of virulence factors in murine models of candidiasis with mutations to these genes show depressed virulence capacity and fungal proliferation into organ tissues and associated tissue damage by measuring Cr release.\(^{13}\)
C. albicans is capable of secreting phospholipases and aspartyl proteases that assist in the diverse spectrum of strategies used by the organism to improve its virulence capacity against host cells. Additionally, heat shock proteins are also fundamental in regulating protein secretions used to resist defense mechanisms employed by the host organism. Hydrolytic enzymes such as the CR3-like receptor and Hsp90 modulate immune cell function and enhance adhesion and tissue destruction. While all the immunologic effects of Hsp90 are not completely understood, its inhibition will interrupt T-cell-linked inflammatory disease pathways and seems to have some therapeutic activity in murine models with autoimmune disease. Additionally, blocking Hsp90 has been found to inhibit activated B cells from proliferation as well as their secretion of IgG.

Pathogenesis of Invasive Candidiasis

C. albicans may produce superficial and systemic infections. Invasive candidiasis, which comprises both candidemia (blood infection) and deep-seated tissue candidiasis, is responsible for the majority of nosocomial fungal disease cases in the developed world; the incidence of candidemia is roughly 6.9 per 1000 patients in ICUs worldwide (Figure 2). While candidemia and deep-seated candidiasis are not mutually exclusive, candidemia accounts for most cases of invasive candidiasis, but blood-culture-negative forms of invasive candidiasis, which includes chronic disseminated (hepatosplenic) candidiasis, occurs typically in patients with hematologic cancers. Deep-seated infection may also occur in other tissue sites including the bones, muscles, joints, eyes, or central nervous system. Deep-seated infection is thought to develop in these distal sites from a preceding or undiagnosed candidemia. In a study of candidemia susceptibility in ICUs, scientists identified three new genes associated with 19-fold increased risk of contracting C. albicans infections.
*C. albicans* is the most common serious fungal pathogen, yet resides harmlessly as a commensal organism in roughly 50% of individuals.\textsuperscript{10} However, currently as the fourth most common pathogen isolated in blood cultures in the United States, *C. albicans* accounts for roughly 6.9 infections per 1000 patients in ICUs worldwide. The pathogen has become increasingly resistant to antifungal therapies and is associated with attributable mortality rates estimated to be between 38% to 49%.\textsuperscript{16,17} In addition to immunosuppression, other risk factors for infection include the presence of central vascular catheters, recent (typically abdominal) surgery, and administration of broad-spectrum antibiotic therapies.\textsuperscript{15} Geographic hot-spots for candidemia – typically in developing countries in Asia, Latin America and Africa – have realized significant improvement when hygiene and disease-management programs were introduced.\textsuperscript{16,18} Candidemia burden estimates are difficult as systematic surveillance studies are typically absent in these regions.\textsuperscript{18}

While rates of candidemia have decreased in ICUs and stem cell transplant units when the antifungal treatment fluconazole is introduced prophylactically, there are increased rates of resistance of nonalbicans candida species including *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*.\textsuperscript{16} This is especially problematic due to a global shift towards nonalbicans candida infections.

Based on recent research, it is hypothesized that fungal overgrowth and its subsequent related pathology occurs in response to a bacterial imbalance associated with antibiotic treatment.\textsuperscript{19} An imbalance between bacterial and fungal species at the mucosal interface has been found to correlate with a metabolic imbalance in the microbial environment that effects the integrity of the mucosal barrier.\textsuperscript{19} Microbial adhesion is responsible for the establishment of polymicrobial colonization on both hard dental and soft mucosal tissues of the oral cavity.\textsuperscript{14} Asymptomatic
colonization by Candida is most typically found around the oral cavity, rectum and vagina with colonization thought to be established at a young age due to passage through the birth canal, during nursing, or from foods. Due to the early established colonization of Candida, circulating IgG and mucosal secretory IgA antibodies to C. albicans are thought to be the reason for the elevated levels in healthy adults. Scientific evidence supports a strong link between Candida species and specific oral bacterial species they often interact with at these colonization sites and these interactions promote adhesion and colonization by mixed-species bacteria.

In some oral infections instigated by close-fitting upper dentures, Candida infections are able to colonize due to the normal oral mucosa being cut off from the protective properties of saliva by the upper denture. Concurrently, the presence of heavy oral bacteria that are not commensal at oral sites such as Staphylococcus aureus and E. coli present on the palatal surface and the fitting surface of the denture, will propagate the inflammatory response at the oral mucosa. Gingivitis is another instance of the cumulative detrimental effects of chronic inflammation and poor oral hygiene resulting in infection at the gingival margins typically involving Candida and S. aureus or Pseudomonas bacteria. Numerous predisposing factors would account for the establishment of these bacterium at sites at which they are not typically present in healthy individuals. Saliva rich in lysozyme, lactoferrin, salivary peroxidase and other host defenses along with the mechanical movement of the tongue often are able to keep adhesion at bay, but may be outcompeted by factors such as xerostomia, the introduction of medical devices, or other immunosuppressing factors and diseases.

Additionally, biofilm formation is common in individuals who receive surgical laryngectomies where voice prosthetics are introduced between the trachea and esophagus. It is hypothesized that Candida colonization on these silicone prosthetics are responsible for their
degradation, which often fail due to polymicrobial biofilm formation involving various strains of streptococci, staphylococci, enterococci, and C. albicans. Roughly 27% of nosocomial C. albicans infections are polymicrobial, with Staphylococcus aureus as the third most common organism found in conjunction with C. albicans. Their dual interaction is characterized by C. albicans forming the underlying scaffolding and S. aureus serving as the outer surface layer of the biofilm as S. aureus does not grow as effectively on abiotic surfaces. The synergistic effects of these two bacteria lead to increased mortality in a murine model utilizing C. albicans SC5314 and S. aureus ATCC 25923 strains.

Fungal-bacterial coinfections are responsible for polymicrobial peritonitis in patients susceptible to infection from surgery, peritoneal dialysis, and those who are in critical condition. Peritonitis is an inflammatory disease of the lining of the abdominal walls and organs often the result of a bowel perforation, laparotomy surgery, intestinal surgeries, or the introduction of a medical device allowing for the colonization of C. albicans and S. aureus.

The association of fungal colonization in the GI tract and the effect on IBD in humans is correlative, mostly because of the lack of comparable animal models for assessing causal relationships. Therefore, it is imperative that risk factors and predictive models are improved to best-target which patients are at highest risk for candidemia/candidiasis so that use of fluconazole treatment may be appropriately targeted.

**Gastrointestinal Immunity**

The human gastrointestinal mucosa comprises the largest surface area in the body extending up to 400 square meters, approximately 200 times greater than the skin. Gut-associated
lymphoid tissue (GALT) is one of the largest lymphoid organs comprising upwards of 70% of the body’s immunocytes.\textsuperscript{23,24} The GALT consists of organized lymphoid tissues, such as the mesenteric lymph node (MLN) and Peyer’s patches (PPs), and more diffusely scattered lymphocytes in the intestinal epithelium and lamina propria (subepithelial tissue).\textsuperscript{24} The human small intestine contains approximately 280 immune sensors called PPs whose quantity peaks during adolescence and wanes in size and number with age.\textsuperscript{23} The number, distribution, shape and size of PPs also vary between individuals.\textsuperscript{23} It is the role of PPs to carefully orchestrate the symbiotic environment with the commensal microbiota while controlling the entry of potentially harmful antigens and microbes. Within the GI tract are approximately 400-500 species that comprise the microflora, translating to $10^{14}$ microorganisms.\textsuperscript{19} Each and every one of these microorganisms contains its own metabolism, which contributes to the host immune system so significantly that it is referred to as an “additional organ”.\textsuperscript{19}

PPs are aggregate lymphoid structures located along the small intestine which serve as sites of immune surveillance; they are central to determining mucosal tolerance or inciting an inflammatory response against these microorganisms.\textsuperscript{23} Mice have roughly 7-9 PPs throughout their small intestine.\textsuperscript{23} The microanatomy and microenvironment of a PP are unique (Figure 1). PPs are clusters of B cell follicle domes with T cell enriched interfollicular regions (IFRs) interspersed amongst them.\textsuperscript{25} Specifically, the interface between the external luminal environment and the PP is called the follicle-associated epithelium (FAE).\textsuperscript{23} The FAE is composed of a single layer of epithelial cells that separates the internal patch and the external luminal environment. The extensive crosstalk that occurs between cells within the lymphoid follicles and the Follicle-Associated Epithelium (FAE) of the small intestine is vital to the signaling process that determines whether to initiate the defense cascade of the mucosal immune system in response to recognizing
pathogens and commensal bacteria as they pass through the intestinal lumen.\textsuperscript{23} Antigens and microbes in the intestinal lumen adhere to the apical surface of M-cells of the PP.\textsuperscript{26} These antigens and microbes are then transcytosed through M cells and into a network of dendritic cells located in the sub-epithelial dome (SED).\textsuperscript{27}

Dendritic Cells (DCs) are key participants in initiating the immune response within the PP.\textsuperscript{23} DCs reside in various epithelial tissues such as the skin, tonsils, and vaginal mucosa as well as in the bronchotracheal epithelium, and the villus epithelium of the small intestine.\textsuperscript{28} Under normal conditions, DCs will reside in these sites, but will migrate when pathogens are present in the form of bacterial products such as enterotoxins and Toll-like receptor agonists.\textsuperscript{28} The myeloid dendritic cells present within the PP are specifically programmed to internalize bacteria and dead cells as well as efficiently present antigens to the PP immune cells (Figure 1).\textsuperscript{23} There are five distinct dendritic cell types in a murine Peyer’s patch: CD11b+/CD8alpha⁻ (myeloid) DCs localized to the subepithelial dome, CD11b⁻/CD8alpha⁺ (lymphoid) DCs in the interfollicular regions, and CD11b⁻/CD8alpha⁻ (double-negative) DCs at both sites.\textsuperscript{29} DCs in the SED region within the FAE of the PP, specifically function to capture incoming pathogens, and these DCs will migrate from the SED into the FAE when pathogens are present.\textsuperscript{28} Peyer’s patches are the first location in the gut where there is T-cell-specific priming and proliferation.\textsuperscript{23} Antigens and microbes are then presented to T cells, stimulating B cells at the germinal center.\textsuperscript{27} Plasma B cells make IgA antibodies that are transcytosed across the lumen via the pIgR receptor and subsequently are able to clear the potential pathogen.

