Murine Models of *Candida* Gastrointestinal Colonization and Dissemination

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Ninety-five percent of infectious agents enter through exposed mucosal surfaces, such as the respiratory and gastrointestinal (GI) tracts. The human GI tract is colonized with trillions of commensal microbes, including numerous *Candida* spp. Some commensal microbes in the GI tract can cause serious human infections under specific circumstances, typically involving changes in the gut environment and/or host immune conditions. Therefore, utilizing animal models of fungal GI colonization and dissemination can lead to significant insights into the complex pathophysiology of transformation from a commensal organism to a pathogen and host-pathogen interactions. This paper will review the methodologic approaches used for modeling GI colonization versus dissemination, the insights learned from these models, and finally, possible future directions using these animal modeling systems.

Infectious diseases are the third leading cause of death in the United States and the leading cause worldwide. Ninety-five percent of all infectious agents enter through mucosal surfaces, most notably the linings of the respiratory, gastrointestinal (GI), and genitourinary tracts (1). The human body is colonized by microbial organisms, with the vast majority colonizing the GI tract. Commensal microbes outnumber host cells by a factor of 10 to 1 and aid in many essential metabolic and immune host functions (2). Not all host-microbiota interactions, however, promote health, and there are some symbiotic microorganisms in the GI tract that can cause human disease under specific gut environmental and/or host immune conditions. The term “pathobiont” has been coined to describe resident microbes with pathogenic potential (3). In contrast to traditional pathogens that may cause disease in a normal healthy host, pathobionts are harmless to the host under normal conditions. *Candida* spp., including *Candida albicans*, are considered to be pathobionts.

One of the “holy grails” of microbial pathogenesis research is trying to understand how and why a harmless commensal can transform into an invasive pathogen. For pathobionts residing in the GI tract, the concern is that these microbes will translocate to extraintestinal organs, notably, the liver and spleen, and ultimately in the bloodstream and thus throughout the host. In cancer patients, *C. albicans* usually colonizes the GI tract with subsequent translocation into extraintestinal organs in the setting of chemotherapy-induced neutropenia and GI mucosal damage (4). In neutropenic patients, the role of the gut as a source for disseminated candidiasis had been postulated based on older autopsy studies (5), and a recent study showed that *C. albicans* blood isolates recovered from patients with candidiasis were often similar in identity (confirmed by molecular methods) to rectal isolates (6). Interestingly, in this same study, *Candida parapsilosis* bloodstream isolates did not correspond to rectal isolates (6), confirming earlier data suggesting that *C. parapsilosis* infections have a different origin and do not typically originate from gut microbiota (5). In animal models, the three primary mechanisms that promote microbial GI translocation are as follows: (i) disruption of the normal GI microbiome equilibrium, allowing intestinal dominance of pathogens; (ii) increased permeability of the intestinal mucosal barrier, and (iii) deficiencies in host immune defenses (notably cellular immune defenses) (7, 8). Not surprisingly, common risk factors for developing candidemia in human patients include neutropenia, mucositis, use of broad-spectrum antibiotics, and invasive medical procedures (9, 10). Therefore, since colonization is a precursor to invasive disease, understanding how host and microbial factors govern fungal GI colonization is essential for our understanding of the pathogenesis of fungal disease. Naturally, many investigators have devised animal models of *Candida* GI colonization and dissemination, with the vast majority of models utilizing mice. The remainder of this minireview will focus on the methodologic approaches used for murine models of *Candida* species GI colonization versus dissemination, the insights learned from these models, and finally, possible future directions using these murine models.

