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(Article begins on next page)

Mucosal Damage and Neutropenia Are Required for *Candida albicans* Dissemination

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***Candida albicans* fungemia in cancer patients is thought to develop from initial gastrointestinal (GI) colonization with subsequent translocation into the bloodstream after administration of chemotherapy. It is unclear what components of the innate immune system are necessary for preventing *C. albicans* dissemination from the GI tract, but we have hypothesized that both neutropenia and GI mucosal damage are critical for allowing widespread invasive *C. albicans* disease. We investigated these parameters in a mouse model of *C. albicans* GI colonization that led to systemic spread after administration of immunosuppression and mucosal damage. After depleting resident GI intestinal flora with antibiotic treatment and achieving stable GI colonization levels of *C. albicans*, it was determined that systemic chemotherapy with cyclophosphamide led to 100% mortality, whereas selective neutrophil depletion, macrophage depletion, lymphopenia or GI mucosal disruption alone resulted in no mortality. Selective neutrophil depletion combined with GI mucosal disruption led to disseminated fungal infection and 100% mortality ensued. GI translocation and dissemination by *C. albicans* was also dependent on the organism's ability to transform from the yeast to the hyphal form. This mouse model of GI colonization and fungemia is useful for studying factors of innate host immunity needed to prevent invasive *C. albicans* disease as well as identifying virulence factors that are necessary for fungal GI colonization and dissemination. The model may also prove valuable for evaluating therapies to control *C. albicans* infections.**

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Introduction

Candida albicans is a ubiquitous commensal organism that can cause serious disseminated infections in cancer patients [1,2]. *Candida* spp. are the fourth leading cause of nosocomial bloodstream infections in the United States, with treatment costs estimated to be more than \$2–\$4 billion annually [3] and with attributable mortality rates estimated to be between 38% to 49% [4]. Among the various invasive fungal infections reported in cancer patients, candidiasis is the most common infection (58%–69%) [5–7], and over the past decade, the incidence of invasive fungal infections in this population has increased significantly [8].

The presumed mechanism for all invasive *C. albicans* disease involves initial mucosal surface colonization followed by invasion into the adjacent tissues and organs. In cancer patients, *C. albicans* usually colonizes the gastrointestinal (GI) tract with subsequent translocation into extraintestinal organs (i.e., mesenteric lymph nodes, blood stream, liver, and spleen) in the setting of chemotherapy-induced neutropenia and GI mucosal damage [9]. The three primary mechanisms that promote pathogenic microbial (bacterial and fungal) translocation in animal models are: 1) disruption of the normal GI microbiologic equilibrium allowing intestinal overgrowth of pathogens, 2) increased permeability of the intestinal mucosal barrier, and 3) deficiencies in the host immune defenses [10,11]. Not surprisingly, common risk factors for developing candidemia in human patients include neutropenia, mucositis, use of broad spectrum antibiotics, and invasive medical procedures [6,12].

The majority of murine models of disseminated candidiasis have employed the administration of a chemotherapeutic agent (e.g., cyclophosphamide) followed by the subsequent intravenous injection of *C. albicans* [13,14] or simply the intravenous injection of high inocula of *C. albicans* [15,16]. Therefore, a murine model that first establishes GI colonization followed by translocation and dissemination via disruptions of select components of the innate host defense would afford valuable opportunities for studying the details of *C. albicans* pathogenesis, as well as delineating the relative roles of the major immune compartments or the actual immune mechanisms responsible for killing and/or controlling translocating *C. albicans* [10].

To evaluate normal host factors that must be disrupted to allow for GI colonization, fungal dissemination and significant morbidity or mortality due to candidemia, we have developed a reproducible mouse model wherein *C. albicans* GI colonization is first established and subsequent fungal dissemination is achieved following induction of immunosuppression and disruption of mucosal integrity. Both neutropenia and GI mucosal damage appear to be necessary

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Author Summary

Candida albicans is a fungus that lives harmlessly in the gastrointestinal (GI) tracts of humans. In cancer patients and patients undergoing bone marrow transplantation, however, the anti-cancer drugs that are administered to these patients also cause the undesired effect of suppressing the human immune system. The treatments allow *C. albicans* to spread into the blood and other organs and cause a severe disease. We found we could colonize the GI tracts of mice with *C. albicans* and then suppress the immune system with anti-cancer drugs to determine which components of the innate immune system (neutrophils, lymphocytes, macrophages, or GI tract integrity) are critical for preventing *C. albicans* from spreading from the GI tract. We found that lowering the neutrophil counts and damaging the GI tract were both needed to cause systemic infection with *C. albicans*. We also found that the ability of *C. albicans* to switch from the yeast (spherical) form to the filamentous form is also important for establishing invasive disease. Our study provides new insights into the process of how a typically harmless microorganism inhabiting the GI tract can cause severe invasive disease once critical components of the host immune system are compromised.

for fungal dissemination in this murine model. Finally, we evaluated *C. albicans* mutants that had varying abilities to switch between the yeast and the hyphal growth form of *C. albicans* and found changes in virulence associated with an inability to switch to the hyphal phase, indicating the utility of this animal model for studying different aspects of the pathogenic process of *C. albicans* in the setting of GI colonization and dissemination.

Results

GI Colonization by *C. albicans*

Wild-type *C. albicans* strains SC5314 (a strain that has been frequently used in various *C. albicans* murine models [17–20]) and CAF2-1 consistently colonized the mouse GI tract at comparable levels: SC5314 (median = 2.24×10^7 cfu/g stool, first quartile = 1.07×10^7 , third quartile = 3.75×10^7) and CAF2-1 (median = 2.60×10^7 cfu/g stool, first quartile = 2.28×10^7 , third quartile = 3.19×10^7 ; Figure 1). When tested up to 21 d later, there was no significant change in fungal colonization levels of the stool.

