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Galectin-3 Expression and Effect of Supplementation in Neonatal Mice with Disseminated *Candida albicans* Infection

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Abstract

Background: Invasive candidiasis is an important cause of fungal infections in immunocompromised patients, including premature infants. The S-type lectin, galectin-3 (gal3), is increasingly recognized for its role in antifungal host defense. This study tested the hypothesis that tissue gal3 expression is affected by disseminated infection with *Candida albicans* and that supplementation with gal3 will provide a benefit in this setting.

Methods: To determine the expression of gal3 at the tissue level in response to disseminated infection with *C. albicans*, adult and neonatal mice were infected using previously established models. End points were chosen that reflected substantive tissue fungal burden but before mortality.

Results: No differences in gal3 were detected in tissues of adult animals relative to uninfected controls. In neonatal animals, gal3 concentration was lower in the spleen of infected animals compared to uninfected. Pretreatment of neonatal mice with recombinant gal3 was associated with reduced mortality and reduced fungal burden in the kidney, spleen and lung at 24 hours following infection.

Conclusion: These findings suggest that gal3 has an active role in host defense against candidiasis and that neonatal animals can benefit from supplementation with this lectin in the setting of disseminated candidiasis.

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AUTHOR CONTRIBUTIONS

Each author has met the authorship requirements as follows: Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data (PV, SSL-N, RT, QM, MED, JMB); Drafting the article or revising it critically for important intellectual content (PV, RT, MED, JMB); and Final approval of the version to be published (PV, SSL-N, RT, QM, MED, JMB). Current affiliation for PV: Children's Hospital Medical Center, Cincinnati, OH

DISCLOSURE

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INTRODUCTION

Disseminated candidiasis remains an important problem among the immunocompromised. Premature infants are among those at risk, and *Candida albicans* is the etiological agent in up to 70% of cases. (1) Despite antifungal treatment, mortality is common and neurodevelopmental impairment occurs in the majority of survivors. (1, 2) The susceptibility of these infants is likely through mechanisms that differ from other populations at risk. (3) Identifying the complex host defense mechanisms against these infections that may be amenable to modification in favor of the host will advance insight of patient susceptibilities and hasten the development of novel therapeutics.

The host immune system responds to invading pathogens via recognition of specific pathogen associated molecular pattern (PAMPs). Fungal PAMPs are generally components of the carbohydrate rich cell wall and include mannan and beta-glucan structures. These PAMPs are identified by pathogen recognition receptors (PRRs) which are found in a wide assortment of effector cells and involve a variety of receptor types including toll-like receptors (TLRs), integrins, and lectin receptors. (4, 5)

The S-type lectin receptor, galectin-3 (gal3), is one of a family of β-galactoside-binding lectins, and has an increasingly apparent role in infection and inflammation. (6, 7) Among its complex and diverse functions, it is involved in the host response to fungi, in part through its recognition of β-(1-2)-linked oligomannans, which are a component of the carbohydrate-rich cell wall in *C. albicans*. (8, 9) Gal3 is expressed at cell surfaces, extracellular matrix and in cell secretions by activated macrophages and damaged cells. (10, 11) It plays an important role in differentiating nonpathogenic from pathogenic fungi. (12, 13) By recognizing similar antigenic elements on a microbe and host cell, but only targeting the microbe; it helps the immune system target those microbes which otherwise would have evaded the immune system due to molecular mimicry. (14-16)

Previous studies have supported a role for gal3 in host defense against disseminated candidiasis. (17, 18) Mice deficient in gal3 had a higher mortality than wild-type mice when infected via tail vein with *C. albicans*. In addition, the fungal burden was higher on day 3 and the distribution of fungal elements in the kidneys was more diffuse in gal3 deficient mice. (17) A potential role for gal3 in susceptibility of preterm infants to candidiasis was suggested by the observation that term and preterm infant express lower gal3 expression in cord blood as compared to adults; and their gal3 expression correlates directly with gestational age. (19) To test the hypothesis that a deficient gal3 response contributes to the susceptibility of premature infants to disseminated candidiasis, murine models were used in the current study to compare the tissue gal3 response in adult and newborn mice infected with *C. albicans*. We also explored whether supplementation of gal3 could attenuate disease in disseminated neonatal candidiasis.

METHODS

Growth, maintenance, and preparation of organisms

C. albicans strain SC5314 was used throughout this study. Yeast cultures were maintained on yeast extract, peptone, dextrose (YPD) plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar). Yeast for injection were prepared by growth for 16 h at 37°C with vigorous aeration in YPD broth. Cells were collected by centrifugation, washed in sterile, hospital grade-saline, enumerated by hemacytometer and adjusted to the desired concentration for injection as described below.