If the primary intraluminal and mucosal defenses become compromised allowing a pathogen to gain an advantage and invade, the pathogen will then traverse the FAE via specialized Microfold (M) cells from the intestinal lumen into the PP. Subsequently, pathogens will be
recognized by a network of Langerin dendritic cells (DCs) and will be processed and presented to T-cells, which then migrate to the germinal center of the patch where they will present the antigen to B cells, which make up roughly 60% of the immune cells in the PP and produce pathogen-specific IgA. Utilizing the pIgR receptor in the FAE, these IgA will transcytose across the patch back into the lumen to neutralize and clear the pathogens in the intestinal lumen.

There are five sets of PP-associated DCs including: plasmacytoid DCs (pDCs), CD8α+ DCs, CD11b+ DCs, double-negative DCs (DN DCs), and lysozyme-expressing DCs (LysoDCs). Monocytes mature into macrophages and LysoDCs, both of which participate in antigen uptake and have strong innate antiviral and antibacterial gene properties. Additionally, if LysoDCs are stimulated by TLR7, they will secrete IL-6 and TNF. pDCs and DN DCs are primarily found in the SED and the IFR, CD8α+ DCs are mostly in the IFR, and the SED is where CD11b+ DCs and LysoDCs are located. Due to shared cell surface markers, CD11b+ and LysoDCs are often confused. Some DCs are capable of additional interaction with immune cells, the DN DCs and LysoDCs are capable of entering the FAE to interact specifically with M cells. LysoDCs are also the most efficient DC cell type at endocytosing pathogenic bacteria, dead cells, and particulate antigens and they are adept at internalizing luminal antigens by extending dendrites through M cell-specific transcellular pores into the gut lumen. In order to continue to expand upon the specific roles of these various DCs, it is imperative that scientists improve upon cell surface markers to distinguish each cell type. Within the FAE, there is increased expression of a macrophage inflammatory protein (MIP)-3alpha mRNA whose receptor CCR6 lies within the SED, whereas CCR7 primary is expressed in the IFR. CCR6 is known to be functionally expressed only by DC subsets present in the SED and splenic DC subsets that did not migrate toward MIP-3alpha, all suggesting MIP-3alpha/CCR6 has a distinct and unique role in recruiting
CD11b+ DCs to the mucosal surface and MIP-3beta/CCR7 in recruiting CD8alpha+ DCs to the T cell-rich regions.³⁰ As such, myeloid DCs from the PP stimulate naïve T cells to secrete high levels of IL-4 and IL-10 compared with those from nonmucosal sites and lymphoid and DN DCs from all tissues stimulate IFN-gamma production suggesting that various DC subsets specifically within mucosal sites are uniquely equipped to induce immune responses.²⁹

*Candida albicans in the context of the gut*

Inflammatory bowel disease (IBD) is a term that encompasses both Crohn’s Disease (CD) and Ulcerative Colitis (UC), both of which are characterized by chronic, relapsing inflammation of the gastrointestinal tract mucosa. The two diseases differ in their pathophysiology. CD is a chronic transmural inflammatory disease that usually affects the distal ileum and colon but may occur in any part of the GI tract. Symptoms include abdominal pain and diarrhea. Abscesses, internal and external fistulas, and bowel obstruction may develop. UC is a chronic inflammatory and ulcerative disease arising in the colonic mucosa and is characterized most often by bloody diarrhea. Both CD and UC may have extraintestinal symptoms, most commonly arthritis and are treated with anti-inflammatory medications, immunomodulators, anticytokines, antibiotics and occasionally, surgery.¹⁹,³²,³³ In the United States as of 2015, roughly 1.3% of adults (~3 million) had reported diagnosis of IBD, a marked increase from 0.9% in 1999 (~2 million).³² The etiology of these diseases is currently unknown, but are thought to be related to a combination of genetic predisposition, environmental factors, and immune dysfunction. Speculations include a dysregulated inflammatory response to intestinal microorganisms, such as *C. albicans*, in an individual who is genetically susceptible or predisposed.³³,³⁴ One of the possible culprits is thought to be a commensal fungus such as *C. albicans*.³³,³⁴ Patients with CD have also been demonstrated
to possess antibodies against *Saccharomyces cerevisiae* mannan linking CD and yeast. These antibodies are present in roughly 50-60% of individuals with CD. In addition, some studies of UC have reported anti-yeast antibody percentages present in as many as 91% of patients.\(^{19,34}\) Additionally, CD patients are more frequently colonized with *C. albicans* than healthy individuals, further linking yeast presence and a disproportionate inflammatory response in the gut.\(^ {33,34}\) Once *C. albicans* causes an infection, it may propagate a range of macroscopic tissue changes analogous to those found in IBD, including ulcerations of the mucosa, loss of the native structure of the colonic crypt, submucosal swelling, necrotic changes, micro clot formations, acute inflammatory neutrophil-induced infiltrations, and necrosis of colonic mucosa and submucosa.\(^ {19}\) Histologically, these tissues have marked inflammation, ulcerations, fibrosis, granulomas, and neutrophil infiltration corresponding with damage to the colonic crypt structure by the hyphal fungus presence.\(^ {19}\) Gastric ulcers in patients who also were colonized with *C. albicans* had ulcers that were larger in diameter and the rate of ulcer healing was also slower than patients who were not colonized.\(^ {33}\)

In light of its importance as a nosocomial microbe and because of the increased incidence of Candida gut colonization in cancer patients, ICU inpatients, and IBD-diagnosed individuals, Koh et al. developed a murine model to replicate and study intestinal colonization and dissemination.\(^ {17}\) He hypothesized that Candida dissemination originated from a primary infection occurring in the GI tract in these patients receiving chemotherapy.\(^ {17}\) He subsequently investigated which components of the host innate immune system in healthy individuals are required to prevent dissemination since *C. albicans* is relatively harmless to healthy individuals.\(^ {17}\) Identified risk factors for invasive Candidiasis included central vascular catheters, recent (typically abdominal) surgery, and broad-spectrum antibiotic use.\(^ {16}\)
At the time of Koh’s research, most murine models of Candida dissemination required pretreatment with cyclophosphamide or a similar chemotherapeutic agent followed by IV injection of *C. albicans* or *C. albicans* inocula. Koh noted that these models were inadequate for describing GI colonization, translocation and eventual dissemination of *C. albicans*. He outlined three necessary components for potentiating bacterial or fungal invasion and infection including: 1) GI microbiome dysbiosis supporting pathogenic overgrowth; 2) disruption of the intestinal mucosal barrier to increase permeability; and 3) immunosuppression. Koh noted that disruptions to the mucosa, application of a mucolytic, induction of neutropenia, and the administration of broad-spectrum antibiotics used in various particular combinations, would afford valuable opportunities for studying the details of *C. albicans* pathogenesis in a model that produced a compromised innate immune system. Additionally, an effective murine model would also serve to further investigation of the immune mechanisms responsible for killing and/or controlling translocating *C. albicans*. Koh’s murine model was constructed under the assumption that inducing both neutropenia and GI mucosal damage are imperative for dissemination of *C. albicans* disease. He confirmed establishment of Candida dissemination by collecting fecal samples and measuring the CFU output of Candida under a variety of treatment combinations.

The Koh model employed a specific antibiotic cocktail that included Penicillin-G and Gentamicin to incite microbiome dysbiosis. His model focused on survivorship under various conditions in order to deduce which aspect(s) of the innate immune system was/were important in disease dissemination and subsequent mortality. After formulating the antibiotic regimen, Koh established the conditions for inducing neutropenia in mice using 200 µg of anti-neutrophil mAb RB6-8C5. In addition to its opportunistic nature in cancer patients, it has been documented that individuals suffering from Crohn’s disease, have significant colonization of *C. albicans* at the
inflammatory sites within the intestinal mucosa. To replicate this condition, Koh utilized a three to seven day treatment of dextran sodium sulfate (DSS) to elicit local areas of inflammation in the cecum. Mucositis was only produced using the seven day regimen of DSS, which was found to contribute to *C. albicans* dissemination only when mice were co-treated with the anti-neutrophil antibody. Koh was able to successfully cause fungal dissemination evident by liver invasion when 5% DSS was introduced in combination with mAb RB6-8C5 which resulted in 100% mortality. Other research, conducted by Jawhara et al has also confirmed the necessity of including DSS in a murine model to mimic human ulcerative colitis conditions and underlined its requirement within the protocol for establishing *C. albicans* colonization of the GI tract. They continued by stating that *C. albicans* on its own is insufficient in inciting enough gastrointestinal inflammation for colonization to occur. Therefore, researchers speculate that a dysbiosis in the microbiota creates susceptibility in individuals to CD and other triggering events culminate in recurrent inflammation, which allows for *C. albicans* to colonize. Once established, *C. albicans* causes additional inflammation and delays healing of ulcers, providing conditions for disseminated or deep-seated Candidiasis.