Dissemination by *C. albicans*

Mice that were colonized with *C. albicans* strains SC5314 and CAF2-1, as well as non-*Candida* colonized control mice that were only decontaminated with antibiotics, were separated into 17 groups (8 mice/group, with the exception of 4 mice/group in Rag^{-/-} mice), treated with immunosuppressive regimens (Table 1), and the effect on dissemination of the different methods of immunosuppression determined (Table 2). Mice that were only decontaminated with antibiotics all survived after administration of immunosuppressive regimens—with the exception of 1 death in the group given mAb RB6-8C5 plus methotrexate (MTX) (Group 7). All groups that were colonized with *C. albicans* strains SC5314 and CAF2-1 then given immunosuppression showed levels of GI colonization comparable to those of mice not given immunosuppression (results shown in Figure 1; data for immunosuppressed mice not shown). Mice that were only given antibiotic

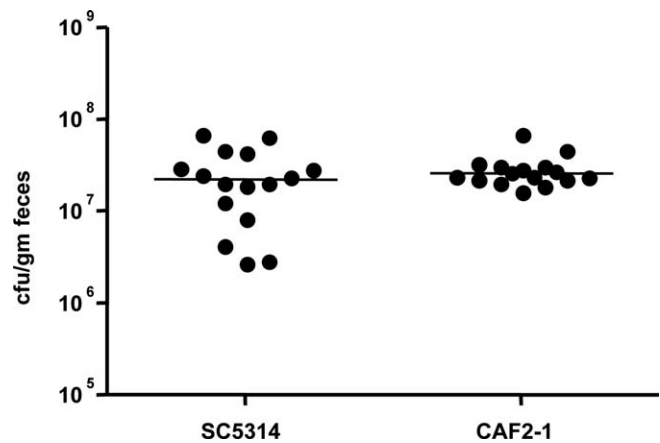


Figure 1. Gastrointestinal Colonization Levels of C3H/HeN Mice Fed *C. albicans* Strains SC5314 and CAF2-1

Mice were maintained on sterile water with 2 mg streptomycin/ml and 1,500 U penicillin G/ml throughout the duration of the experiment, and 0.2 mg gentamicin/ml was added once colonization was confirmed. Points represent results from individual animals, and horizontal lines represent the medians. $n = 16$ for each strain. doi:10.1371/journal.ppat.0040035.g001

decontamination and no immunosuppression after colonization with *C. albicans* (Group 1) also all survived.

Chemotherapy. When either Cy alone (Group 2), Cy plus MTX (Group 3) or mAb RB6-8C5 plus MTX (Group 5) were administered, fungal dissemination occurred in all mice associated with a high level of mortality. Interestingly, when MTX alone (Group 4) was given, 3 of 8 mice in the group colonized with strain SC5314 and 4 of 8 mice in the group colonized with strain CAF2-1 died from disseminated disease. In contrast, when MTX was given concurrently with mAb RB6-8C5 (Group 5), all mice died following fungal dissemination.

Neutrophil depletion. Mice that were made neutropenic after a single injection of 200 μ g of anti-neutrophil mAb RB6-8C5 (mAb $\times 1$) survived (Group 6). Even when given two additional doses of mAb RB6-8C5 (mAb $\times 3$; Group 7) and thus made neutropenic for 15 d, there was no evidence of *C. albicans* dissemination in these mice. The four mice that died in this group only showed evidence of bacterial (e.g., *Enterobacter* spp.), not fungal, dissemination.

Macrophage depletion. By immunohistochemical analysis, macrophages in both the liver and spleen were reduced after liposomal clodronate administration (Figure S1). By FACS analysis, F4/80 and CD11b double-stained macrophages were reduced in the spleen and bone marrow after liposomal clodronate administration from 26% to 16% (Figure S2). Mice given liposomal clodronate (Group 8), clodronate plus mAb RB6-8C5 (Group 9), or clodronate plus DSS (Group 10) all survived.

Lack of functional lymphocytes. RAG^{-/-} mice colonized with strains SC5314 or CAF2-1 showed no evidence of dissemination (Group 11)—not even when mAb RB6-8C5 (Group 12), DSS (Group 13), or liposomal clodronate (Group 14) were subsequently administered to different groups of RAG^{-/-} mice.

GI mucosal disruption. There was no histologic evidence of any GI mucosal injury or damage in the ceca of mice given mAb RB6-8C5 or DSS for 3 d; in contrast, mice given DSS for

Table 1. Immunosuppressive Regimens

Agent	Dose	Route of Administration	Frequency	Vendor	Reference
CL ₂ MBP (liposomal clodronate)	200 μl (2 mg)	IP	Once	Roche Diagnostics, Mannheim Germany	[61]
Cyclophosphamide (Cy)	150 mg/kg/dose	IP	Three times every other day	Sigma-Aldrich	[63]
Dextran sulfate sodium (DSS)	2.5% or 5% (wt/vol) in sterile water	Oral (via drinking water)	7 d	Sigma-Aldrich	[52,53]
Methotrexate (MTX)	150 mg/kg/dose	IP	Once	Bedford Labs, Bedford, OH	[29]
RB6-8C5 monoclonal antibody (mAb)	200 μg	IP	Once	—	[64]

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7 d had ulcerations and focal areas of inflammation noted in the cecum (Figure 2). The cecums of the mice that had received DSS for 7 d and one dose of mAb RB6-8C5 showed diffuse autolysis and sloughing. Mice given DSS alone showed no evidence of *C. albicans* dissemination (Group 15); however, when both DSS (2.5%) and mAb RB6-8C5 were given, disseminated disease and mortality was achieved (50% to 87.5% mortality). Increasing the DSS concentration from 2.5% (Group 16) to 5% (Group 17) while still giving a single 200 μg/mouse dose of mAb RB6-8C5 achieved 100% mortality associated with fungal dissemination to the liver.

When mice were found moribund or died, the levels of *C. albicans* in the livers were comparable among animals given the different treatment regimens when both neutropenia and mucosal damage were induced (Figure 3). With the exception of the four mice noted previously in Group 7 (mAb × 3), there was no evidence of concurrent bacterial infection.

Translocation Studies

We analyzed the effects of immunosuppression and/or mucosal damage treatments on translocation of *C. albicans* from the GI tract. All mice in these studies were colonized with *C. albicans* SC5314 and had levels of GI colonization comparable to that shown in Figure 1 (data not shown). Mice given no immunosuppression (Group A) all survived, and none appeared moribund and none grew *C. albicans* from the blood, spleen or MLN. Of note, fungi were found at very low levels in the livers of 4 (out of 20) healthy-appearing mice (9, 42, 208, and 369 cfu/g). The mice given mAb RB6-8C5 treatment only (Group B) all survived, and none appeared moribund. Similar to Group A, *C. albicans* was not found in the blood, spleen or MLN of mice only given the neutropenia-inducing mAb, but fungi were occasionally found in the liver (5 out of 20; median 9.88×10^2 cfu/g, first quartile = 4.09×10^2 , third quartile = 2.68×10^3); these are clearly higher levels than those found in the livers of mice in Group A which did not receive any immunosuppression. In contrast, 3 of the 4 mice from the group given neutropenia-inducing mAb RB6-8C5 plus MTX (Group C) died by day 4 post-immunosuppression, and 4 of 4 of these mice had died by day 5. All of these mice showed significant levels of *C. albicans* in the liver (median 8.77×10^3 cfu/g, first quartile = 3.45×10^3 , third quartile = 2.51×10^4). Finally, the mice given Cy only (Group D) all had *C. albicans* in the liver by days 4 and 5: (median 7.71×10^4 cfu/g, first quartile = 3.87×10^4 , third quartile = 1.13×10^5).