Animal models

All animal studies were reviewed and approved by the Lifespan Institutional Animal Care and Use Committee, which oversees the animal care facility where animals were housed for this study. For adult experiments 4-8 week-old wild-type female BALB/c mice were obtained from Charles River Laboratories (Shrewsbury, MA). They were maintained in standard animal facility conditions with unlimited access to food and water. Mice were randomized to receive either 1×10^5 cfu of *C. albicans* or vehicle (sterile saline) by tail-vein injection of 200 μl. Animals were euthanized 48 hours post infection.

For neonatal experiments timed-pregnant 4-6 week-old BALB/c mice were obtained. Pregnant dams were maintained in individual cages with unlimited access to food and water. Mice were monitored to determine the date of parturition. On day 2 following delivery, pups were randomized to receive intraperitoneal (i.p.) injections in 20 μl of either 5×10^6 cfu of *C. albicans* or vehicle (sterile saline). Animals were euthanized 24 hours post infection. In a subsequent experiment, pups were delivered and randomized to receive 5 μg carrier-free recombinant mouse gal3 (R&D Systems, Minneapolis, MN) or saline in 20 μl i.p. injection prior to infection with *C. albicans* as described above. This dose of gal3 was selected because it far exceeds the physiologic concentration of gal3 in mouse pups and would therefore maximize the likelihood of detecting an effect should one exist. The timing of infection with *C. albicans* following gal3 administration was as short as possible given the logistics of administration and allowing for a short recovery period between i.p. injections. These pups were monitored closely every 3-8 h for signs of illness and were euthanized if moribund. Surviving pups were euthanized at 72 h following infection. To better elucidate the kinetics of dissemination, a time-course experiment was also performed. Pups (n=5 per treatment group for each time point) were administered gal-3 or saline and infected as described above, and then euthanized at 24 and 36 hours after infection.

In all experiments, at the time of death or euthanasia, organs (kidney, liver, spleen, lung, and brain) were harvested and serum was collected from individual mice. In neonatal mice, sera were pooled from animals in the same group because of low volumes from individual pups. Organs were homogenized by a FastPrep-24 Instrument (MP Biomedical, Inc., Solon, OH) using Lysing Matrix D (Qbiogene; MP Biomedical, Inc.) in 1-mL sterile saline, and appropriate dilutions were plated on YPD containing streptomycin (100 μg/ml) and ampicillin (50 μg/ml). Colonies were enumerated after an overnight incubation at 37°C.

Fungal burden was expressed as the colony forming units (CFU)/ml/gram of harvested organ.

Quantification of Galectin-3, Chemokines and Cytokines

Gal3 levels were quantified using a commercially available gal3 Mouse ELISA kit (Abcam, Cambridge, MA) according to the manufacturer's instructions. Cytokines and chemokines in tissue homogenates were quantified using the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay according to the manufacturer's instructions and analyzed using a Bio-Plex 200 instrument (Bio-Rad, Hercules, CA). The following analytes were interrogated: Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, KC, MCP-1 (MCAF), MIP-1α, MIP-1β, RANTES and TNF-α.

Statistical Analysis

Gal3 concentration in tissues was analyzed by ANOVA and inter-group comparisons were made by the Student-Newman-Keuls Method. Differences in fungal burden were analyzed using a negative binomial model to account for the variability in these data. Survival analysis was by log-rank test. Cytokine/chemokine expression was compared in gal3-treated and control animals by t-test. Sigma plot version 13.0 and SAS 9.3 (SAS Institute, Cary, NC) were used for statistical calculations. P values < 0.05 were considered significant.

RESULTS

Galectin-3 response to disseminated candidiasis in adult mice

Adult mice were injected via tail-vein with *C. albicans* or with vehicle (sterile saline) and were euthanized 48 h after infection. Fungal burden and gal3 were measured in tissue homogenates and gal3 was also measured in serum. The infective dose and time point were selected to represent a time when the infection would be well established but before the onset of mortality, based on previous work with this model. Fungal burden was highest in kidney followed by the spleen, which was consistent with previous experiments (Figure 1A). Despite differences in fungal burden, the concentration of gal3 in serum, kidney, liver, spleen, lung and brain was very similar among infected vs uninfected animals (Figure 1B).