*The Role of the Peyer’s patch in the Uptake Dissemination of C. albicans*

In a study conducted by De Jesus et al, *C. albicans* was gavaged into different mouse strains, such as WT Swiss Webster, BALB/c, and C57BL/6 mice. A specific type of PP dendritic cell (DC) that expresses the C-type lectin receptor, Langerin, was identified which is responsible for the uptake of *C. albicans*. The study also found that M-cells were at least partly responsible for the uptake of *C. albicans* into the patch. However, while uptake was found to be possible, *C. albicans* never gained an advantage and they were unable to achieve dissemination.
The goal of this project is to assess which conditions from Koh’s model, i.e. depletion of microbiota, elimination of intestinal mucous, neutropenia and DSS-induced intestinal inflammation are necessary to produce invasion and disseminated infection while specifically preserving the ability to study PP function (Figure 2). In our effort to elucidate what happens to \textit{C. albicans} within PPs, we seek to identify the conditions required that allow \textit{C. albicans} to gain an opportunistic advantage while maintaining the integrity of PPs. While the lens through which Andrew Koh was investigating candidiasis was survivorship after eliciting fungal colonization and dissemination, in our model we are not using survivorship as a predictive measure for disseminated disease. Instead, we examined blood and body tissues including kidneys, spleen, brain, heart, lungs, stomach, liver and PPs for the presence of \textit{C. albicans} to document dissemination. Both the kidney and spleen are known to support the growth of \textit{C. albicans} systemic infections, and therefore it is appropriate to determine the level of microbial burden based upon CFUs in the kidney and spleen.\textsuperscript{21} This approach allowed us to more specifically identify the specific requirements for uptake into PPs and what factors facilitate dissemination.

\textbf{Our aim was to improve upon a mouse model of intestinal inflammation such that \textit{C. albicans} is provided an advantage and becomes opportunistic for systemic dissemination and not cleared by the innate immune system.} The innovative aspect of this research will be the development of a new model that employed fewer hits to the innate immune system than Koh initially speculated were necessary to cause invasive candidiasis. Specifically, we will assess the effect of eliminating the induction of neutropenia and the use of DSS, while preserving PP integrity and function through the inclusion of N-acetyl cysteine to deplete the mucous layer and the use of Penicillin-G, Streptomycin and Gentamycin.
To address this, we are systematically determining the specific conditions that contribute to giving *C. albicans* as an opportunistic advantage and eliminating those that do not by 1) reducing the microbiome with antibiotics; 2) removing the mucous layer with N-acetylcysteine (NAc); 3) disrupting the mucosal barrier by inciting colitis with Dextran sodium sulfate (DSS); and 4) ablating Langerin+ Dendritic Cells with Diphtheria Toxin (DT) (Table 1).

This study will also assess whether the DNA of non-viable, heat-killed *C. albicans* can be detected in the stomachs, PPs and deep tissues of mice exposed orally. Specifically, this work will attempt to establish whether antigen from heat-killed organism can be recognized, taken up and distributed to the deep tissues in our murine model. If successful, this has implications for evaluating the biological and physiological effects of true infection in future studies of invasive infection. Additionally, this investigation will be evaluating whether or not dissemination is an active or passive process by comparing the outcomes of introducing heat killed versus live *C. albicans* via the oral route. The process of heat-killing the *C. albicans* will preserve the surface receptors and allow us to ascertain the involvement of the fungus as a live organism to the dissemination process.
Figure 1. The microanatomy of a Peyer’s patch allows for selective sampling immune sensing that is specific to the gut. Sampling by Peyer’s patches (PP) is a selective process as antigens and microbes must first cross the microbiota, mucous, and the follicle-associated Epithelium (FAE) in order to be sampled. If these barriers are insufficient against a pathogen or become compromised, the pathogen will then traverse the FAE via specialized Microfold (M) cells from the intestinal lumen into the PP. Dendritic cells also have the capability of stretching through the epithelial layer to recognize and bind pathogens in the lumen and bring them into the patch. Pathogens recognized by a network of Dendritic Cells (DCs) will be processed and presented to T-cells. Peyer’s patch T cells will migrate to the Germinal Center of the patch where they will present the antigen to B cells. These B cells make up roughly 60% of the immune cells in the Peyer’s patch, and make pathogen-specific IgA. By binding the plgR receptor in the FAE, these IgA will transcytose the patch back into the lumen to neutralize and clear the pathogens in the intestinal lumen.
Figure 2. **Organ and tissue sites associated with pathogenesis of invasive candidiasis in humans.** Invasive candidiasis has become the most common nosocomial fungal disease in the developed world.\textsuperscript{16} Mortality rates remain upwards of 40\% plaguing hospitals with a disease gaining resistance against various antifungal therapies.\textsuperscript{16} Invasive candidiasis, is comprised of both candidemia and deep-seated candidiasis.\textsuperscript{16} Koh investigated which components of the host innate immune system in healthy individuals are required to prevent dissemination, since \textit{C. albicans} is relatively harmless to healthy individuals.\textsuperscript{17} Risk factors include central vascular catheters, recent (typically abdominal) surgery, and broad-spectrum antibiotic use for invasive candidiasis.\textsuperscript{16}
Table 1. Detailed outline of the six approaches that were used to optimize *C. albicans* dissemination in a murine model. Treatments included: 1) Diphtheria toxin injection to ablate Langerin Dendritic Cells (DCs); 2) an anti-neutrophil antibody mAb-Rb68c5; 3) Dextran sodium sulfate (DSS) in various concentrations to incite colitis; 4) antibiotics to deplete the intestinal microbiome: 5) N-acetylcysteine as a mucolytic agent: and 6) either live or Heat-killed *C. albicans*. Weights were collected throughout the experiments, fecal pellets were obtained for Colony-Forming Unit (CFU) analysis, and blood and tissue samples were collected for matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF), Polymerase-Chain Reaction (PCR), and histopathology analysis.
CHAPTER 2: MATERIAL AND METHODS

Animals. Male and female transgenic Langerin DTR-EGFP mice with a C57 background and inbred C57 mice were either bred and maintained at the Wadsworth Centers for Laboratories and Research (WCLR) or obtained from the Jackson Laboratory. Female outbred Swiss Webster mice were obtained from Taconic Biosciences. Animals were housed under conventional, specific pathogen-free conditions and treated in accordance with the Wadsworth Center’s Institutional Animal Care and Use Committee (IACUC) guidelines. As per IACUC standards of care, mice losing a minimum of 20% of their body weight in a short period of time are considered severely ill and are required to be subjected to human termination from the protocol. Based on this standard, mice were weighed daily throughout various trials.

Ethics Statement. Experiments described in this study that involve mice were reviewed and approved by the Wadsworth Center’s Institutional Animal Care and Use Committee (IACUC) under protocol #15-450. The WCLR complies with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and was issued assurance number A3183-01. Moreover, the WCLR is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Obtaining this voluntary accreditation status reflects that Wadsworth Center’s Animal Care and Use Program meets all the standards required by law and goes beyond the standards as it strives to achieve excellence in animal care and use.

Fungal strain. We utilized the laboratory strain of Candida albicans SC5314 from Aaron Mitchell at Carnegie Mellon, a strain which is known to be virulent in a mouse model of systemic infection.\textsuperscript{38} C. albicans colonies were stored in a horizontal shaking incubator at 37°C in a Sabouraud dextrose broth. To ensure that the colonies remain viable and did not die from using up their nutrient broth, the colonies were reinoculated every two weeks. Reinoculation was performed
under sterile technique starting by sterilizing the laboratory bench with 10% bleach followed by 70% ethanol. A sterile flask and the flask containing the culture medium were opened at a 45° angle and both passed through an open flame to minimize airborne contamination. After passing the sterile flask through the open flame and doing the same with the nutrient broth, 30mL of nutrient broth was poured into the empty flask before both being resealed. Then, passing the pipette tip through the flame and re-sterilizing the flask mouth, 500ul of *C. albicans* from the culture medium was pipetted into the nutrient broth. The new inoculate was covered and placed in the shaker at 37°C and the old culture medium was properly discarded.

**Heat-killed *C. albicans***. The same strain of *C. albicans* SC5314 was utilized for the heat-killed trial. 2mL of *C. albicans* suspension was pipetted into fifteen 2mL tubes. Samples were then spun at 2000 RPM for 2 minutes. The supernatant was discarded, and the pellet was washed with 2mL of PBS. It was spun at 2000RPM for 2 minutes and discarded the supernatant. We then resuspended the pellet in 300ul PBS and placed samples in the horizontal shaker at 90°C for 60 minutes at 1000 RPM. Every 15 minutes, the vials were removed to be vortexed, spun, and again vortexed before returning to the shaker 90°C all the time ensuring the sample does not come into contact with the lid of the tube. Vials were vortexed, spun, and finally resuspend with a pipette. Two spots of 20µL sample plated from each of 15 tubes to confirm the sample was successfully heat-killed and would not grow on nutrient Sabouraud dextrose media.