C. albicans Morphogenesis as a Virulence Determinant

In order to assess the role of fungal morphogenesis as a virulence determinant in our murine model, we tested three *C. albicans* strains for their ability to colonize and disseminate following immunosuppression: strain CAF2-1 is a wild-type organism that was chosen because it, like the additional mutants we tested, only has one copy of the *URA3* gene [21]; strain HLC54 (*cph1/cph1 efg1/egf1*) exhibits decreased filament formation, hereafter referred to as $\Delta efg1/cph1$ [22]; and strain BCa-210 (*tup1/tup1*, hereafter referred to as $\Delta tup1$) exhibits constitutive filamentous growth [23]. As shown in Figure 4, both CAF2-1 (median = 2.99×10^7 cfu/g, first quartile = 2.38×10^7 , third quartile = 3.30×10^7) and strain $\Delta efg1/cph1$ (median = 5.32×10^7 cfu/g, first quartile = 4.69×10^7 , third quartile = 1.03×10^8) were able to colonize the GI mucosa at comparable levels, while the $\Delta tup1$ strain colonized at a level 2-logs lower (median = 7.71×10^4 cfu/g, first quartile = 5.0×10^4 , third quartile = 1.66×10^6 , $p = 0.0003$ by Mann Whitney test compared with other two strains). This discrepancy is explained by the fact that the maximum concentration achievable of $\Delta tup1$ in water was approximately 2-logs lower than the levels for the wild-type and $\Delta efg1/cph1$ strains—a difference which was consistent throughout the experiment. Interestingly, strain $\Delta efg1/cph1$ caused less mortality compared to wild-type *C. albicans* (8 of 16 mice given $\Delta efg1/cph1$ died versus 14 of 16 mice infected with wild-type, $p = 0.02$, Fisher's exact test), whereas in spite of the 2-log lower levels of strain $\Delta tup1$ in feces it caused mortality comparable to that of wild-type (7 of 8 mice died; Figure 5). In preliminary experiments with wild-type *C. albicans* strains SC5314 and CAF2-1, if we administered immunosuppression (e.g., mAb RB6-8C5 plus MTX) before final levels of GI colonization (between 10^7 and 10^8 cfu/g) had been established, dissemination was not induced, and all mice survived (data not shown). All of these surviving mice continued to be colonized with their respective strains of *C. albicans* at levels comparable to that achieved before administration of mAb and MTX.

Eight additional mice were administered higher concentrations of $\Delta tup1$ (5.5×10^5 cfu/ml) in the drinking water, and GI colonization levels achieved with this higher dose were comparable to those of the wild-type *C. albicans* strain (median = 1.48×10^7 cfu/g, first quartile = 5.20×10^6 , third quartile = 4.08×10^7), indicating that the level of GI colonization can be increased for this strain. To determine if this constitutively filamentous strain could by itself induce mucosal damage sufficient to achieve dissemination, we

Table 2. Survival of Mice after Administration of Immunosuppressive Regimens

<i>C. albicans</i> Strain	None	Cy	Cy + MTX	MTX	mAb + MTX	mAb × 1	mAb × 3	Liposomal Clodronate	Clodronate + mAb	Clodronate + DSS
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9	Group 10
None	—	8/8	8/8	8/8	7/8	8/8	8/8	8/8	8/8	8/8
SC5314	8/8	0/8	0/8	5/8	0/8	8/8	4/8 ^b	8/8	8/8	8/8
		(<i>p</i> = .00008) ^a	(<i>p</i> = 0.00008) ^a	(<i>p</i> = 0.10) ^a	(<i>p</i> = 0.0007) ^a					
CAF2-1	8/8	0/8		4/8	2/8	8/8	5/8 ^b	8/8	8/8	8/8
		(<i>p</i> = 0.00008) ^a		(<i>p</i> = 0.038) ^a	(<i>p</i> = 0.020) ^a					

Abbreviations: mAb, RB6-8C5 monoclonal antibody; Cy, cyclophosphamide; MTX, methotrexate; DSS, dextran sulfate sodium; RAG^{-/-}, recombinase activating gene deficient mice. Mice were observed for a total of 7 days after the administration of immunosuppression, with the exception of Group 5, which was observed for periods of 5 days after each RB6-8C5 mAb dose for a total of 15 days.

^a*p*-value by Fisher's exact test compared to untreated group.

^bBacterial (*Enterobacter* spp.) dissemination only.

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administered the mice 200 µg of mAb RB6-8C5 only. No enhanced mucosal disruption sufficient to allow fungal dissemination in most mice was achieved by constitutive hyphal expression, as 7 of 8 colonized mice made only neutropenic still survived.

Discussion

In this study, we attempted to devise a mouse model to study *C. albicans* pathogenesis and host factors leading to susceptibility to disseminated infection that emulate the pathophysiology that takes place in a human host, wherein receipt of broad-spectrum antibiotics, extensive hospitalization, or administration of immunosuppressive agents makes patients more vulnerable to invasive disease, often associated with intercurrent mucosal disruption owing to surgery, tumor invasion, or chemotherapy [24,25]. In neutropenic patients, the role of the gut as a source for disseminated candidiasis has been supported from autopsy studies [26].

Several murine models of GI-derived *C. albicans* fungemia and sepsis have been reported previously [27–32]. The levels of GI colonization we obtained were substantially higher than those achieved by prior investigators (1 to 3 log-fold higher) [31,33] and were maintained at least up to 21 d (our latest verification date). This higher colonization level is most likely due to the addition of penicillin to the drinking water. Penicillin most likely led to reductions of endogenous anaerobic bacterial flora, promoting subsequent intestinal overgrowth by *C. albicans* [34]. One other model did achieve comparable colonization levels by also administering adjunctive oral antibiotics [32]. The need for reduction of indigenous flora, particularly gram-negative bacteria, is critical given that bacteria are just as likely as the *C. albicans* to translocate and disseminate when immunosuppression is administered [35].