Galectin-3 response to disseminated candidiasis in neonatal mice

Two-day-old mouse pups were injected intraperitoneally with *C. albicans* or with vehicle (sterile saline), euthanized at 24 h, and fungal burden and gal3 were measured in tissue homogenates. Gal3 was also measured in a single pooled serum sample from all the pups in each group. As with the adult model, the infective dose and time point were selected to represent a time when the infection would be well established but before the onset of mortality, based on previous work with this model. Fungal burden was highest in spleen and liver, which is characteristic of this model (Figure 2A). Overall, gal3 expression was similar among infected vs control pups, with the exception of the spleen, where gal3 expression was significantly reduced in infected relative to control pups ($p=0.04$).

Effect of supplementation with recombinant galectin-3 in neonatal mice infected with *C. albicans*

The reduction in gal3 in spleens of mouse pups infected with *C. albicans* together with the previous observation that gal3 deficient mice have increased susceptibility to *C. albicans* infection (17) led us to hypothesize that supplementation with gal3 may afford an advantage to infected neonatal mice. To test this hypothesis, mouse pups were injected intraperitoneally with recombinant gal3 or vehicle followed by infection with *C. albicans* 2 hours later. Animals were closely observed following infection and surviving animals were euthanized at 72 hours. Organs were harvested at the time of death or at 72 hours when they were euthanized. Treatment with recombinant gal3 led to a significant decrease in mortality (100% to 60%) with an increase in median survival from 36 to 70 h as compared to control (Figure 3, p=0.02). Uninfected pups that received only gal3, had no signs of illness. Fungal burden at the time of death is depicted in Figure 4. Although trends toward reduced fungal burden with gal3 treatment could be identified, the only significant reduction in fungal burden at the time of death was seen in the lungs (median 8.7×10^4 cfu/g in gal3 group vs. 1.1×10^5 cfu/g in control, p=0.048).

Determination of tissue fungal burden at the time of death (or euthanasia) represents a heterogeneous sample in terms of time from infection. These data are therefore unlikely to capture the kinetics of disease progression, particularly considering the difference in survival curves between gal3-treated and control animals (Figure 3). To better delineate the kinetics of infection, the experiment was repeated and groups of animal were euthanized at specific time points following infection. Fungal burden in each tissue at 24 hours and at 36 hours are depicted in Figure 5. One animal from each group assigned to the 36 hour time point died prior to 36 hours and was excluded. Significant reductions in fungal burden were noted in the kidney, spleen and lung at 24 hours. The reduction in the kidney was still noted at 36 hours, but the lung had increased fungal burden in the gal3 group at 36 hours. Fungal burden in the brain was not detectable in the majority of animals at these time points. As expected, the fungal burden at the time of death (Figure 4) was greater in all tissues examined than was seen at these time points.

To provide a broad assessment of alterations in the inflammatory response associated with gal3 supplementation, a multiplex cytokine/chemokine assay was performed on the kidney and spleen at 24 and 36 hours to capture the time points that showed reduced fungal burden with gal3 treatment. No statistically significant differences were found for any of the 23 proteins measured at either time point (data not shown). Three proteins showed a trend toward lower levels in the spleens harvested at 24 hours in gal3-treated mice relative to untreated; G-CSF (mean 499 pg/ml vs. 1812 pg/ml, p=0.057), KC (mean 203 pg/ml vs. 416 pg/ml, p=0.077) and MIP-1α (mean 21 pg/ml vs. 203 pg/ml, p=0.089). At the 36 hour time point splenic concentrations of each of these proteins had decreased in the control animals and were more similar between gal3-treated and control animals.

DISCUSSION

Disseminated candidiasis is a life threatening complication among immunocompromised patients. These infections occur in the setting of severe illness requiring intensive care, in

patients with malignancy and undergoing chemotherapy, and in bone-marrow transplant units (20). The importance of the neutrophil in host defense is apparent since neutrophil dysfunction is associated with increased risk, either due to neutropenia or functional impairments in neutrophil effector mechanisms (21). Our focus has been on prematurity as a risk factor for disseminated candidiasis. The characteristics of this patient population that put them at risk are likely to be unique relative to other immunocompromised patients, and therefore potentially informative in the development of strategies to reduce risk. The observation that infections caused by the non-*albicans* species, *C. parapsilosis*, are more common in premature infants than in other populations at risk may be one manifestation of this unique host-pathogen interface (22). Additionally, despite the known importance of neutrophil dysfunction, preterm infants are rarely neutropenic when they develop these infections. Further, studies of ex vivo neutrophils isolated from cord blood of term and preterm infants found no differences in either phagocytosis or the capacity to generate an oxidative burst when co-incubated with *C. albicans* or *C. parapsilosis* (23). These findings were the basis for the hypothesis that premature infants manifest risk for disseminated candidiasis due to alterations in the developing immune system that indirectly impact neutrophil function. Gal3 is one such candidate. In a variety of experimental settings, gal3 has been shown to be a chemoattractant and to function as an opsonin (24, 25). In regard to neutrophil function, exogenous gal3 improves migration, phagocytosis, and the production of reactive oxygen species and cytokines (26-30). In a mouse model gal3 deficient mice infected with a low-lethal dose of *C. albicans* or with *C. parapsilosis* had more severe disease manifestations than wild-type mice (17). Finally, we and others have found reduced gal3 concentrations in infant serum relative to healthy adults (17, 19).