**Fungal Diversity in the Mammalian GI Tract**

Before delving into the specifics of murine models, it is important to review what is known about the fungal diversity in the human GI tract. Although the human GI tract is dominated by bacteria, microbes from other domains, notably *Archaea* and *Eukarya*, are present in the human intestine (11). While high-throughput sequencing technology has provided great insight into the characterization of the vast bacterial communities in the human GI tract, the eukaryotic component of the human gut remains relatively unexplored. In one study using a multiligo pyrosequencing approach (using the pan-fungal internal transcribed spacer [ITS] region as a target) to characterize fungi present in the oral cavities of 20 healthy individuals, 74 culturable and 11 nonculturability fungal genera were noted. *Candida* species were the most frequent (isolated from 75% of participants), followed by *Cladosporium* (65%), *Aureobasidium* (50%), *Saccharomycetales* (50%), *Aspergillos* (35%), *Fusarium* (30%), and *Cryptococcus* (20%) (12). Using a similar pyrosequencing approach, commensal fungi have been
different antibiotics to adult mice result in different colonization levels, and germfree mice (29) can be sustainably colonized with a notable absence of Trichosporon genera (notably Aspergillus, Mucor, Cryptococcus, Rhodotorula, and Trichosporon) are occasionally detected in the gut, but since these fungi are most often residents of the respiratory tract or skin, they are thought to be only transient in the gut.

**MURINE MODELS OF CANDIDA GI COLONIZATION**

*Candida albicans* does not have an environmental reservoir (17) and is almost always associated with humans or other mammals. When modeling *Candida* GI colonization and dissemination in mice, one must always be cognizant that *C. albicans* and some other human-pathogenic *Candida* spp. are not natural commensals or even pathogens in mice and in many other animals (18). The immune system (19–21) and resident gut microbiota (13) differences between mice and humans have been well documented. Thus, investigators should take great caution in extrapolating pathogenesis and mechanistic insight from animal models to human patients. If anything, the differences between mice and humans (e.g., why are adult mice *C. albicans* colonization resistant?) should be used to help us understand the basic mechanisms of GI commensalism and pathogenesis, with hopes that mechanistic insight can help us ultimately find novel methods for preventing and/or treating invasive fungal infections in humans.

**Microbial competition in the GI tract.** There is growing evidence that disturbances in a healthy microbiome community can result in severe imbalances in immune regulatory systems resulting in pathological states, most notably inflammatory bowel disease (IBD). Thus, it is very important to look simultaneously at the bacterial and fungal communities to determine whether a shift in the normal (bacteria predominant) microbiota is correlated with a rise in opportunistic fungi. This concept is most readily elucidated when examining murine GI fungal commensal populations. As with humans and other mammals, mice appear to be predominantly colonized with *Ascomycota*, particularly *Candida* spp., but with a notable absence of *C. albicans* and often a predominance of *Candida tropicalis* (13). In fact, *C. albicans* is not a natural commensal or pathogen of mice (18), and adult mice are naturally resistant to *C. albicans* GI colonization (5, 22). Yet, the GI tracts of neonatal mice (23, 24), antibiotic-treated adult mice (22, 25–28), and germfree mice (29) can be sustainably colonized with *C. albicans*, suggesting that the composition of the bacterial gut microbiome is an important determinant of *Candida albicans* colonization.

**Antibiotic-treated adult murine models.** Many murine models of GI-derived *C. albicans* fungemia have been reported previously (22, 27, 28, 30–34). The levels of GI colonization and duration of sustained colonization are widely variable. In general, however, pretreatment with oral antibiotics prior to *Candida* inoculation leads to substantially higher GI colonization levels and longer sustained colonization. The choice of which oral antibiotics is critical when using these murine models. Administration of different antibiotics to adult mice result in different *C. albicans* colonization phenotypes: notably, administration of penicillin results in markedly higher *Candida* GI colonization rates than administering regimens without penicillin (22). Penicillin has been shown to dramatically reduce endogenous murine anaerobic GI bacteria resulting in overgrowth of Gram-negative bacteria (7, 35); therefore, anaerobic bacteria may also be essential for maintaining *C. albicans* colonization resistance in adult mice. The need for depletion of other microbiota, namely, Gram-negative bacteria, is critical given that bacteria are just as likely as *C. albicans* to translocate and disseminate when an immunosuppressive drug is administered (36). *Candida* is administered via the drinking water or via oral gavage, with comparable GI colonization levels. Maintenance of antibacterial antibiotics in the drinking water is necessary for both sustained GI colonization and dissemination studies. Dissemination is typically achieved via administration of immunosuppressive agents (i.e., cyclophosphamide, methotrexate) (22). As opposed to models of intravenous *Candida* inoculation where the kidney is used as confirmation of disseminated disease, the liver is a much more reliable organ to confirm that systemic infection is achieved in GI colonization and dissemination models (22, 34). *Candida* may translocate to the liver via the portal circulation or via the biliary tree in these models.