**Antibodies, antibiotics, and reagents.** The anti-neutrophil antibody administered to treatment mice was mAb-Rb68c5 which is a monoclonal antibody that reacts strongly with mouse Ly6G and weakly with mouse Ly6C. The antibiotics administered to treatment groups included Penicillin G (G+ > G-), Streptomycin (broad-spectrum antibiotic) and Gentamycin (G-). N-acetylcysteine was introduced into the water source as the mucolytic agent, affective by means of
disrupting disulfide bonds in the mucous structure. Our culture plates for growing *C. albicans* colonies were comprised of Chloramphenicol, Penicillin, Gentamycin, and Streptomycin. Blood samples were collected utilizing Heparin-treated capillary tubes. The antibodies used for staining prior to confocal microscopy included our primary antibodies PA17206/PA127158, a polyclonal antibody derived from rabbit IgG, and our secondary antibody which was Goat/Anti-rabbit. Our confocal microscopy images were imaged on a DAPI background. Dextran sodium sulfate (DSS) is a known chemical for inciting intestinal inflammation mimicking colitis in mice. Throughout various experiments 2.5%, 3%, and 5% concentrations were administered to the mice through their water source. The inflammation is caused by DSS establishing linkages with medium-chain-length fatty acids (MCFAs) in the lumen of the colon. Then, DSS and MCFAs complex to form vesicles that fuse with the colonocyte membranes, thereby activating signaling pathways that activate intestinal inflammation.

**Colony-Forming Unit.** The presence or absence of *C. albicans* in the organs was first determined by plating serial dilution samples from organs and tissues with varying degrees of association, or lack thereof, with the gastrointestinal system including stomach, kidney, liver, spleen and Peyer’s patches (PPs); and lungs, heart, and brain. Additionally, to confirm that *C. albicans* had come into contact with the intestines successfully after oral introduction of the fungus, we collected fecal samples that were processed and plated to confirm presence by means of colony growth. Our plates were made by the Media Core at the WCLR and in addition to agar, contained Chloramphenicol, Penicillin-G, Gentamycin, and Streptomycin. A colony-forming unit (CFU) is a unit to estimate the number of viable fungal cells in a sample. The term “viable” refers to the ability of that unit to multiply by binary fission in controlled conditions. The purpose of plating is to estimate the number of cells present based upon their ability to produce a colony under
specific conditions including nutrient medium, temperature, and time. During necropsy, whole organs were extracted and cut in half; half an organ was utilized for plating, except for lungs and kidneys where one whole organ was designated for plating. Each sample was placed in 1mL HBSS and ground through a strainer. The resulting solution was recollected and 20µL were plated in triplicate in serial dilutions $10^0$, $10^{-1}$, $10^{-2}$, and $10^{-3}$. For the fecal and stomach samples, additional dilutions were plated to include $10^{-4}$, $10^{-5}$, and $10^{-6}$. The colonies were counted after 24 hours and were normalized based upon the concentration under which growth was present and visually countable.

**Polymerase chain reaction (PCR).** Polymerase chain reaction (PCR) is a technique to make large numbers of copies of short sections of DNA from a small sample as an amplification technique. Polymerase chain reaction (PCR) was run on blood samples from mice that received live *C. albicans* and mice that received heat-killed *C. albicans* since it is the *C. albicans* DNA that is detected in this assay regardless of the viability of the cell (Table 3). Therefore, PCR was also utilized to evaluate the organs of mice that received heat-killed *C. albicans*. *C. albicans* DNA was detectable in all organs and tissues evaluated. We sent blood samples to the mycology laboratory for PCR analysis in order to test for the presence of specifically *C. albicans* in these samples and ensure that there was no fungal contaminant that was growing on our agar plates. PCR analysis was performed on all blood samples in Approaches 3, 4, 5, and 6 as well as on organs for mouse 1 of each group in Approach 6. 1.5mL tubes containing three beads that will be used to break open *C. albicans*’ cell wall. Reagents added in included 400ul buffer AL, 40µL proteinase K solution and 3ul reagent DX (an antifoaming agent). After sterilizing the cabinet, original samples were centrifuged for 30 seconds to remove any sample from the cap. 400µL of blood was pipetted into the beads containing the hard beads and then vortexed. These tubes were then placed into the
Thermomixer C block shaker at 56 degrees Centigrade for 10 minutes and then vortexed for 30 seconds. We homogenized our samples at 6500rpm for two minutes followed by vortexing for 30 seconds. Simultaneously, into empty 1.5mL tubes was pipetted 5µL of E. coli bicoid gene from Drosophila species which functioned as the inhibition control into which we transferred 843µL of the prepared samples. Tubes were labeled for the rotor adaptors that went into the QIACube for DNA extraction, which ran ten samples at a time for 55 minutes. Upon completion of the DNA extraction, the resulting sample was transferred into a 96-well plate for PCR analysis. The C_T is the value at which the sample’s curve intersects the threshold on the application plot, which is set at 26,516 as determined by the control lot of genomic DNA. PCR results were interpreted based upon C_T values and were significant and therefore labeled as positive (+) if less than 38. Our negatives were confirmed via inhibition control via a bicoid plasmid from the bicoid gene isolated from drosophila. Since hemoglobin is inhibitory to amplification, the inclusion of this inhibition control allows for the conclusion that our PCR is reflecting true amplification.

Matrix-assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF). Finally, to parallel our verification of C. albicans presence in the blood, the mycology laboratory also processed streaks of fungal growth that had been plated from various organs and samples utilizing Matrix-assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF) technology. This technology is a specialized form of mass spectrometry utilizing the time of flight as the charged ions leave the matrix and fly up into detector upon irradiation by a pulsed laser. MALDI-TOF is species-specific and owing to its utilization of ribosomal proteins, is a highly accurate assay. These samples were prepared by streaking a single colony for the preparation of matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) for confirmation of specifically C. albicans spp. Plates where fungal
colonies were grown after tissue samples were plated, a single colony representing a pure isolate was selected from the plate and streaked on a new SAB plate. Plates were left for 24 hours to incubate at 37°C. The new fungal growth was collected via wand transfer into a 1.5mL conical tube containing 300µL of LCMS water and the wand was stirred in the LCMS water until it became cloudy with the sample. 900µL of ethanol was added and then the solution was vortexed briefly and then centrifuged for 2 minutes at 13.2 x1000 RPM. Excess liquid was removed with a bulb pipette leaving the pellet and the centrifugation and drying was repeated a second time. Then, 25µL of 70% formic acid was added to each sample tube and vortexed briefly. 25µL of acetyl nitrile was added, vortexed, and centrifuged for 2 minutes before the sample was spotted onto the MALDI-TOF plates in 1µL spots. Samples were spotted in duplicate and then covered with a 1µL spot of matrix before being placed into the equipment for analysis.

**Cryosectioning.** Organs and Peyer’s patches were collected during necropsy and placed into Optimum Cutting Temperature compound (OCT) in cassettes and dropped immediately into liquid nitrogen. The cassettes were stored at -20°C until they were removed for cryosectioning. Cryosectioning is performed on a ThermoFisher Scientific CryoStar NX70 cryostat to ensure cryogenic temperatures are maintained during the process of cutting samples onto slides for future staining and imaging. Samples were mounted in OCT and placed on a stage. The cryostat block remains at -21°C and the blade is set at -20°C. For sectioning Peyer’s patches, the cutting thickness was set to 18mm and 25mm for organs, and two slides of four samples, minimum, were collected for staining and confocal microscopy imaging.

**Confocal microscopy.** Images were captured utilizing a Leica SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany). Confocal, or conjugate focus, microscopy is a specialized microscope system creates an image via a point source of light in conjugate focus at the specimen
on the image plane.\textsuperscript{40} An opaque pinhole aperture allows only light coming from the focal point on the specimen to access the detector, and eliminates incoming light from above or below the plane that is not in focus.\textsuperscript{40} Confocal microscopes utilize a variety of arrangements, lasers, and editing tools depending on whether or not the tissue is live, the specimen fixation and processing, and the spectrum of the fluorescent tags being utilized, and the quality of the results depends largely on the expertise of the microscopist.\textsuperscript{40} We used a polyclonal Rabbit/IgG antibody that is known to be reactive against fungi and our secondary antibody was a Goat/Anti-rabbit antibody with a fluorescent tag. DAPI is one of the stains that we used specifically for confocal microscopy since it is a fluorescent nuclear and chromosomal counterstain and emits blue fluorescence upon binding to A-T rich regions of DNA. DAPI is excited with UV light and detected through a blue/cyan filter and has a broad emission peak, which is convenient when aiming to stain the same sample with multiple fluorescent stains. One of the most important benefits to a confocal microscope system is the ability to manipulate the various components and tailor the technology to your needs. Aside from the immense cost of lasers and yearly maintenance, one of the downsides to confocal microscopy is the risk for bleaching the specimen due to the finite number of photons available for image formation.\textsuperscript{40}

**Histopathology.** Organs are collected during necropsy and placed immediately into cassettes that are dropped into a formalin solution to preserve the tissues. These samples are catalogued and sent to a histopathology core for slide preparation and staining. Samples were stained with Periodic Acid Schiff (PAS) staining technique as well as Haemotoxylin and Eosin (H&E). PAS stains are used for staining fungus and glycogen in tissue sections. H&E stains are utilized for recognizing a variety of tissue types and stains cytoplasmic, nuclear, and extracellular matrix features.\textsuperscript{41} Hematoxylin stain nucleic acids a deep blue-purple color, and stain cytoplasm
and the extracellular matrix various shades of pink.\textsuperscript{41} The benefit to utilizing H&E is its ability to elucidate structure of a tissue.\textsuperscript{41} PAS staining will stain the live fungal cell walls. The positive control utilized was HistoGel blocks of \textit{Candida albicans} culture, while negative controls included for the antibody: Primary antibody (7206) replaced by normal rabbit serum, tissues: negative control animals, and cross-reaction: Histogel blocks of \textit{Saccharomyces cerevisiae}. Slides were deparaffinized in Citrosolv and re-hydrated by processing them through graded alcohols. Antigen retrieval was performed by digesting the tissues in PK (details in provided SOP). Rodent Block M (Biocare Medical) was used as a blocking agent. The primary antibody (Rabbit polyclonal-Thermo Fisher #7206) was incubated on the tissue sections at a dilution of 1:10,000 for 1 hour at room temperature. The signal was amplified and detected using an anti-rabbit alkaline phosphatase polymer (Biocare Medical) and Warp Red as chromogen (Biocare Medical). Sections were counterstained in Tacha’s hematoxylin and mounted using eco-mount (Biocare Medical), a non-aqueous mounting medium. Negative antibody controls for each slide included replacing the primary antibody with normal rabbit serum and omitting the use of a primary antibody.
CHAPTER 3: RESULTS

Our **specific aim was to** improve upon a mouse model of intestinal inflammation such that *C. albicans* is provided an opportunistic advantage.