In the current study, we sought to examine the gal3 response to disseminated candidiasis in adult and infant mice at the tissue level. There are important differences between the adult and neonatal models employed in this study. The overall goal was to examine gal3 expression at a time point in each model when they would have substantial fungal burden but before death. The intraperitoneal route was used in neonatal mice because it more closely mimics the mechanism of dissemination that occurs following peritoneal seeding of *Candida* with intestinal perforation in human preterm infants; an important and common route of infection in this population. This route is also technically more feasible. When adult animals are infected intraperitoneally, they clear the infection with limited dissemination and no mortality (31), so the intravenous route was used to ensure disseminated disease. Adults were euthanized at 48 h and neonates at 24 h based on experience with each model and our goal for measurable fungal burden and no mortality. Because of these limitations, however, comparisons between adult and newborn animals are unlikely to be meaningful, and we focused our analysis on infected vs. uninfected animals in each group.

Adult animals infected intravenously did not manifest any differences in tissue concentrations of gal3 compared to uninfected animals at the infective dose and time point studied. Because this study interrogated gal3 expression at the tissue level rather than at the cellular or molecular level, a lack of change in gal3 with infection does not imply that the lectin lacks a role in the host response. On the contrary, a role for gal3 is well supported by the literature and is likely to be quite complex. A role for gal3 in discrimination between pathogenic yeast such as *C. albicans* and the non-pathogenic *Saccharomyces cerevisiae* was

suggested by a study of macrophage responses. TNF-α production was more strongly induced by *C. albicans* than *S. cerevisiae* and gal3 in association with TLR2 was required for this effect.(12) The importance of gal3 in fungal recognition and response in coordination with both TLR2 and dectin-1 was consistent in other experimental settings.(13, 32) Studies of gal3 in neutrophils suggest that the effects of extracellular and intracellular gal3 may differ. Whereas exogenous gal3 augments recruitment, migration, phagocytosis and the production of IL-8 and reactive oxygen species, (18, 26, 30, 33) intracellular gal3 has been shown to negatively regulate ROS-dependent killing of *C. albicans* induced by complement receptor 3 (CR3).(34) Using neutrophil adoptive transfer, this study also suggested that intracellular gal3 down-regulates neutrophil effector functions. Further, gal3 deficient mice had reduced mortality and tissue fungal burden than wild-type animals as had also been seen in a previous study.(35) These outcomes in gal3 deficient mice are at odds with our results showing gal3 deficient mice to be more susceptible to mortality and higher fungal burdens than wild-type (17) and with the results of a study involving mannosylation mutants that also showed increased virulence of the wild-type strain in gal3-deficient mice following i.p. injection.(36) As suggested by the authors, the discrepant findings may reflect differences in the derivation of the knockout mouse strain and/or effect of different infective *C. albicans* strains, routes or doses. Our study employed a “low-lethal dose” that was 5 to 10-fold less than that used in the other studies.

A number of additional studies of host defense against fungal pathogens have supported a positive role for gal3. A role for gal3 has been identified in generation of a protective Th-17 response in experimental models of both candidiasis and crytococcosis (37, 38). It has also been shown to trigger TNF-α production by macrophages and contribute to a fungicidal effect (9). Similar to our studies with disseminated candidiasis in mice, gal3 deficient mice had increased mortality when infected with *Cryptococcus neoformans* (38). These mice also lacked the increase in IL-17/IL-23 cytokines that was seen in wild type animals.