**Neonatal models.** *Candida* spp. are the second leading cause of deaths related to infectious diseases in extremely premature infants (37). There are several reasons why the neonatal gut may be more prone to colonization by *Candida* spp. and dissemination than the adult gut. Newborn human and mice infants have a “leaky” gut epithelium, making them more prone to microbial gut translocation (38). In terms of gut microbiota populations, the bacterial phyla *Firmicutes* and *Bacteroidetes* account for >98% of bacteria in the distal guts of adult mice and humans (39) whereas the neonatal gut microbiota is dominated by *Proteobacteria* (Gram-negative enteric bacteria such as *Escherichia* and *Shigella*) with very little *Bacteroidetes* and *Firmicutes* (40). Interestingly, a number of human diseases, including inflammatory bowel disease and necrotizing enterocolitis, have been shown to be associated with gut *Proteobacteria* dominance (41). Numerous animal studies have shown that the normal anaerobic commensal GI bacteria provide an important defense mechanism against infections by inhibiting growth of potentially pathogenic organisms, a concept known as colonization resistance (42–44). Gut commensal microbes stimulate the GI epithelium to produce antimicrobial proteins (AMPs) that act as a first line of defense against invading bacteria (45) and fungi. When commensals are depleted after antibiotic administration, select pathogenic microbes can overgrow and cause invasive disease (46). Commensal anaerobes also directly inhibit translocation of microbes, including Gram-negative bacteria and *C. albicans* (47), from the GI tract (35)—but the exact mechanisms are not known. In the neonatal mammalian host, expression of these gut immune effectors (i.e., AMP, cytokines) is notably different than in the adult. For instance, in mice, small intestinal crypts, a source of many AMPs, develop 10 to 12 days after birth, and this is accompanied by increased epithelial cell renewal (48). Of note, changes in the composition of antimicrobial peptides and in antimicrobial activity have also been noted in the intestinal lumen of human neonates (49).

All these differences from the adult gut make the neonatal murine gut much more amenable to *Candida* GI colonization and dissemination. Neonatal models have been established for over 30 years. Most neonatal models (23, 50) utilize infant mice (~5 to 7 days of age) and administer the *Candida* via oral gavage (10⁷ to 10⁸ CFUs).
CFU). Antibiotics are not needed to establish GI colonization. GI colonization has been reported to persist up to 20 days after the initial gavage (10² to 10⁵ CFU/g intestinal tissue), with levels dropping precipitously (by 3 to 4 log units) within the first 24 h. These results are not surprising, as the infant mouse matures and develops a gut microbiota more similar to adults, one would expect that the Candida colonization phenotype would closely resemble the adult phenotype. We have actually documented that C. albicans levels not only decrease as the infant mouse ages but become undetectable in the GI tract once the mouse reaches adolescence (4 to 6 weeks of age) (unpublished observation). Translocation to extraintestinal organs, such as the livers, kidneys, and spleens, occurs almost uniformly within the first 6 to 72 h of infection but appears to be transient. As for virulence, mortality using this model appears to be dependent on the C. albicans strain used, with mortality rates ranging from 17 to 47% (23). A more recent neonatal model utilized younger infant mice (2 days old) and intraperitoneal injectional administration of C. albicans (51). Antibiotics and immunosuppressive agents are not used. Dissemination to the kidneys, lung, and brain are consistently uniform, and mortality occurred in a dose-dependent manner at doses higher than 10⁶ CFU/g infant mouse. The clinical relevance of this model is somewhat less clear, although the authors state that direct inoculation of the peritoneum via perforated bowel is another mechanism of Candida GI translocation.

Germfree and immunocompromised host models. Admittedly, the previously described “conventional” models of Candida GI colonization and dissemination are challenging to work with given the requirement of broad-spectrum antibiotics and administration of immunosuppressive agents. Thus, an alternative approach is to use germfree (GF) or gnotobiotic mice that do not require antibiotics and, in some instances, immunosuppression. GF mice have no Candida-inhibitory microbiota. Furthermore, GF mice with defined, innate, and/or acquired immune system deficits are available (i.e., mice deficient in NK, T-cell function, pattern recognition receptor knockout, etc.), and these models have been shown to be susceptible to mucosal and systemic candidiasis of endogenous origins (52–57). The major limitation of these models is the requirement of highly specialized germfree animal facilities which are not available to all investigators. Therefore, further details on utilizing GF models will not be discussed in this minireview.