This study will assess three associated sub aims:

1.1 **Intraluminal barriers-microbiome and mucous:** Assess the role of antibiotics and N-acetylcysteine in fostering infection while preserving the ability for the Peyer’s patch to take up *C. albicans*.

1.2 **Eliciting inflammation:** The role of DSS in establishing a model for *C. albicans* dissemination.

1.3 **Gross signs of disease:** The role of weight as a predictor of *C. albicans* dissemination:

Andrew Koh’s model heavily relied on weight as a predictive tool for dissemination, a requirement for illness used as a standard in the current literature. This study will assess the consistency of this finding.

The protocol modifications employed in this study showed that production of disseminated infection did not require induction of all three conditions outlined by Koh, i.e., dysbiosis, elimination of GI mucous, and mucosal injury. In addition, we found that induction of neutropenia using the anti-neutrophil antibody mAb-Rb68c5 was also not necessary for production of disseminated infection. In each of our four trials with mice exposed to live *C. albicans*, disseminated infection was successfully produced in a majority of mice, including at lower proportions of Dextran sodium sulfate (DSS), and among those that were exposed to antibiotics alone or in combination with N-acetylcysteine (NAc).
Organ and fecal samples were prepared by streaking a single colony for the preparation of matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) for specific confirmation *C. albicans* spp. Polymerase chain reaction (PCR) was run on blood samples from mice that received live *C. albicans* and mice that received heat-killed *C. albicans* since it is the *C. albicans* DNA that is detected in this assay regardless of the viability of the cell. Therefore, PCR was also utilized to evaluate the organs of mice that received heat-killed *C. albicans*. *C. albicans* DNA was detectable in all organs and tissues evaluated. Colony-forming units (CFUs) confirmed *C. albicans* presence in all organs and tissues evaluated, while fungal elements were only visualized with Periodic acid–Schiff (PAS) staining from the stomach of mice given live *C. albicans*. Overall, of the 60 mice surviving the various treatment protocols throughout the experiment, 40 (66.7%) had documented disseminated candidiasis including 29 with organ involvement, 2 with positive blood samples, and 9 with *Candida albicans* identified in both blood and organ specimens (Table 2, Figure 3A/B). 24 (82.8%) of the 29 with organ involvement had infection detected in the kidneys and spleen. Heart samples were obtained from 46 mice including 27 with disseminated infection; 8 (29.6%) were positive. Brain samples were obtained from 37 mice including 25 with disseminated infection; 10 (40%) were positive. Lung and liver samples were obtained from 29 mice including 17 with disseminated infection; 6 (35.3%) lung samples and 2 (11.8%) liver sample were positive. Of the 27 negative liver samples, disseminated infections were detected in 14 (51.9%) mice via brain (8), lung (5), kidney (4), blood (3), heart (2), and spleen (2) samples. In addition, of the 23 with negative kidney samples, disseminated infections were detected in 10 (43.5%) mice via brain (6), spleen (3), blood (2) heart (1), lung (1), and kidney (1) samples.
In our model, disseminated *C. albicans* was documented in 30 (76.9%) of 39 mice exposed to DSS; no mice were exposed to DSS alone. Alternately, disseminated *C. albicans* was found in 10 (47.6%) of 21 mice that were not exposed to DSS. Thus, while the proportions of disseminated infection were highest among mice exposed to DSS, mucosal disruption with DSS was not required to produce disseminated *C. albicans* infection. Although, overall the number of deaths of study mice was small in our trials, by eliminating the DSS from the protocol we could further reduce and perhaps eliminate the risk of mortality.

In Approach 2 we looked at mice with a C57 background vs Swiss Webster to evaluate the strain that provided the most efficacious model of dissemination based upon the mice our laboratory already had established tools with which to work. After running the experiment in parallel between both strains with pretreatments including various combinations of antibiotics, NAc, DSS, and an anti-neutrophil antibody prior to *C. albicans* exposure, C57 mice had 1 (33%) of 3 positive in the heart, 9 (82%) out of 12 mice CFU positive in the spleen, and 7 (64%) of 11 CFU positive in the kidney. Swiss Webster mice had 0 of 4 CFU positive in the heart, 5 (42%) of 12 CFU positive in the spleen, and 4 (33%) of 12 CFU positive in the kidney. Therefore, subsequent experiments were conducted utilizing mice with a C57 background.

1.1 Intraluminal barriers-microbiome and mucous: Assess the role of antibiotics and N-acetylcysteine in fostering infection while preserving the ability for the Peyer’s patch to take up *C. albicans*

Evidence of disseminated *C. albicans* was found in 26 (52.0%) of 50 mice (9 of 9 groups) exposed to antibiotics; this includes 5 of 6 mice exposed to antibiotics as the sole pretreatment; and 4 of 9 exposed to antibiotics in conjunction with NAc (excluding DSS) (Approach 2, 3, 4, 5).
Also, evidence of disseminated *C. albicans* infection was present in 25 (62.5%) of 40 mice (5 of 5 groups; Approach 2, 3, 4, 5) exposed to NAc. This included 1 of 6 mice exposed only to NAc in Approach 4.

Approach 3 employed interventions aligning more closely to that of Koh’s and we utilized his antibiotic protocol which included Streptomycin, Penicillin-G, and Gentamycin. Additionally, we gave select treatment groups NAc, a mucolytic agent. In this trial, we transitioned from gavaging fluorescently-labeled Candida to introducing *C. albicans* into the drinking water at a concentration of $1 \times 10^8$ CFU/mL as did Koh. We also included two concentrations of 2.5% and 5% DSS treatments in the drinking water to determine the lowest possible concentration of DSS that would still prove efficacious in disrupting the mucosal layer in the intestines.

We observed dissemination in both the 2.5% and 5% DSS concentration treatments. One mouse receiving 5% DSS and antibiotics (but not NAc) did not survive until the end of the trial, and the other two groups had visible signs of illness, these mice were therefore terminated from the trial early (Figure 3B).

In Approach 3, we repeated the antibiotic protocol as well as treatment with NAc, but eliminated the anti-neutrophil antibody mAb RB6-8C5 by IP injection. We included controls for Candida and DSS in this trial. Groups 1 and 4 did not receive any DSS in their water, and group 4 was also not administered any Candida in their water. Upon tissue analysis and fecal CFU counts, treatment group 1 that did not receive any DSS in their water, experienced dissemination of Candida in not only gut-associated organs like the stomach, kidney and spleen, but also the heart and brain (Figure 3B). Group 4 was our control for measuring the length of the small intestine as
intestinal inflammation leads to a decrease in its length. We observed no correlation between colon length and pre-treatment/treatment protocol for these mice.

Approach 3 also encompassed a larger scope of confirmatory testing to include PCR of blood samples from infected mice. The results reveal the specific presence of *C. albicans* suggesting transient shedding and deep-seeded infection. We did, however, confirm *C. albicans* presence in gut-associated and distal organs in all mice receiving *C. albicans* treatment. This experiment demonstrated that NAc and 2.5% DSS were sufficient for dissemination of *C. albicans* into a multitude of organs while maintaining viability of the mice.

Three mice were exposed to heat-killed *C. albicans*, 2.5% DSS, and either antibiotics, N-acetylcysteine, or both. *C. albicans* DNA was identified by PCR in the tissues of all three mice including the heart (3), brain (2), lungs (1), spleen (1), kidney (1), liver (1), and Peyer’s patch (1). Each mouse had at least two deep tissue samples positive (range 2 – 4) and one with 4 positive deep tissue sample also had DNA detected in its PP sample. All three mice appeared healthy at the sacrifice on the 18th day of the trial; two mice gained weight during the trial and one with DNA recovered from 5 tissues experienced a 5.8% decline in weight from baseline.

1.2 Eliciting Inflammation: The role of DSS in establishing a model for *C. albicans* dissemination

Evidence of disseminated *C. albicans* was found in 30 (76.9%) of 39 mice represented by 8 out of 8 groups, exposed to DSS (Figure 3B). No mice were exposed to only DSS (Approach 2, 3, 5). However, only 10 (47.6%) of 21 mice (3 of 3 groups) had evidence of disseminated disease in protocols where they were NOT pretreated with DSS (Approach 3, 4). Confirmatory tests including matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-
TOF) of the organ and fecal CFUs and Polymerase Chain Reaction (PCR) of blood samples were used to demonstrate that infection was due to *C. albicans*.