The notion that exogenous gal3 may promote effector functions against candidiasis is well supported by our observations with the neonatal mouse model. Infection of mouse pups with *C. albicans* was associated with decreased expression of gal3 in the spleen. Because mouse pups were infected intraperitoneally, the spleen is likely to be an important route of dissemination in this model. This hypothesis is supported by the observation that the spleen bears the highest fungal burden, and by previous work with this model showing hyphal elements penetrating the spleen capsule by microscopy (39). These findings led us to speculate that *C. albicans* may deplete splenic gal3, leading to increased fungal burden and promoting dissemination, and that supplementation of gal3 may reduce disease. Administration of exogenous gal3 substantially reduced mortality in subsequent experiments. Futhermore, the reduction in mortality was associated with reduced fungal burden in kidney, spleen and lung at 24 hours after infection that persisted to 36 hours in the kidney. Together, these data suggest that gal3 supplementation delayed the progression of infection in these animals.

The mechanism by which gal3 provided benefit is likely multifactorial and is the focus of ongoing investigation. As a first step to characterize how gal3 impacts the early inflammatory response to infection, a wide array of cytokines and chemokines were assessed

in tissues showing differences in fungal burden. In general, the inflammatory profiles were similar. The majority of information regarding the inflammatory response to disseminated candidiasis in mice has come from adult animals infected via tail-vein, and data from newborn mice are extremely limited. Most relevant to the current study, evaluation of the early response to infection in adult mice has highlighted the importance of KC production and its association with progression of pathology in the kidney; the organ most consistently affected in this model (40). Given its role in neutrophil recruitment (41), it is likely that KC expression is important in recruiting leukocytes to the source of infection. The trend toward reduced KC and G-CSF associated with reduced fungal burden that we observed in the spleen at 24 hours suggests that the reduction in viable yeast may not be due to more efficient neutrophil recruitment or function but rather may reflect less stimulation for neutrophil recruitment. Because gal3 can directly bind to and kill *C. albicans* through recognition of β-1,2-linked oligomannans (9), this interaction represents one possible mechanism by which fungal growth may have been inhibited, reducing fungal burden and leading to less inflammatory stimulus. Given the multifaceted effects reported for exogenous gal3 on various components of the innate immune system, multiple mechanisms are possible and likely. Additional experiments focused at the cellular and molecular level within different tissues will better inform the mechanisms at play. Further, defining the timing at which administration of gal3 will alter the course of infection is an important element with potential clinical implications.

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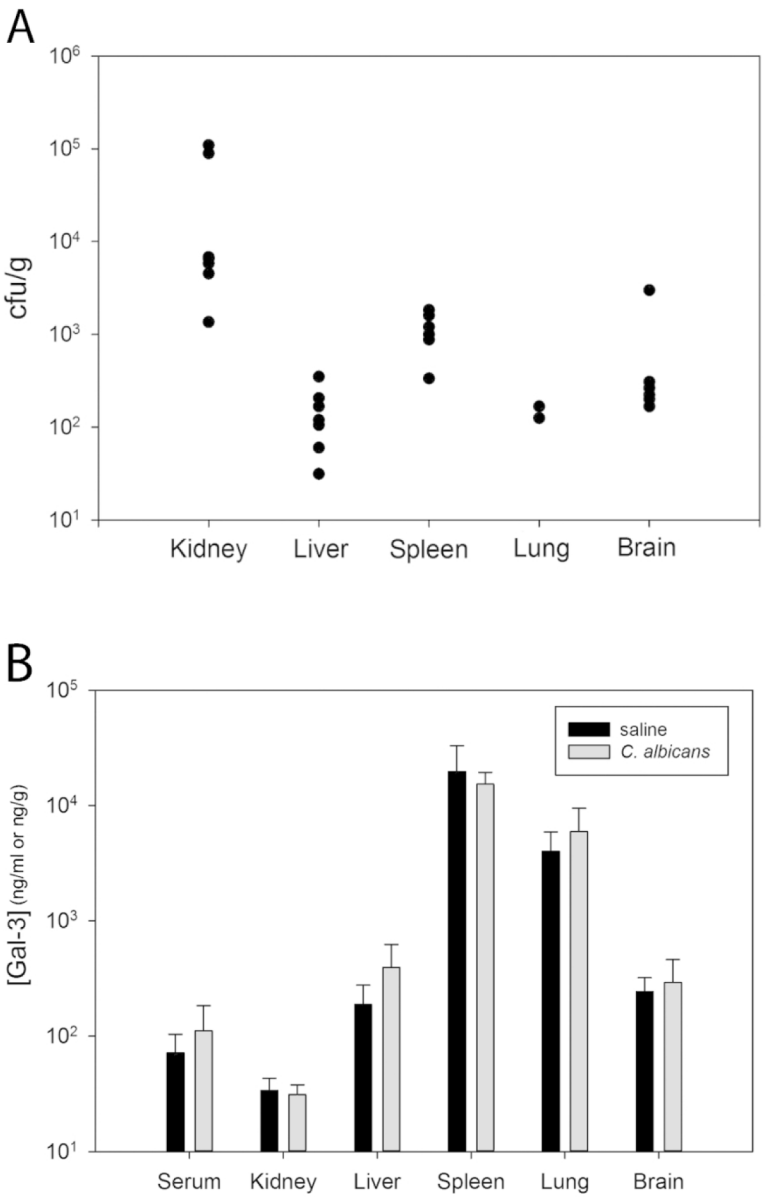