We were able to show this model recapitulates important aspects of human susceptibility to candidiasis, although there are some limitations to consider. The model was developed using adolescent/young adult 6- to 8-wk-old female C3H/HeN mice, and its applicability to other mouse strains or mice of other ages is not fully known. It would be difficult to use neonatal or infant mice in this model as others have done

with acute *C. albicans* infections [28–30] due to the need for the animals to be able to drink both antibiotic water and fungi in the water and then give the immunosuppressive/mucosal disrupting agents, which would not be feasible with mice prior to weaning. C3H/HeN mice were used because of experience in a previous model of *P. aeruginosa* GI colonization and neutropenia-induced dissemination [35], and the fact that this strain has no major underlying immune deficit and is highly susceptible to DSS-induced colitis [36]. While we have not extensively studied other mouse strains, initial studies using Swiss Webster and C57/BL mice showed that comparable levels of GI colonization, mortality, and liver dissemination can be achieved when using Cy as the immunosuppressive/mucosal damaging agent (AYK and GBP, unpublished data). *C. albicans* administration via drinking water limits the ability to control the infecting dose as might be achieved by administering fungal cells by gavage. However, administering *C. albicans* in the drinking water for 5 d led to a reproducible and consistent GI colonization level (Figure 1) associated with dissemination following the disruption of specific host defenses. Additionally, we did not confirm the entire spectrum of bacterial decontamination achieved by the antibiotics, as we did not utilize anaerobic culture conditions or media specific for all types of aerobic and anaerobic flora. Therefore, we cannot be absolutely sure that we truly eradicated all indigenous aerobic and anaerobic flora. In any case, whatever residual aerobic or anaerobic flora that remained after antibiotic decontamination does not appear to have any pathogenic significance, as evidenced by the fact that mice given only antibiotic contamination and subsequent immunosuppression all appeared healthy and exhibited 0% mortality.

We also chose to measure fungal levels in livers as an indicator of dissemination. In other murine models of intravenous *C. albicans* infection fungal levels in the kidneys were used to confirm systemic infection was achieved. In preliminary experiments, we found that in mice that were colonized with *C. albicans*, given subsequent immunosuppression (cyclophosphamide or RB6-8C5 + MTX), and had no other detectable infectious cause of death (e.g. bacterial dissemination) that the liver (presence of *C. albicans* in 100% of livers from deceased mice) was a more reliable organ for confirmation of dissemination compared to the kidneys

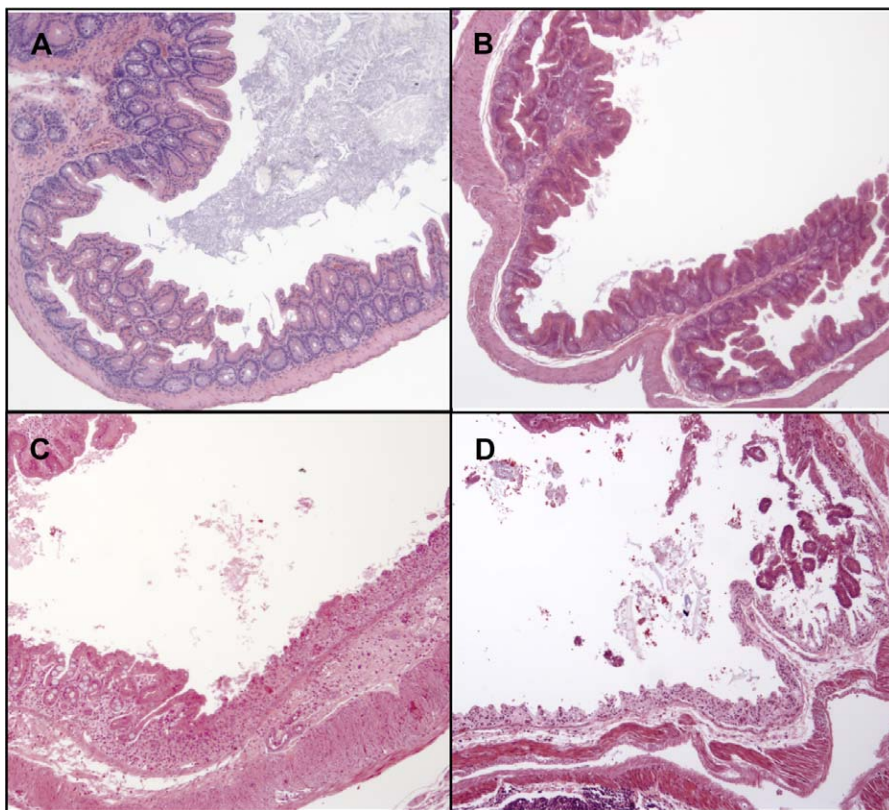
Table 2. Extended.

RAG ^{-/-}	RAG ^{-/-} + mAb	RAG ^{-/-} + 2.5% DSS	RAG ^{-/-} + Clodronate	DSS (2.5%, 5%) (<8 days)	2.5% DSS + mAb	5% DSS + mAb
Group 11	Group 12	Group 13	Group 14	Group 15	Group 16	Group 17
—	—	—	—	8/8	8/8	8/8
8/8	4/4	4/4	4/4	8/8	1/8 (<i>p</i> = 0.0007) ^a	0/8 (<i>p</i> = 0.00008) ^a
8/8	4/4	4/4	4/4	8/8	4/8 (<i>p</i> = 0.038) ^a	0/8 (<i>p</i> = 0.00008) ^a

(presence of *C. albicans* in 50%–80% of kidneys in deceased mice; AYK and GBP, unpublished data). One other model using *C. albicans* GI colonization and chemotherapy-induced dissemination also noted 100% recovery from the livers but significantly less recovery from the kidneys [32]. Finally, for practical purposes we had to use organs for CFU enumeration from moribund then euthanized mice or mice that died between observations whose carcasses were frozen as close to the time of death as feasible. However, in limited studies we did compare the *C. albicans* yields from organs resected after

freezing and storage with yields from organs resected from freshly euthanized mice and found the differences in CFU were not statistically different (AYK and GBP, unpublished data). Nonetheless, because there could be effects from post-mortem fungal growth or losses upon storage at -20°C one must be cautious in using levels of *C. albicans* in the organs as a measure of virulence.

We also attempted to determine how *C. albicans* might spread from the GI lumen to internal organs. Given the sporadic and low levels of hepatic dissemination in mice

**Figure 2.** DSS-Induced Murine Gastrointestinal Mucosal Damage

Histological sections of cecum from mice colonized with *C. albicans* strain SC5314 with continuation of penicillin, streptomycin, and gentamicin in the drinking water then given water with 2.5% DSS for various periods with or without induction of neutropenia.

(A) No DSS, 200 μg of RB6-8C5 IP once.

(B) DSS for 3 d.

(C) DSS for 7 d.

(D) DSS for 7 d and after the third day of DSS mice were given 200 μg of RB6-8C5 IP once and observed for 96 h after the RB6-8C5 dose. Magnification: Objective 10 \times .

Sections in (A) and (B) have normal histologic appearance, whereas in panels (C) and (D) disruption of the epithelial barrier integrity was clearly seen.