**1.3 Gross signs of disease: The role of weight as a predictor of *C. albicans* dissemination**

Weight loss of more than 20% of baseline was observed in only one trial (Approach 3) in which mice received live *C. albicans*, N-acetylcysteine (NAc), and 5% Dextran sodium sulfate (DSS). No significant weight loss occurred in the remaining trials under other combinations of treatments and during other trials involving either live or Heat-killed *C. albicans*, including those with documented invasion and dissemination (Figure 5).

A total of only 5, including 2 mice with borderline significant weight loss, of 63 mice exposed to *C. albicans*, representing 2 out of 11 treatment groups, experienced weight loss of or near 20%. 4 of the 5 mice with organ tissues tested had evidence of disseminated *C. albicans* based on Colony-forming unit (CFU) organ analysis. Subsequently 33 of the remaining 56 mice (58.9%), with clinically subthreshold weight loss, stable weight, or weight gain had evidence of disseminated infection represented in Approaches 2,3,4, and 5. Finally, 62.2% of mice (10 out of 16 from four groups in Approach 2 and 5) receiving DSS, antibiotics, and NAc in addition to *C. albicans*, had evidence of disseminated *C. albicans*. It was therefore concluded that gross signs of disease such as the typical standard of losing 20% of their initial weight was not predictive of fungal dissemination.
Table 2. Localization of Candida albicans to tissue-specific sites. Throughout tissue processing and analysis, various assays were utilized for detecting the presence of C. albicans throughout the mouse organs. Blood, feces, Peyer’s patches (PPs), stomach, spleen, lung, liver, heart, brain and kidney were evaluated in mice receiving live and heat-killed C. albicans. Specimens were plated for tissues and organs for all mice and colony-forming units were counted when growth was observed. PCR results were interpreted and were significant and therefore labeled as positive (+) if the C_T was less than 38.
Figure 3A. Colony-forming unit in collected tissues. (A) During necropsy, tissue and fecal samples were obtained from mice that were and were not given *C. albicans* treatments, as well as 0 to 5% DSS, antibiotics, and NAc. These samples were processed in serial dilutions and plated for the recovery of colony forming units (CFU). The colonies were counted after 24 hours and were normalized based upon the concentration under which growth was present and visually countable.
Figure 3B. Colony-forming unit in collected tissues. (B) An average was calculated between the triplicate dots plated and the log value was calculated and graphed as above across all trials. A total of 108 mice cumulatively comprised the 6 trials, 75 of which received *Candida albicans* as part of their treatment protocol.
Table 3. *Candida* species Multiplex Real-Time PCR Assay Primers and Probes. Polymerase chain reaction (PCR) was run on blood and tissue samples from mice that received live *C. albicans* and mice that received heat-killed *C. albicans* since it is the *C. albicans* DNA that is detected in this assay regardless of the viability of the cell. *C. albicans* DNA was detectable in all organs and tissues evaluated. PCR analysis was performed on all blood samples in Approaches 3, 4, 5, and 6 as well as on organs for mouse 1 of each group in Approach 6 not only to confirm presence in blood from mice treated with *C. albicans*, but also to evaluate for contamination.
Figure 4. Histopathologic evaluation of invasion of *Candida albicans* into the lumen of the stomach. These histologic sections were collected from the stomach tissue of mouse from group 2 which received antibiotics and 7 days after a 5-day treatment of *C. albicans*, but NO N-Acetylcysteine (NAc). (A)(B) These images illustrate local invasion of the mucosa and submucosa, mostly at the non-glandular/glandular junction of the stomach. (C) Indicates an inflammatory response around the hyphae involving mostly neutrophils and macrophages definitive characteristics of tissue invasion(D) Denotes hyphal form of *C. albicans* in the tissue.
Figure 5: Initial and final weights for mice receiving Heat-Killed and Live *Candida albicans* not a predictor for dissemination. To standardize average initial weights, all mice were weighed and grouped by weight before being redistributed evenly throughout treatment groups. Paired, one tailed Student's t-test between Heat Killed Initial and Final showed significant weight gain, P=0.0281. There was no significant weight change for mice receiving live *C. albicans* throughout the experiment, P=0.0598. There was no significant difference between initial weights between both treatment groups, P=0.3909. N(Heat-killed *C. albicans*)=12. Final weights between treatment groups were significantly different, with mice receiving live *C. albicans* weighing significantly less, P=0.0068. N(Live *C. albicans*) =12. The mean and standard deviation are represented.
Figure 6: Proposed model of live and heat-killed *Candida albicans* distribution in a human based upon a mouse-model. Under various conditions designed to compromise the mucosal immune system, both live and heat-killed *C. albicans* colonies and hyphae were observed by both Colony-forming unit (CFU) and histopathology Periodic acid-Schiff (PAS) staining in all organs and tissues collected. Mice were given Dextran sodium sulfate (DSS) to incite inflammation in the intestines, N-acetylcysteine (NAc) a mucolytic agent, and antibiotics to deplete the microbiota. Following these disruptive measures, it was possible to establish an advantage over the adaptive immune system and establish fungal presence in organs unassociated with the route of administration. Live and heat-killed *C. albicans* were detected in the blood, feces, brain, lungs, liver, spleen, heart, and stomach throughout all six approaches.
Figure 7: Future directions for researching the components for creating and manipulating a tailored murine model of candidiasis. Future experiments proposed include: (A) a control group in which all mice receive no treatments and no Candida, (B) a second set of healthy mice that receive live and heat-killed Candida, and (C) a third set pretreated with various combinations of N-acetylcysteine (a mucolytic agent), Dextran sodium sulfate and an antibiotic cocktail that subsequently receive live and heat-killed Candida as described by Andrew Koh. Also proposed would be the inclusion of a neutropenic agent, a known requirement in humans for dissemination to occur.
CHAPTER 4: DISCUSSION

With growing concern regarding the morbidity and mortality of candida infection in immunocompromised patients, there is a great need to understand the mechanisms of invasion and dissemination. Current models that utilized pretreatment with chemotherapeutic agents followed by IV injection of *C. albicans* or *C. albicans* inocula models were deemed inadequate for describing GI colonization, translocation and eventual dissemination of *C. albicans* by Andrew Koh of the University of Texas Southwest Medical Center.\(^{17}\) Koh postulated that there were three necessary components for potentiating bacterial or fungal invasion and infection including: 1) GI microbiome dysbiosis supporting pathogenic overgrowth; 2) disruption of the intestinal mucosal barrier to increase permeability; and 3) immunosuppression.\(^{17}\) In his 2008 study, Koh employed a multidimensional murine model that used 16 different treatment combinations including chemotherapeutic medications and agents producing neutrophil depletion, macrophage depletion, and GI mucosal disruption as well as a subset of mice with dysfunctional lymphocytes to investigate the mechanisms responsible for invasion and dissemination of *C. albicans*.\(^{17}\) Unlike previous studies, Koh used oral exposure to candida rather than intravenous inoculation in his pretreatment regimen along with orally administered antibiotics to produce GI candida colonization and dysbiosis. His model focused on mortality combined with detection of *C. albicans* in the liver as indicative of “significant” dissemination under these various conditions in order to deduce which aspect(s) of the innate immune system was/were important. Only six of his treatment groups produced dissemination and death including all four groups exposed to two chemotherapeutic agents, cyclophosphamide and methotrexate, either alone or in combination, or methotrexate with 200 µg of the anti-neutrophil mAb RB6-8C5. The remaining two groups had received dextran sodium sulfate (DSS) for 7 days, which causes GI mucosal disruption, in
combination with mAb RB6-8C5. Regimens that produced neutropenia alone, macrophage
depletion alone or with either neutropenia or mucosal disruption, or which utilized mice with
lymphocyte dysfunction did not result in dissemination. From these results, Koh concluded that
neutropenia and GI mucosal damage are imperative for dissemination of *C. albicans* disease.\(^{17}\)
Other research, conducted by Kumamota\(^{31}\) and Jawhara, et. al.\(^{33}\) confirmed the necessity of
including DSS in a murine model to mimic human ulcerative colitis conditions and underlined its
requirement within the protocol for establishing *C. albicans* colonization of the GI tract. They
continued by stating that *C. albicans* on its own is insufficient in inciting enough gastrointestinal
inflammation for colonization to occur.\(^{33,35}\)

The clinical definition of invasive candidiasis in humans includes both candidemia (blood
infection) and deep-seated tissue candidiasis including blood-culture-negative forms. Deep tissue
forms include chronic disseminated (hepatosplenic) candidiasis, as well as in other tissue sites such
as the bones, muscles, joints, eyes, or central nervous system.\(^{16}\) Deep-seated infection is thought
to develop in these distal sites from a preceding or undiagnosed candidemia.\(^{16}\) Koh’s model
appears to have relied on a narrow definition of disseminated infection; i.e., *C. albicans* detected
in the liver plus mortality. In his single control and 16 exposure groups, however, only survival
data was provided. Data on the level of or hepatic involvement was not included.