Figure 1.

Tissue fungal burden and galectin-3 concentrations in adult mice with disseminated candidiasis. Mice (n=7) were infected via tail-vein injection with *C. albicans* and euthanized at 48 h after injection. Panel A: Tissue fungal burden. Panel B: Mean gal3 concentration in tissue homogenates compared to animals receiving saline (n=10). Error bars represent standard deviation. No differences in tissue gal3 concentration were detected by ANOVA with inter-group comparisons by the Student-Newman-Keuls Method.

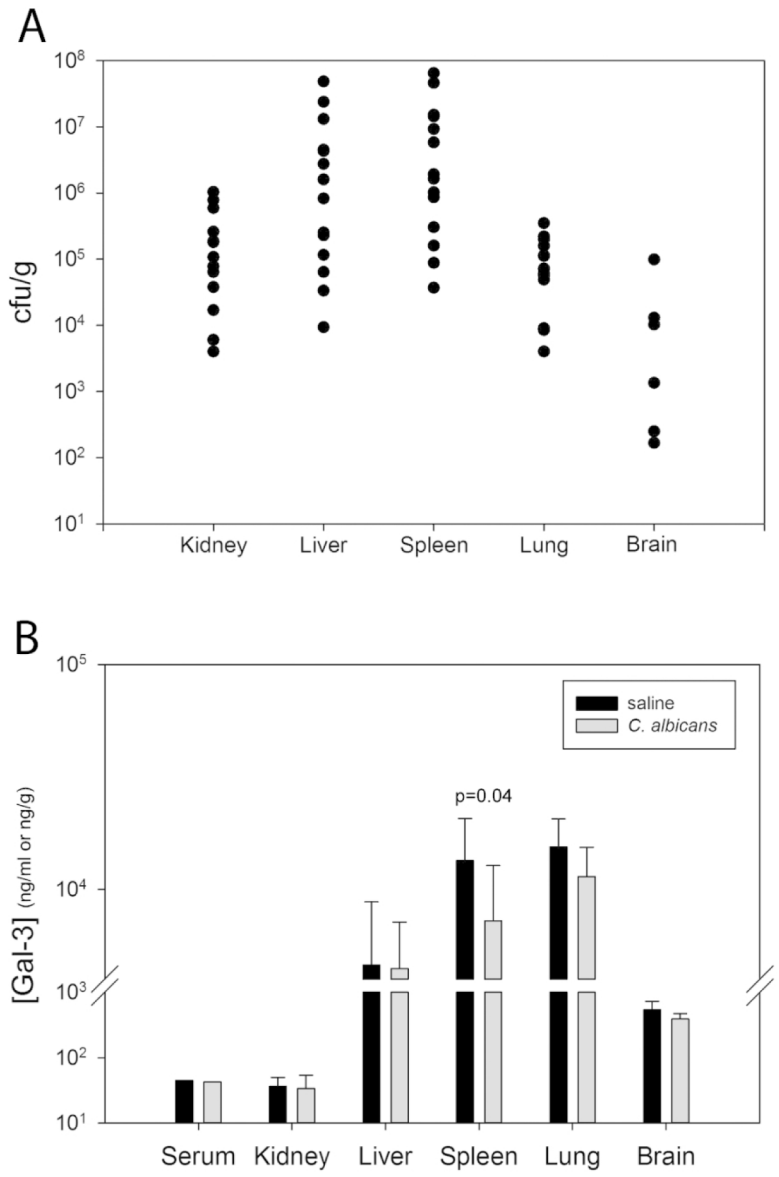

Figure 2.