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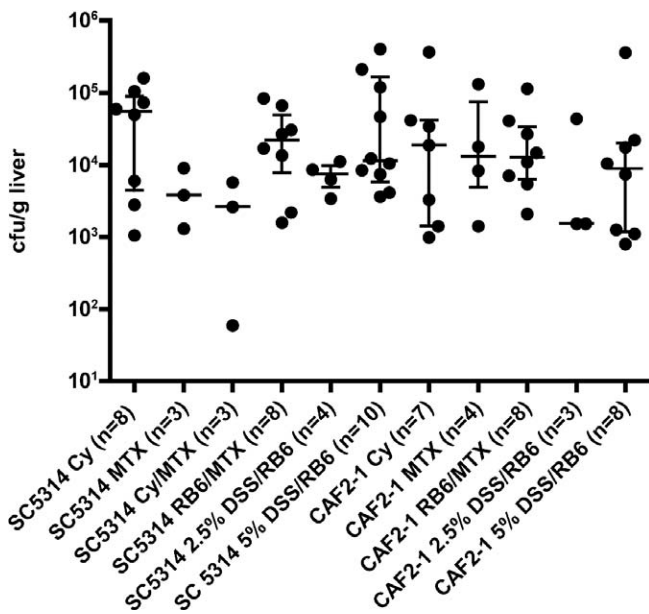


Figure 3. Viable Counts of *C. albicans* Strains SC5314 or CAF2-1 in the Livers of Deceased C3H/HeN Mice that Were Previously Colonized in the GI Tract with *C. albicans* and Subsequently Given Various Forms of Immunosuppression

Cy, cyclophosphamide; MTX, methotrexate; RB6, RB6-8C5 mAb; DSS, dextran sulfate sodium. Points represent results from individual animals. Horizontal lines with bars represent median with interquartile range when $n > 3$, otherwise only the median is shown.
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colonized with *C. albicans* but not receiving any immunosuppression, it is conceivable 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. In our study, mice colonized with *C. albicans* and given only RB6-8C5 mAb also showed sporadic levels of hepatic dissemination, albeit at higher levels than in mice that did not receive immunosuppression. The higher fungal burden is most likely secondary to the lack of neutrophils and thus the subsequent diminished ability of the immunosuppressed animal to clear the *C. albicans* from the liver. But even in the absence of neutrophils, the fungal burden was not enough to cause frequent morbidity or death. Not surprisingly, when we administered systemic chemotherapeutic agents such as cyclophosphamide (resulting in both neutropenia and GI mucosal damage) we were able to reproducibly achieve widespread dissemination with *C. albicans*.

In the final analysis we found both neutropenia and disruption of the integrity of the GI mucosa were needed for fungal dissemination. In several intravenous murine models of *C. albicans* systemic infection, neutrophil depletion notably increases fungal burden and mortality [37,38]. In this murine model, however, neutropenia alone is not sufficient for extra-intestinal dissemination most likely due to the fact that *C. albicans* is still unable to breach the intact GI epithelium.

Although one prior study implicated the importance of CD4⁺ lymphocytes in protective immunity to systemic *C. albicans* infection [39], several studies in SCID mice have

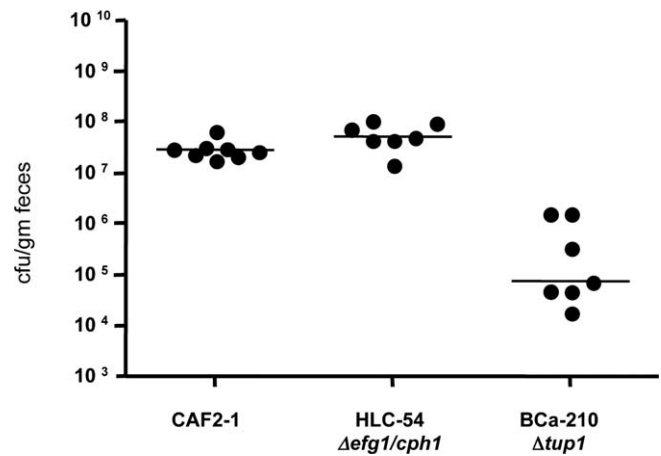


Figure 4. Gastrointestinal Colonization Levels of Female 6- to 8-Wk-Old C3H/HeN Mice Fed *C. albicans* Strains CAF2-1, HLC-54 ($\Delta efg1/cph1$), and BCa-210 ($\Delta tup1$)

Points represent results from individual animals, and horizontal lines represent the medians. $n = 8$ for CAF2-1, $n = 7$ for $\Delta efg1/cph1$ and $\Delta tup1$. Colonization levels with strain $\Delta tup1$ were significantly lower ($p = 0.0003$ by Mann Whitney test) compared with other two strains.
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shown that a defect in the TH1 CD4⁺ T-cell response to *C. albicans* results in mucosal or esophageal candidiasis but not in systemic dissemination. Even in C5 deficient DBA/2 mice [38] and a recent murine HIV model [40], T cell depletion also does not result in disseminated *C. albicans* disease. Although the RB6-8C5 mAb at the dose used also induces some depletion of lymphocytes [35], we found that both C3H/HeN and RAG^{-/-} mice that lack mature lymphocytes were not susceptible to fungal disease following GI colonization and neutropenia alone. Thus, our murine model correlates with these previous studies: lymphopenia alone or in combination with macrophage depletion, neutrophil depletion, or GI mucosal disruption without neutropenia did not result in fungal dissemination or death of the colonized mouse.

Similarly, depleting macrophages only did not lead to any susceptibility to systemic fungal infection. Although murine peritoneal macrophages [41,42], pulmonary alveolar macrophages [43], Kupffer's cells [44] and human peripheral blood monocytes [45] have the ability to phagocytose and kill *C. albicans* in vitro, in vivo studies on disseminated candidiasis have generated evidence both supporting [42,46–48] and refuting the importance [49–51] of macrophages. Of the two studies utilizing selective macrophage depletion [48,50], only one administered liposomal clodronate followed by intravenous fungal injection to produce a disseminated candidiasis. Spleens from the mice treated with clodronate lost their ability to trap yeast [48]. Furthermore, when macrophage-depleted mice were systemically challenged with *C. albicans*, not only was clearance of yeast decreased in blood, but kidneys had higher fungal burdens and overall mouse survival was decreased. Although we used the same dose of clodronate, we also utilized FACS analysis to quantify the macrophage depletion (37% depletion) rather than measuring peripheral blood monocyte counts (30%–85% depletion). Ultimately, our lack of finding a significant role for macrophages in host resistance to *C. albicans* may be related to the use of different models. In an intravenous model with direct