Interestingly, in the same paper, Koh reported unpublished data from a preliminary study
of four exposure groups of mice including: 1) controls with no immunosuppression; 2) neutropenia
only; 3) neutropenia plus methotrexate; and 4) cyclophosphamide alone. In this analysis samples
of blood, mesenteric lymph nodes (MLN), kidney, liver and splenic were assayed. The latter two
groups experienced 100% morality with high levels of *C. albicans* detected in 100% of the liver
specimens (Grp 3: median 8.77 × 10³ CFU/g; Grp 4: not reported); the kidneys were positive in
only 50-80%. Data for blood, MLN and splenic samples were not reported. Alternately, among the controls he reported observing non-fatal sporadic (4 of 20 mice) and low level hepatic dissemination (CFU not reported) in mice colonized with C. albicans. Finally, mice colonized with C. albicans and receiving only RB6-8C5 mAb also showed sporadic (5 of 20 mice) levels of hepatic dissemination, but at higher levels (median 9.88 ×10^2 CFU/g) than in mice that did not receive immunosuppression. Blood, MLN, and splenic samples were negative in these two groups, however, no data for kidney samples were included.

Given the spectrum of pathology of disseminated infection in humans, we felt the sampling frame used by Koh was probably too narrow and the inclusion of the mortality criterion for the definition of significant infection potentially too limiting. Specifically, the former might miss instances of deep tissue dissemination where the liver or kidneys are not involved while the latter might exclude either an infection that is perhaps in an early phase or, as will be proposed, a possible mechanism of invasion and dissemination that might be more readily evident in a non-lethal scenario. In this context, our protocol in trials 4, 5, and 6 included sampling of several tissues, i.e., stomach, kidneys, spleen, liver, lung, brain, heart, PP, feces, and blood (trial 5 only). Furthermore, Koh included exposure with antibiotic pretreatment alone or in combination with either a single chemotherapeutic agent, RB6-8C5 mAb, or DSS. Thus, all groups were subjected to GI dysbiosis prior to oral candida exposure. The latter group received only a 3-day exposure to 5.0% DSS, which did not produce mucosal disruption. In contrast, our study included more groups exposed to a single agent without antibiotic pretreatment including a 5-day exposure to two different concentrations of DSS (2.5% and 5.0%).

Our findings demonstrated that DSS in combination with antibiotic pretreatment and either N-acetylcysteine (mucolytic agent) or mAb-Rb68c5 (anti-neutrophil antibody) were our most
useful tools for establishing *C. albicans* colonization and invasive infection. Additionally, while the proportions were lower, we were able to document deep tissue infection in mice exposed to antibiotics or N-acetylcysteine alone.

Our research was able to document isolation of *C. albicans* from multiple organs including the heart, brain, lungs, spleen and liver. Twenty-four (60.0%) of the 40 mice with disseminated infection had *C. albicans* detected in the kidneys. A similar proportion of infections were identified in the spleen. Heart samples were obtained from 46 mice including 27 with disseminated infection; 8 (29.6%) were positive. Brain samples were obtained from 37 mice including 25 with disseminated infection; 10 (40%) were positive. Lung and liver samples were obtained from 29 mice including 17 with disseminated infection; 6 (35.3%) lung samples and only 2 (11.8%) liver samples were positive. Of the 27 negative liver samples, disseminated infections were detected in 14 (51.9%) of mice via brain (8), lung (5), kidney (4), blood (3), heart (2), and spleen (2) samples. In addition, of the 23 with negative kidney samples, disseminated infections were detected in 10 (43.5%) of mice via brain (6), spleen (3), blood (2) heart (1), lung (1), and kidney (1) samples.

Only 4 (10%) of the 40 mice with disseminated infection died. In addition, this study also evaluated Koh’s observation that loss of 20% or more of body weight could be used as a marker of disseminated infection. Our findings identified only five (7.9%) of 63 mice exposed to *C. albicans* that experienced weight loss of or near 20% from baseline including two mice with borderline weight loss of 18.3% and 19.7%, respectively. Four of the 5 with organ tissues tested had evidence of disseminated *C. albicans*. 33 of the remaining 56 mice (58.9%) without threshold level weight loss had evidence of disseminated *C. albicans*. 10 (62.2%) of 16 mice without threshold weight loss and who received DSS, antibiotics, and NAc had evidence of
disseminated *C. albicans*. Thus, death and weight loss of 20% or more were not reliable as predictors of disseminated *C. albicans* infection as evident by isolation of *C. albicans* in tissue. These results support our contention that mortality or 20% weight loss combined with hepatic detection of *C. albicans* is adequate for documentation of disseminated infection.

Based on the preliminary study findings summarized above, Koh hypothesized that *C. albicans* may be able to translocate to the liver via the portal circulation or via the biliary tree in the absence of immunosuppression but does not cause widespread disease because of a competent immune system that is able to prevent significant dissemination of the fungi. Again, our findings suggest that non-lethal infection may occur in other organs even in the absence of liver or renal involvement. However, Koh’s hypothesis does raise the very interesting possibility that translocation via the portal circulation may be an important possible alternate mechanism that contributes to seeding of the deep tissues, which, in turn, may serve as an important precursor to overt morbidity and mortality once immunosuppression is added into the mix.

The continued study of gut microorganisms related to the larger gut microbiome is a rapidly expanding field as we continue to learn about the significance in regulation of normal human protein biology and metabolism, as well as the role that these microorganisms play in a variety of pathogenic conditions including obesity, hypertension, inflammatory bowel disease, asthma, and autism. In terms of the pharmacokinetics and therapies being tailored specifically to each patient, the role of microorganisms plays a significant role in influencing drug metabolism, dietary caloric bioavailability, immune system conditioning and response, and post-surgical recovery. Scientists acknowledge, “analysis of the gut microbiome and its activities is essential for the generation of future personalized healthcare strategies and that the gut microbiome represents a fertile ground
for the development of the next generation of therapeutic drug targets. It also implies that the gut microbiome may be directly modulated for the benefit of the host organism”.

Continued study of intestinal *C. albicans* is very relevant to caring especially for immunocompromised patients due to the high risk for systemic infection and increased rates of strains that have become resistant to our current antifungal medications. Intestinal *C. albicans* colonization has been associated with several gastrointestinal diseases, such as stomach and duodenal ulcers, as well as irritable bowel syndrome. There is also delayed healing of inflammatory lesions due to colonization and inflammation being tightly intertwined processes; however, it is currently poorly understood which process initiates the cycle.

Thus, it is important to study intestinal candida because of its significance as an opportunistic pathogen and its emerging role in intestinal immune function. Also, the mechanisms of invasion and dissemination are still poorly understood including whether the local immune surveillance structures (i.e., PPs) are playing a major role in translocation or whether another underlying mechanism (e.g., portal circulation) is involved.

Among the original goals of this work was to determine the point of recognition and uptake of *C. albicans* by the PP and assess its possible role in the pathogenesis of disseminated infection. Koh described the presumed mechanism for dissemination as beginning with Candida exposure at the gastrointestinal surface where, in cancer patients, the organism translocates into extraintestinal organs via mesenteric lymph nodes, migrates to the blood stream, and is then transported via the blood stream to the liver and spleen. Our question included not only the mechanism by which *C. albicans* is taken up by the M-cells at the PP, but also its mode of travel throughout the body into the organs. We hypothesized that *C. albicans* follows a similar path of *Salmonella typhimurium* and Poliovirus as the infection translocates the M-cells, passes through the PP and makes its way
to the blood and nerves to disseminate throughout the host (Figure 6). One possible explanation for not capturing histopathologic images of *C. albicans* in the tissues may relate to the timing of imaging relative to intestinal exposure to *C. albicans*. Specifically, we may have missed *C. albicans*’ passage through the patch during imaging, which occurred 8 days following cessation of exposure. To assess this possibility, future studies could be designed to obtain samples sequentially both during and immediately following cessation of Candida administration (Figure 7). Alternately, our inability to pinpoint *C. albicans* uptake at the patch, may point to the possibility of a mechanism other than the PP M cells and Langerin DCs for uptake and dissemination, perhaps via mesenteric lymph nodes as suggested by Koh or another pathway that we have not yet identified.

We were able to demonstrate that DNA fragments of heat-killed *C. albicans* administered orally in 3 mice treated with DSS combined with antibiotics and/or N-acetylcysteine could be detected in samples of the Peyer’s patch (PP) and deep tissues. All three mice had at least two of the deep tissue samples positive (range 2 – 4) and one with 4 positive deep tissue samples also had DNA detected in its PP sample. This small sample needs to be confirmed, but the findings suggest that the non-viable organism may be uptaken and disseminated to deep tissues possibly either through the blood or lymphatic system. Further studies are needed to illuminate the exact mechanism of uptake and distribution of heat-killed organism and its effects on the tissues to innumerate the differences from true disseminated infection.

Our results differed from those documented by Koh due to a few variations in methodology and terminology. We expanded upon Koh’s research by performing wider sampling, isolating single agents, and by utilizing an entire trial to explore the outcome of introducing live versus Heat-killed *C. albicans* to observe for dissemination.
Our laboratory followed the definition of invasive candidiasis described by Campion EW, Kullberg BJ, and Arendrup MC in the New England Journal of Medicine as, “Invasive candidiasis, which comprises both candidemia (blood infection) and deep-seated tissue candidiasis…” “blood-culture-negative forms of invasive candidiasis, which includes chronic disseminated (hepatosplenic) candidiasis...”16 “Deep-seated infection may also occur in other tissue sites including the bones, muscles, joints, eyes, or central nervous system.16 Deep-seated infection is thought to develop in these distal sites from a preceding or undiagnosed candidemia.16 This definition of invasive candidiasis requires the fungal organism to be extraintestinal, since that was the route of systemic introduction to the mouse. Koh is combining mortality plus presence of *C. albicans* in the liver as his definition of dissemination and appears to be equating liver presence but survivorship as colonization but not dissemination. He is inconsistent as to his definition. In his preliminary study, he hypothesized, “that *C. albicans* is able to translocate to the liver via the portal circulation or via the biliary tree in the absence of immunosuppression but does not cause widespread disease because of a competent immune system that is able to prevent significant dissemination of the fungi”.17

Subsequently, we decided to isolate each specific variable to document the impact of a single agent on the ability of *C. albicans* to disseminate, invade tissue, and incite an immune response. We sent our specimens to pathology for staining and analysis to illuminate tissue damage and neutrophil presence at the sites of candida invasion aside from just the intestines.