INSIGHTS GAINED FROM CANDIDA GI COLONIZATION AND DISSEMINATION MODELS

Gut microbiota. With the advent of next-generation microbial pyrosequencing techniques, it has become much easier to study the complex bacterial-fungal interactions that transpire within the mammalian gut. Recently, Lactobacillus has been shown to displace Candida from GI epithelium (58), prevent germ tube formation (59, 60), and inhibit hyphal invasion (58, 61). Lactobacillus acidophilus given to animals with Candida-induced gastric ulcers resulted in not only a decrease in ulcer size but a 60% reduction in Candida GI colonization (62). A double-blind placebo-controlled study with 80 premature babies found a significant reduction in Candida GI colonization following oral administration of Lactobacillus rhamnosus for 12 months (63), and an analogous study in elderly adults showed a similar decrease in Candida GI colonization (64). While these results are intriguing, unfortunately the mechanisms by which bacteria diminish or prevent fungal GI colonization are poorly understood. Conversely, it has been shown that fungi can also affect bacterial GI colonization: the oral introduction of C. albicans can prevent Lactobacillus species repopulation and actually promote Enterococcus faecalis population growth in both the stomach (65) and cecum (66). This has immense clinical ramifications, as intestinal bacterial dominance with Enterococcus spp. can dramatically increase the incidence of bacteremia (5-fold) in bone marrow transplant patients (67). Thus, fungal GI colonization may not only predispose to fungal infections but may also make bacterial infections more likely. Follow-up studies need to be completed to elucidate these complex bacterial-fungal interactions.

Host immune factors. (i) Innate cellular immunity. Lymphocyte deficiency, in particular CD4⁺ Th1 deficiency, results in oral and esophageal candidiasis (68, 69)—best illustrated by the fact that children with athymic dysplasia (70) and patients with HIV and AIDS (71, 72) are more susceptible to oral candidiasis. In contrast, lymphocytes do not appear to be important for modulating distal GI Candida colonization levels, as noted by older studies using athymic mice (73) and recent studies using recombinase-activating gene-deficient mice (22). Neutrophils have been shown to be critical for controlling disseminated fungal disease (22, 74–76), their role in controlling GI colonization levels appears to be negligible (22). Similarly, macrophages do not appear to affect Candida GI colonization (22), and to date, there have been no studies that have examined the role of NK cells alone on GI colonization. A combination of defective T-cell-mediated immunity and phagocytic cell defects (in neutrophils, macrophages, and NK cells), however, predisposed mice (bg/bg nu/nu mice) to severe mucosal and systemic candidiasis of endogenous origin without prior antibiotic therapy or additional immunosuppression (53).

In terms of disseminated disease, isolated depletion of neutrophils, macrophages, or mature lymphocytes does not lead to C. albicans translocation from the murine gut (22). In fact, only a combination of intestinal mucosal damage and neutropenia was sufficient for C. albicans dissemination from the murine gut (22).

(ii) Pattern recognition receptors. Mammalian innate immune systems recognize fungi via pattern recognition receptors (PRRs). The Toll-like receptor (TLR) family is the best studied of all PRRs, with TLR2 and TLR4 being implicated in fungal recognition (77). Members of the C-type lectin receptor (CLR) family, and dectin-1, however, appear to play the primary role in host defense against a number of pathogenic fungi, including C. albicans, Aspergillus fumigatus, and Pneumocystis jirovecii (78–80). In murine models, dectin-1 is necessary for controlling Candida infections in both the oral and vaginal mucosa (81, 82). Similarly, humans that are dectin-1 deficient have an increased incidence of chronic mucocutaneous candidiasis, which affects the skin, nails, and oral and vaginal mucosa (83). The role of dectin-1 in fungal GI colonization is somewhat unclear. Two studies have implicated the importance of dectin-1 in controlling C. albicans GI colonization (81, 84). Yet, in another recent study, there was no significant difference in Candida GI colonization between wild-type and dectin-1-deficient mice (85), but this was observed only when the wild-type and dectin-1-deficient mice were housed together in the same cage. The disparate results reported from these studies may be due to variations in the microbiota of the mice: there was no cohousing in the first two experiments and different sources for these animals. It has been well established from the bacterial mi-
crobiome literature that the same inbred strain of mice from different locations, separate housing (86), and different vendors (87, 88) can result in specific but notable differences in gut microbiota populations.