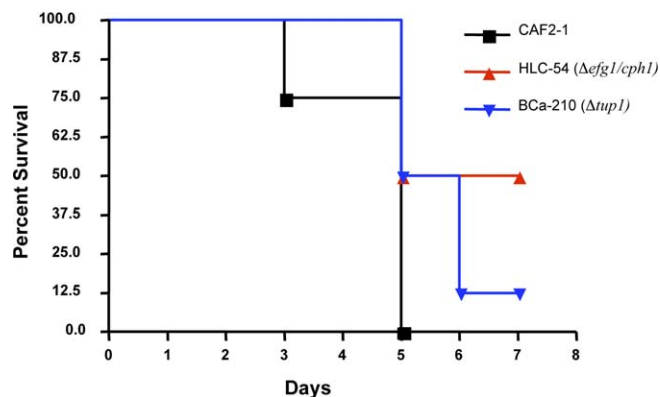


Figure 5. Survival Curve of C3H/HeN Mice Fed *C. albicans* Strains CAF2-1, HLC-54 ($\Delta efg1/cph1$), or BCa-210 ($\Delta tup1$) and Subsequently Given mAb RB6-8C5 (200 μ g IP) and Methotrexate (150 mg/kg/dose IP)

Median survival of mice colonized with strain $\Delta efg1/cph1$ was significantly higher than that of mice colonized with wild-type *C. albicans* CAF2-1 ($p = 0.01$, log rank test). Each group contained 8 mice. doi:10.1371/journal.ppat.0040035.g005

inoculation, this degree of macrophage depletion may be enough of a deficit to lead to more severe disseminated disease. In our murine model, it may be that macrophages do play a role, but more substantial macrophage depletion may be necessary to see this effect.

Finally, while DSS has been used for experimental murine models of human inflammatory bowel disease [52,53], the histopathologic damage induced by DSS has some similarities to chemotherapy-induced mucositis in that both result in denudation and ulceration. Interestingly, when we maintained the same degree of neutropenia but increased the GI mucosal damage (by changing DSS from 2.5% to 5%), we notably increased the mortality to 100%, and this is most likely secondary to the increased mucosal damage caused by the higher concentration of DSS. These studies further support our hypothesis that it is a combination of neutropenia and GI mucosal damage that are critical for *C. albicans* dissemination in our murine model.

In the host, *C. albicans* grows as all known morphologic forms (budding yeast, pseudohyphal filaments, and true hyphae), and it has been postulated that the ability to induce hyphal formation is a critical virulence determinant [22]. Mutant strains of *C. albicans* that are incapable of hyphal formation have been found to be avirulent in murine models of disseminated candidiasis [54–56]. Whether it is simply that hyphae formation leads to an increased ability to invade host epithelial cells, or induces greater cytotoxicity, or whether the advantage of filamentous growth is in providing greater resistance to phagocytosis [22], the definitive mechanistic explanation linking hyphal formation to virulence is lacking. When testing the morphogenesis mutants, $\Delta efg1/cph1$, which exhibit decreased filament formation [22], and $\Delta tup1$, which exhibits constitutive filamentous growth [23], we noted a nearly 2-log lower GI colonization level of the $\Delta tup1$ mutant compared to the wild-type strain, CAF2-1. As noted earlier, this discrepancy in colonization level was most likely due to the difficulty of suspending $\Delta tup1$ in drinking water and achieving higher concentrations. All of the mice colonized with strain $\Delta tup1$ had colonization levels lower than the 10^7 to 10^8 cfu/g achieved with *C. albicans* strains SC5314 and CAF2-1,

yet 7 of the 8 mice colonized with the $\Delta tup1$ strain died following immunosuppression and GI mucosal damage. Since it is difficult to titrate inocula in our murine model, this finding of dissemination and mortality in the setting of significantly lower GI colonization levels may suggest that the $\Delta tup1$ mutant is more virulent in this setting. The appropriate quantification of filamentous strains, however, is confounded by the fact that cells may not be properly separated, and thus CFUs are underestimated. In addition, filamentous strains such as $\Delta tup1$ may adhere to the GI mucosa more tenaciously, and thus levels in the stool could be an underestimation of actual GI colonization levels. However, increased filamentous growth of the $\Delta tup1$ mutant alone is not sufficient to disrupt the GI mucosa and allow for fungal dissemination even in the setting of neutropenia.

Our findings with CAF2-1 and $\Delta efg1/cph1$ were consistent with previous investigators that utilized an intravenous model of *C. albicans* dissemination [22,57,58]. However, in a model of hypoxic-induced GI translocation [59] and another study using oral inoculation followed by intraperitoneal (IP) injections of dexamethasone for immunosuppression [57], $\Delta efg1/cph1$ was found to be more invasive compared to the wild-type CAF2-1. Whereas both of these studies used the presence of *C. albicans* in extraintestinal organs to define invasiveness or virulence, we used death attributable to *C. albicans* dissemination. Furthermore, we utilized a different means of immunosuppression and attempted to verify that bacterial translocation was not a confounding factor. Therefore, our findings with $\Delta efg1/cph1$ support the hypothesis that the ability to form filaments is important for translocation and dissemination with *C. albicans*.

In conclusion, we have developed a murine model of *C. albicans* GI colonization following anti-microbial agent reductions in the indigenous flora and systemic spread during neutropenia that additionally requires GI mucosal damage. Neutropenia alone is not sufficient to produce disseminated *C. albicans* disease in this murine model. Being able to control these host factors should allow for a more detailed study of host and fungal factors needed to achieve GI colonization and systemic dissemination. These factors should thus be useful for evaluating pathogenesis as well as therapies to control *C. albicans* invasive infections.

Materials and Methods

Fungal strains and growth. The strains of *C. albicans* used are listed in Table 3. *C. albicans* strains were grown overnight at 37°C in yeast extract-peptone-dextrose (YPD) broth, harvested by centrifugation, washed with PBS, and resuspended in PBS. *C. albicans* concentration was determined by use of a hemocytometer.

Murine model of antibiotic-induced GI tract colonization by *C. albicans* and immunosuppressive-induced fungemia. Six- to 8-wk-old female C3H/HeN mice (Harlan, <http://www.harlan.com/models/c3h.asp>) were housed as groups of 4 in sterilized cages equipped with filter hoods. In some experiments, 6-wk-old female recombinase activating gene deficient mice ($Rag^{-/-}$, <http://jaxmice.jax.org/strain/002216.html>) were used. Mice were supplied with sterile bedding, sterile water and sterile mouse chow and maintained under specific pathogen-free conditions at the ARCM-MCP animal facility at Harvard Medical School in compliance with the Harvard Medical Area Institutional Animal Care and Use Committee guidelines. To deplete the indigenous GI bacterial and fungal flora, mice were fed sterile water with 2 mg streptomycin/ml (Research Product International, Mt. Prospect, IL), 1500 U penicillin G/ml (Sigma-Aldrich, St. Louis, MO), and 0.250 mg fluconazole/ml (Roxanne Laboratories, Columbus, OH) for 3 d, then switched to the same concentrations of streptomycin and penicillin G in their drinking water for one more