Tissue fungal burden and galectin-3 concentrations in neonatal mice with disseminated candidiasis. Two-day-old mouse pups (n=14) were infected via intraperitoneal injection with *C. albicans* and euthanized at 24 h after injection. Panel A: Tissue fungal burden. Panel B: Mean gal3 concentration in tissue homogenates compared to pups receiving saline (n=14). Error bars represent standard deviation. Mean gal3 concentration was reduced in the spleen of infected pups relative to controls based on ANOVA with inter-group comparisons by the Student-Newman-Keuls Method (p=0.04).

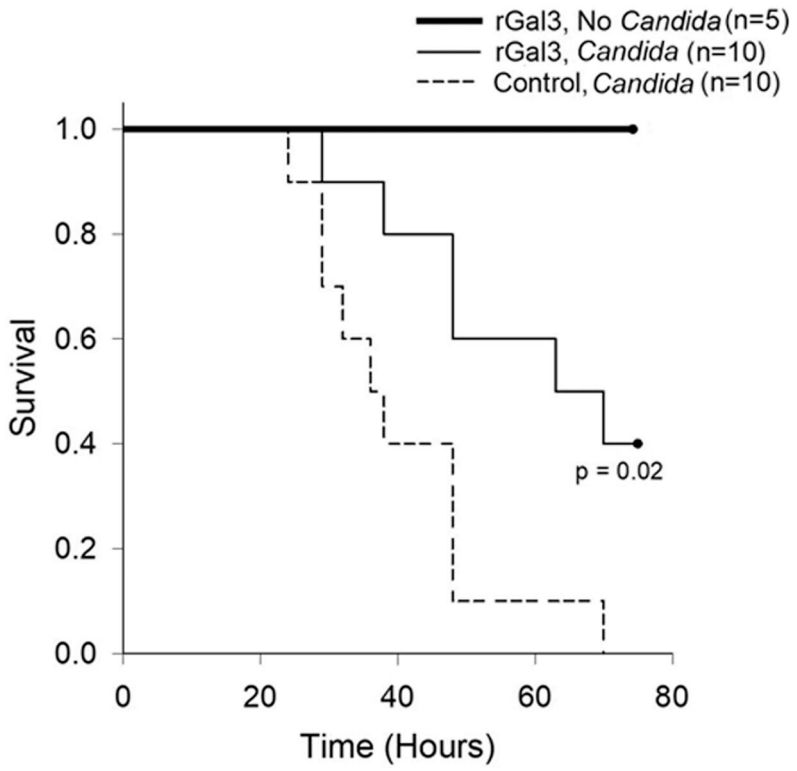

Figure 3.

Survival curve of neonatal mice with disseminated candidiasis after pretreatment with recombinant galectin-3. Two-day-old mouse pups were given intraperitoneal injections of either saline or recombinant gal3, 2 h prior to infection with *C. albicans*. Uninfected pups receiving gal3 only were included as a control. Pretreatment with gal3 reduced mortality in infected compared to saline treated pups based on log-rank test (p=0.02).

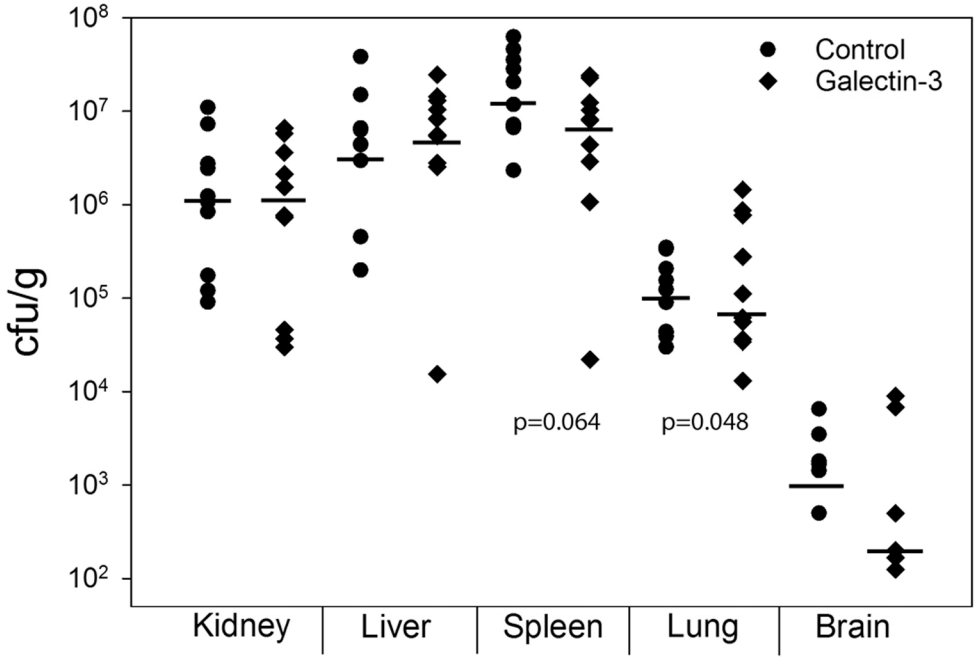

Figure 4.