Finally, mice lacking dectin-1 (Clec7a<sup>-/-</sup> mice) exhibited increased susceptibility to dextran sodium sulfate (DSS)-induced colitis, the most well-established murine model of inflammatory bowel disease, and a result that was ultimately attributed to altered host responses to indigenous fungi (13). Analysis of wild-type (C57BL/6) and Clec7a<sup>-/-</sup> intestinal fungal microbiomes revealed that 97.3% of all the fungal sequences identified belonged to 10 fungal species, with 65.2% of the sequences belonging to a single fungus, *Candida tropicalis*. Mice given *Candida tropicalis* and then subjected to DSS showed significantly more severe colitis than mice given *Saccharomycopsis fibuligera*, a nonpathogenic fungus. Fluconazole treatment in these mice led to improvement in IBD symptoms: reduced weight loss and milder histological disease characteristics. The investigators also identified a polymorphism in the gene for dectin-1 (*CLEC7A*) in humans that is strongly linked to a severe form of ulcerative colitis.

**Cytokines.** Although the Th17 response has been best characterized in models of chronic inflammation and autoimmunity, more recently it has been implicated in mucosal host defense by augmenting neutrophil recruitment and/or function, inducing antimicrobial peptide formation, and maintaining epithelial barrier function. The interleukin 17A (IL-17A) receptor (IL-17RA) (89) and IL-22 (90) appear to be essential for host defense in disseminated *C. albicans* infection. A functional Th17 response also appears to be critical for host defense against oropharyngeal candidiasis in mice (91). The role of a Th17 response in regulating *C. albicans* GI colonization is somewhat unclear. Zelante et al. have demonstrated that the Th17 response is actually detrimental to the host’s ability to control *C. albicans* infection with intragastric inoculation (92). IL-22 appears to mediate early host defense against bacterial enteric pathogens in the gut, and IL-22 knockout mice showed increased GI epithelial damage and increased rates of systemic bacterial burden and infection-related mortality (93). However, studies using Th17 phenotype cytokine-deficient mice (i.e., IL17A, IL17RA, IL-22, IL-23), similar to those done with the oropharyngeal candidiasis model, have not been done using a distal GI colonization murine model. As for other cytokines, IL-10-deficient mice are actually more resistant to *C. albicans* GI colonization (94), whereas mice lacking IL-12 are colonized at higher levels (92).

Interestingly, bacterial pathogens, such as *Salmonella enterica* serotype Typhimurium, have been shown to induce inflammation and subsequently induce changes in the resident microbiota populations, resulting in enhanced *S. Typhimurium* GI colonization (95). An *S. typhimurium* mutant strain that fails to induce inflammation is also defective in colonizing the GI tract. Similarly, there is some evidence that inflammation alone can promote *C. albicans* GI colonization. Jawhara et al. showed that oral inoculation of *C. albicans* in mice previously treated with dextran-sulfate sodium (a chemical that injures epithelial cells and causes inflammation) could result in sustained *Candida* GI colonization, whereas mice not treated with DSS could not be colonized with *Candida* (95).

**Fungal virulence determinants.** Phenotypic variation is well-known in *C. albicans* (i.e., white versus opaque, yeast versus filamentous forms) and has been shown to impact virulence in intravenous disseminated candidiasis models. Very little, however, is known about fungal factors that influence GI colonization. *In vivo* *C. albicans* transcription profiling experiments in both a piglet and murine model of GI colonization revealed that the EFH1 gene was highly expressed during growth in the mammalian GI tract; yet, efh1 null mutants actually exhibited increased colonization in the murine GI tract and EFH1-overexpressing strains exhibited discordant reduced colonization levels (28). EFH1 does not appear to be important for systemic infection. In contrast, genes important for invasive infection (i.e., RBT1 and RBT4) were not required for intestinal colonization. The purported role of EFH1 expression may be for population self-regulation, thereby favoring commensalism and forgoing a pathogenic state.