Table 3. Fungal Strains Used in This Study

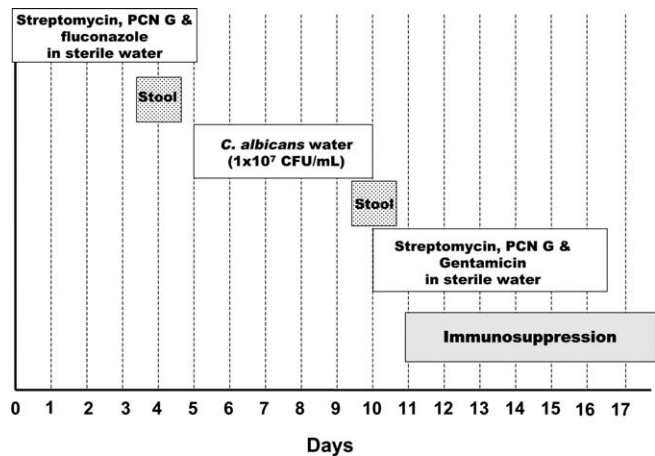
Strain	Description	Reference
SC5314	Wild-type strain	[21]
CAF2-1	Wild-type strain	[21]
HLC-54	<i>cph1/cph1 efg1/efg1</i> (Δ <i>cph1/efg1</i>)	[22]
BCa-210	<i>tup1/tup1</i> (Δ <i>tup1</i>)	[23]

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day. Stool was collected from individual mice (0.030–0.050 g per stool pellet), homogenized in 1 ml 1% protease peptone, and 100 μ l of the homogenate was spread and plated on yeast extract-peptone-dextrose (YPD), trypticase soy (TSA), or MacConkey agars to verify reduction of the targeted indigenous GI microbial flora. *C. albicans* strains (grown as described above) were added to sterile water with 2 mg streptomycin/ml, and 1,500 U penicillin G/ml at approximately 10^7 cfu/ml, and then *C. albicans* was administered via the drinking water to mice for 5 d. Candidal levels were constant in the drinking water and therefore water bottles were not changed during this time. After 5 d of exposure to *Candida*, stool was again collected, homogenized in 1 ml 1% protease peptone, serially diluted and plated on YPD agar with 0.010 mg vancomycin/ml and 0.100 mg gentamicin/ml to measure levels of GI colonization by *C. albicans*. We then induced immunosuppression by treating the mice with the immunosuppressive agents listed in Table 1. After administration of immunosuppressive agents, mice were fed sterile water with 2 mg streptomycin/ml, 1,500 U penicillin G/ml, and 0.2 mg gentamicin/ml for the remainder of the experiment and monitored for morbidity for 7 d. Moribund mice were euthanized and along with mice found dead between observation periods, the carcasses were frozen at minus 20°C, later thawed, livers resected, homogenized in 1 ml 1% protease peptone, serially diluted and 100 μ l of the homogenate was spread-plated on YPD with 0.100 mg gentamicin/ml (Research Product International, Mt. Prospect, IL) and 0.010 mg vancomycin/ml (Sigma-Aldrich), and additional 100 μ l amounts plated onto TSA and MacConkey agar plates. Growth media were incubated at 37°C overnight under aerobic conditions. The presence of a homogeneous population of creamy-white colonies on YPD with gentamicin and vancomycin was used for confirmation of *C. albicans* systemic dissemination (Figure 6).

Production or RB6-8C5 monoclonal antibody and induction of neutropenia. The RB6-8C5 monoclonal antibody (mAb) specific for the Ly6 antigen highly expressed by polymorphonuclear neutrophils (PMN) was produced by growth of hybridoma cells in culture (Dulbecco's modified Eagle's medium with 10% fetal calf serum [FCS]) followed by purification of antibody by affinity chromatography, as previously described [35]. A single dose of 200 μ g of RB6-8C5 was administered to C3H/HeN mice to produce a severe neutropenia (absolute neutrophil count < 100/mm³) for 5 d [35].

In vivo macrophage depletion. CL₂MBP (clodronate) was a gift from Roche Diagnostics (Mannheim, Germany). Preparation of liposomes containing CL₂MBP was done as described previously [60]. For assessment of macrophage depletion, three uninfected mice per group were inoculated IP with 200 μ l of PBS liposomes or with CL₂MBP liposomes (2 mg) [61]. At 2 d later, macrophages were quantified in the bone marrow and spleens using FACS analysis. Bone marrow and spleens were harvested from euthanized mice. To prepare single cell suspensions, spleens were sliced into small pieces with a scalpel, and the pieces placed at the ends of autoclaved frosted-glass microscope slides (Fisher Scientific, Pittsburgh, PA), previously immersed in Hank's balanced salt solution (HBSS; Gibco/Invitrogen, Carlsbad, CA) with 10% FCS. Splenic fragments were disrupted by opposing the slide ends and applying gentle pressure in round circular movements. The resulting splenic cell suspension was collected in a Petri dish and finally run over a 70 μ m nylon cell strainer (BD Biosciences, San Diego, CA). For bone marrow cells, single cell suspensions were prepared by running bone marrow cells resuspended in HBSS with 10% FCS over a 70 μ m nylon cell strainer. Cells from the three mice were pooled. Erythrocytes were eliminated using lysing solution (BD Biosciences, San Diego, CA). The remaining hematopoietic cells were washed and resuspended in PBS supplemented with 2% FCS, 1% bovine serum albumin, and 0.1% NaN₃ (FACS buffer). Cells were blocked with Mouse Fc Block (BD

**Figure 6.** Schema for Murine Model of *C. albicans* Gastrointestinal Colonization and Dissemination after Administration of Immunosuppression

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Biosciences, San Diego, CA) at 1 μ g per 10^6 cells for 5 minutes at 4°C. Alexa-488-conjugated antibody to the mouse macrophage cell surface glycoprotein F4/80 (mAb BM8, Caltag/Invitrogen, Carlsbad, CA) and/or phycoerythrin-conjugated antibody to mouse CD11b (BD Biosciences, San Diego, CA) at 10 μ g/ml/test were added to 1×10^6 cells in 100 μ l of FACS buffer then incubated at 4°C for 30 min followed by washing with FACS buffer. To determine background levels of fluorescence, non-antigen specific isotype controls (Alexa-488-conjugated-IgG_{2a} or phycoerythrin-conjugated-IgG_{2b} antibodies) were used as primary antibodies. After staining, cells were fixed with 1% paraformaldehyde. Fluorescence was detected on a Dako MoFlo High Performance Cell Sorter (Dako, Fort Collins, CO), and data analysis was performed using Summit Software v 4.3 (Dako, Fort Collins, CO). Gates were set to include CD11b and F4/80 double positive cells.