Tissue fungal burden in neonatal mice with disseminated candidiasis pretreated with galectin-3. Two-day-old mouse pups were given intraperitoneal injections of either saline or recombinant gal3, 2 h prior to infection with *C. albicans* (n=10 pups per group). Tissues were collected at the time of death or at 72 h in surviving animals. Fungal burden is depicted with the bars representing median values. P values were derived based on analysis using a negative binomial model to account for the variability in these data which are not normally distributed.

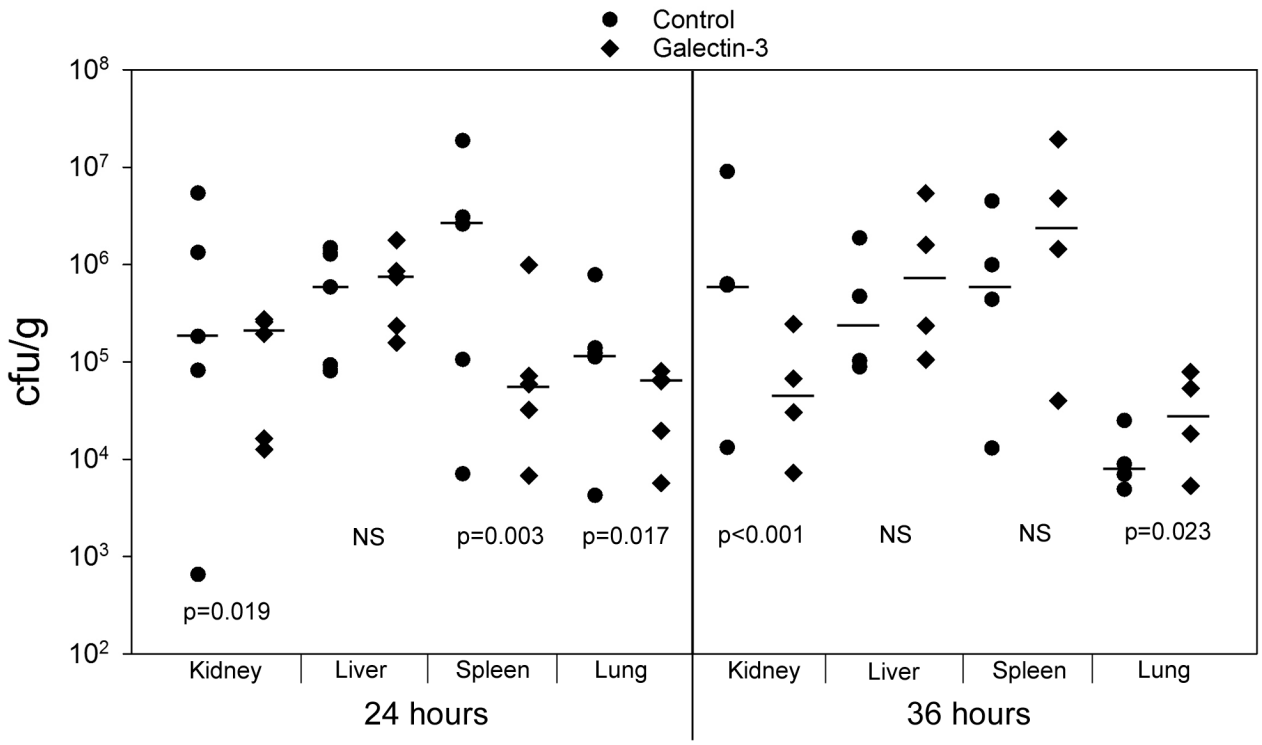

Figure 5.

Tissue fungal burden in neonatal mice with disseminated candidiasis at early time points following infection and pretreatment with galectin-3. Two-day-old mouse pups were given intraperitoneal injections of either saline or recombinant gal3, 2 h prior to infection with *C. albicans* (n=5 pups per group). Pups were euthanized and tissues were collected at 24 and 36 h following infection. Fungal burden is depicted with the bars representing median values. P values were derived based on analysis using a negative binomial model to account for the variability in these data which are not normally distributed. NS – not significant.