In follow-up studies analyzing gene expression in *C. albicans* cells colonizing the murine GI tract, these investigators noted that cells colonizing the GI tract expressed many genes that were characteristic of cells growing *in vitro* in exponential phase (growth genes) and genes characteristic of postexponential phase (stress-induced genes) (96). Surprisingly, gene expression in colonizing cells and invasive cells were often very similar. These results are important for dispensing the belief that certain genes are turned “on” only in certain states (commensalism versus pathogenic).

**Choice of fungal and murine strains.** It is absolutely critical to emphasize that *C. albicans* strains vary considerably in their ability to colonize mucosal surfaces (97, 98) and in their ability to disseminate (22, 23). Whereas many studies have used common “laboratory” strains, such as SC5314, it may be prudent to use several *Candida* strains, ideally including a clinical isolate. Before making generalizable conclusions, it is always best to confirm findings with additional *Candida* strains to eliminate the possibility of strain-specific phenotypes (99).

Similarly, as previously mentioned, the choice of the murine strain is just as important. The same inbred strain of mice (C57BL/6) obtained from different vendors (Jackson versus Tacconic) have been shown to have completely different gut bacterial microbiota (absence or presence of segmented-filamentous bacteria) that ultimately determines whether the host exhibits a particular immunologic phenotype (CD4<sup>+</sup> T helper cells that produce IL-17 and IL-22 [Th17 cells] in the lamina propria of intestinal cells) (88). Similarly, the same inbred strain of mice purchased from different vendors can have vastly different *Candida* GI colonization phenotypes as well (unpublished observation). Finally, housing conditions (86) and diet can also have profound effects on gut microbiota populations.

**FUTURE DIRECTIONS**

With microbes outnumbering human cells by 10 to 1 and the microbial genetic repertoire approximately 100 times greater than that of the human host, the term superorganism, that conglomerate of mammalian and microbial cells that comprise a human being, has gained significant traction in the scientific and lay press. Commensal microbiota contribute to the metabolic, nutritional, and immunological status of the mammalian host. Commensal bacteria are the most abundant members of the gut microbiota and have received the greatest degree of scientific scrutiny, but other domains, including fungi, are normal residents of the mammalian host. The number of studies investigating the human fungal microbiome is quite limited, but studies of both the oral and distal GI tract have shown that *Candida* spp. are the most predominant. Interestingly, other mammals have similar fungal microbiota populations but can vary significantly at the species level—the
most notable example being mice, which exhibit Candida species dominance in the gut but have an absence of C. albicans in the gut and are in fact naturally resistant to C. albicans GI colonization.

The growing emergence of invasive fungal disease in humans in the 20th and 21st centuries is a direct result of the advances in medical care. Antibiotics, cancer chemotherapy, invasive surgical procedures, and advanced life-support devices all result in a human host disequilibrium (whether it be deficits to the immune system, breaks in integumentary boundaries, or disturbances in the normal commensal microbiota) that result in fungal disease. The immunocompetent human host with its resident microbiota is remarkably good at maintaining a healthy symbiotic relationship, so teasing out and understanding the mechanisms by which disruptions in host homeostasis results in both enhancement of proinflammatory commensals and dampening of the growth of proinflammatory commensals. Finally, we may gain better insight into these vastly intricate relationships, with the ultimate goal of devising novel therapies or preventive measures to help prevent invasive fungal infections in the human host.

One can envision monitoring patients’ gut bacterial and fungal microbiomes in a real-time prospective fashion, and intervening once abnormalities are noted. For instance, if a clinician were to notice a rapid expansion and dominance of a particular Candida species in the gut of a patient undergoing bone marrow transplant, one might administer selective antifungal antibiotics to decrease colonization levels. One could administer probiotic treatments such as animal models that involve host immune effectors, host microbiota, and the microbial commensal/pathogen. By utilizing animal models that attempt to approximate the complex physiology in the human host, we may gain better insight into these vastly intricate relationships, with the ultimate goal of devising novel therapies or preventive measures to help prevent invasive fungal infections in the human host.

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