Livers and spleens from mice inoculated IP with 200 μ l of PBS liposomes or CL₂MBP liposomes (2 mg) 48 h prior to sacrifice were resected, fixed in Bouin's solution, and paraffin-embedded sections cut and mounted on slides. 5 μ m sections of hepatic and splenic tissue were deparaffinized in xylene and rehydrated in a series of reverse ethanol washes (100%, 95%, and 80% ethanol sequentially). The samples were then blocked by incubation in histology blocking buffer (PBS containing 1% bovine serum albumin and 2% normal rat serum [Sigma-Aldrich]) for 15 min at 37°C. Samples were then incubated with 1 μ g Alexa-488-conjugated antibody to the macrophage F4/80 antigen/ml for 90 min at 37°C. To detect background fluorescence, an appropriate isotype control antibody (1 μ g rat IgG_{2a}/ml) was used. The samples were then washed and sections visualized with the 10 \times alpha-plan lens on a Zeiss Axioplan 2 microscope, fitted with filter sets for GFP fluorescence, and a cooled CCD Hamamatsu Orca camera. Images were acquired and processed using MetaMorph (Molecular Devices Corporation, Sunnyvale, CA) and Adobe Photoshop software [62].

In vivo gastrointestinal mucosal disruption. To verify we had induced GI mucosal damage/disruption with dextran sodium sulfate (DSS), C3H/HeN mice (6- to 8-wk-old females) colonized with *C. albicans* strain SC5314 and maintained on sterile water with streptomycin, penicillin, and gentamicin to prevent bacterial recontamination were divided into four groups and sacrificed for cecal histology after the following specific treatments: Group A was given RB6-8C5 mAb once; Group B was given 5% DSS for 3 d; Group C was given 5% DSS for 7 d; and Group D was given 5% DSS water for 7 d, and after the third day of DSS water, mice were given mAb RB6-8C5 once, and observed for 96 h after the RB6-8C5 dose. Ceca were resected and immediately fixed in Bouin's solution. Sections were stained with hematoxylin and eosin and reviewed by a veterinary pathologist.

Translocation studies. To determine if the *C. albicans* colonizing the GI tract was translocating to extra-gastrointestinal sites, mice were colonized with *C. albicans* strain SC5314 using the protocol described above then organized into the following four groups containing 24

Table 4. Translocation Study

Group	<i>C. albicans</i> Strain	Immunosuppression	Route	Dose	Frequency
A	SC5314	None	—	—	—
B	SC5314	RB6-8C5 monoclonal antibody (mAb)	IP	200 µg	Once
C	SC5314	RB6-8C5 mAb, methotrexate (MTX)	IP, IP	200 µg, 150 mg/kg/dose	Once, once
D	SC5314	Cyclophosphamide (Cy)	IP	150 mg/kg/dose	Three times every other day

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mice per group: Group A, no immunosuppression; Group B, neutropenia induced by IP injection of 200 µg RB6-8C5 mAb once; Group C, neutropenia and GI mucosal disruption induced by one IP injection of 200 µg RB6-8C5 mAb and a contemporary, single IP injection of 150 mg/kg MTX; and Group D, neutropenia and mucosal damage induced by three IP injections of 150 mg/kg Cy given every other day (Table 4). Four mice from each group were sacrificed on days 0, 1, 2, 3, 4, and 5 after immunosuppression was initiated. Blood (100 µl) was drawn from an ethanol-cleaned tail vein and spread-plated onto YPD agar with vancomycin and gentamicin, TSA, and MacConkey agar plates. Mice were then sacrificed. Mesenteric lymph nodes (MLN), spleens, and livers were immediately resected; organs were homogenized in 1 ml of 1% protease peptone; and 100 µl of homogenate was spread-plated on YPD agar with vancomycin and gentamicin, TSA, and MacConkey agar plates. The presence of a homogeneous population of creamy-white colonies on YPD with gentamicin and vancomycin were used for confirmation of the presence of *C. albicans*. Serial dilutions and plating for fungal enumeration were also done to obtain quantitative data (CFU per gram tissue and CFU per ml blood).

Statistical analyses. Survival data were analyzed by Fisher's exact test and the survival curve was analyzed by the Kaplan-Meier log rank test using the GraphPad Prism software (San Diego, CA). Two-way comparisons of GI colonization levels were carried out using the Mann-Whitney tests and Prism software, and when multiple comparisons or more than two groups were analyzed, Bonferroni's correction to the significance level α was invoked.

Supporting Information

Figure S1. Immunohistochemical Staining with Alexa-488 Anti-Mouse F4/80 of Murine Spleens and Liver after Liposomal Clodronate Administration

- (A) Isotype control (Alexa-488 rat IgG_{2a}) staining of normal murine spleen.
 (B) Alexa-488 rat anti-mouse F4/80 staining of normal murine spleen.
 (C) Isotype control (Alexa-488 rat IgG_{2a}) staining of normal murine spleen 48 h after IP clodronate.
 (D) Alexa-488 rat anti-mouse F4/80 staining of murine spleen 48 h after IP clodronate.
 (E) Isotype control (Alexa-488 rat IgG_{2a}) staining of normal murine liver.

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(F) Alexa-488 rat anti-mouse F4/80 staining of normal murine liver.
 (G) Isotype control (Alexa-488 rat IgG_{2a}) staining of normal murine liver 48 h after IP clodronate.

(H) Alexa-488 rat anti-mouse F4/80 staining of murine liver 48 h after IP clodronate. Magnification: Objective 10X.

Found at doi:10.1371/journal.ppat.0040035.sg001 (3.9 MB TIF).

Figure S2. Expression of F4/80 and CD11b on Murine (C3H/HeN) Bone Marrow and Spleen Cells

Alexa-488-conjugated antibody to the mouse macrophage cell surface glycoprotein F4/80 and/or phycoerythrin-conjugated antibody to mouse CD11b were used. Cells from three mice were pooled for each analysis.

- (A) Bone marrow from normal mice.
 (B) Bone marrow from mice treated with liposomal clodronate 200 µl (2 mg) 48 h prior to analysis.
 (C) Splenocytes from normal mice.
 (D) Splenocytes from mice treated with liposomal clodronate 200 µl (2 mg) 48 h prior.

Found at doi:10.1371/journal.ppat.0040035.sg002 (4.9 MB TIF).

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Author contributions. AYK conceived and designed the experiments, performed the experiments, analyzed the data, and wrote the paper. JRK provided *C. albicans* microbiological instruction and support. KTC performed the experiments. NVR provided the liposomal clodronate and instruction on dosing and methods for verification of macrophage depletion. GPB conceived and designed the experiments and analyzed the data.

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Competing interests. The authors have declared that no competing interests exist.

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