Additionally, in Koh’s group 7 mice that were treated with three injections of mAb, he noted that 4 mice died of Enterobacter infections which brings up the question whether any subsequent results of mortality could be attributed to undetected bacterial infections and not solely dissemination of *C. albicans*. Enterobacter are commensal in the gut microbiome and an
incomplete antibiotic sterilization of the murine gut due to possible resistance may have attributed to it acting as an opportunistic infection and overwhelming the mouse’s immune ability to fight the fungal infection. Koh did not discuss any possible sources of bacterial contamination.

In Koh’s murine model, he defined dissemination as liver presence followed by mortality. Koh commented on the presence of *C. albicans* in the liver establishing not necessarily due to disruption of the mucosa at the PP, but perhaps as a result of translocation via the portal circulation or the biliary tree. The role of the portal circulation providing a conduit for *C. albicans* is perhaps more significant than previously thought. Perhaps *C. albicans* will establish in the liver in an immunocompetent patient “but does not cause widespread disease because of a competent immune system that is able to prevent significant dissemination of the fungi”; however, if the patient becomes immunocompromised (neutropenia or anti-cancer meds) then the immune system may lose competency to control the infection allowing it to disseminate and invade additional tissue sites. Our research supported that any extraintestinal *C. albicans* presence was indicative of dissemination and that isolating single agents was imperative to determining if the treatment was more significant to the mortality than the disseminated infection.

*Limitations and Recommendations for Future Research*

This series of experiments would have benefited by inclusion of unexposed and healthy controls within each trial as well as groups of mice exposed to each of the pre-treatments: DSS alone, antibiotics alone, NAc alone, DT ablation of Langerin DCs alone, along with the various combinations of these treatments as well as weighing organs immediately after tissue collection to be able to determine fungal load per gram of tissue (Figure 7). Additionally, I would have flushed vasculature with buffer until the buffer ran clear to eliminate the possibility of blood contamination.
causing our results to reflect CFU and PCR-positive specimens that had been negative for histopathology. This would have allowed more specific assessment of the relative individual contributions of and relationships among the gut microbiome, mucous layer, intact mucosa, and mucosal immunity in protecting against invasive *C. albicans* infections.

Secondly, as histopathologic evaluation was performed on a limited sample of mice, it could not be definitively ascertained whether organ positivity in brain, heart, spleen, kidney, and liver was due to true tissue infection or candidemia (blood contamination). Therefore, in future experiments there should be a uniform outcome evaluation protocol for all trials that includes: blood and tissue samples for CFU, PCR, and MALDI-TOF analysis along with tissue histology to rule out blood contamination as the source of tissue sample positivity by CFU, PCR, and MALDI-TOF. However, the fact that we had mice with positive tissue samples and negative blood specimens in Approach 3 suggests we had examples of true tissue involvement.

Thirdly, it is possible that there was a slight undercount of the number of mice with disseminated disease due to lack of sampling of brain, heart, lung, liver and blood in all treatment groups. Specifically, in Approach 2, brain and lung specimens were not sampled and heart specimens were obtained on only one mouse from each of the treatment groups in that trial. In that trial, there were 7 mice with negative spleen and kidneys (including one with a negative heart specimen). In subsequent trials, there were 6 cases of disseminated *C. albicans* identified where the spleen and kidney samples were negative. These included 4 brain positive only; 1 brain and lung positive; and 1 heart and lung positive. Furthermore, in Trail 5, two disseminated infections were identified solely by positive blood samples. However, given the high proportion of disseminated infection identified in Trial 2 (65.2%), which was similar to Trail 5 (63.6%) plus the
fact that most cases with positive blood samples also had multiple positive organ samples, it is likely that very few infections were misclassified as negative.

While there were significant findings throughout our various trials and the pretreatments were modified in accordance with our results, we were unsuccessful at evaluating the immunological role of the Peyer’s patch and therefore were unable to determine the mechanism nor pathway taken by C. albicans from the GI tract to the blood. With CFU and PCR-positive blood samples, it is suggested that deep-tissue infection arises from hematogenous spread of the fungus, but how it arrives in the vasculature has evaded our research. One hypothesis arises from the recent research conducted by the collaboration of various scientists and clinicians in the Department of Medicine, Division of Digestive Diseases, Mount Sinai Beth Israel Medical Center, Icahn School of Medicine at Mount Sinai, identified a previously unrecognized submucosal, fluid-filled interstitial space which is a primary source of lymph and serves as a major compartment of body fluid. This structure, which collectively the authors referred to as the “human interstitium,” is a reticular-patterned part of the submucosa surrounding the entire gastrointestinal tract (including the stomach) and urinary bladder, bile duct, muscles, dermis, peri-bronchial and peri-arterial soft tissues and fascia. This structure is comprised of fibroblast-like cells lacking cell-type specific structures and basement membranes, and scientists hypothesize the absence of the basement membrane allows these cells to adhere directly to the collagen bundles underneath. The authors hypothesized that the interstitium may be important in the mechanical functioning of tissues and organs as well as the pathophysiology of edema, fibrosis, and the metastasis of cancer cells from the epithelial tissue to the lymph and other tissue sites. One clinical example of its function was the isolation of cancer cells in metastatic melanoma with the primary site located in the upper arm being isolated within the collagen bundles of the interstitium and metastasizing to
the axillary lymph node with no other lesions or routes having been identified to explain its pathway. Most relevant to our experiment was the authors’ histopathology of a gastric carcinoma present at the mucosal surface that was shown to be invading the submucosa. While they did not identify deeper invasion of the muscular wall or lymphovascular invasion, poorly differentiated tumor cells were clearly shown singly and in clusters infiltrating the interstitial space within the gastric submucosa. We were able to observe local tissue invasion of *C. albicans* in our histopathologic analysis of the mucosa and submucosa of the gastric tissue at the non-glandular/glandular junction. This location mirrors the histopathologic findings of the infiltration of cancer cells into the interstitium from the gastric tissue. I hypothesize that there may also be a role for this structure in the translocation of *C. albicans* into the lymph, how an immune response commences with the macrophage recognition there, and how the organism ultimately disseminates.

Their methods which employed a rapid freezing technique without fixation often destroys the architecture on biopsy. Their research findings suggests that the interstitial space plays a role in inflammatory conditions such as primary sclerosing cholangitis, chronic pancreatitis, inflammatory bowel disease, and scleroderma. Additionally, it may be relevant to the recognition, uptake and immunogenicity surrounding candidiasis or candidemia since macrophages have been shown to traffic into the space when pigment uptake studies were conducted. Considering our experiments were unable to localize *C. albicans* uptake, recognition and regulation of an immune response to the Peyer’s patch, I recommend incorporating the tissue collection protocol employed by the Mt. Sinai researchers to see if following oral introduction of *C. albicans*, we would be able to visualize the organism within these structures.
Finally, to continue the natural progression of this research incorporating investigation of the role of mucosal-associated lymphoid tissue (MALT) including the PP in the process of invasion and dissemination, I recommend constructing a multi-faceted approach that incorporates the expanded pretreatment exposures and evaluation suggested above, the sequential sampling of PPs at times during and after intestinal exposure to *C. albicans*, and sampling of additional MALT tissue including mesenteric lymph nodes to determine the route and the time point at which the infection changes from candidemia to deep-seated candidiasis. The future experiments purposed would serve to isolate which components of the mucosal immune system are necessary for preserving homeostasis and inhibiting dissemination. This will address what are the vital components of the mucosal immune system that, when interrupted, provide an opportunity for *Candida albicans* to invade the tissue and disseminate to various organs. Future experiments proposed include: (A) a control group in which all mice receive no treatments and no Candida, (B) a second set of healthy mice that receive live and heat-killed Candida, and (C) a third set pretreated with various combinations of N-acetylcysteine (a mucolytic agent), Dextran sodium sulfate and an antibiotic cocktail that subsequently receive live and heat-killed Candida as described by Andrew Koh. Also proposed would be the inclusion of a neutropenic agent, a known requirement in humans for dissemination to occur.

I learned the incredible complexity of the myriad factors stemming from the host innate immune system, the fungal pathogen, and the host microbiome that contribute to the recognition, uptake, dissemination and tissue invasion of a typically commensal organism. It was fascinating to hypothesize the various pathways which could be taken by *C. albicans* based upon other known disease pathologies and mechanisms for migration within the host to provide insight into factors that contribute to disease prognosis, treatment, and predicting factors.
REFERENCES CITED


doi:10.1016/j.celrep.2015.03.067


doi:10.4049/jimmunol.166.8.4884

doi:10.1084/jem.191.8.1381


33. Kumamoto CA. Inflammation and gastrointestinal Candida colonization. *Curr Opin*


