MyD88 and TLR2, but not TLR4, are required for host defense against Cryptococcus neoformans

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We investigated here the potential role of Toll-like receptors (TLR) and the adaptor protein MyD88 in innate immunity responses to Cryptococcus neoformans, a pathogenic encapsulated yeast. Peritoneal macrophages from MyD88–/– or TLR2–/– mice released significantly less TNF-α, compared with wild-type controls, after in vitro stimulation with whole yeasts. In contrast, no differences in TNF-α release were noted between macrophages from C3H/HeJ mice, which have a loss of function mutation in TLR4, relative to C3H/HeN controls. When MyD88- or TLR2-deficient mice were infected with low doses of the H99 serotype A strain, all of the control animals, but none of MyD88–/– and only 38% of the TLR2–/– animals survived, in association with higher fungal burden in the mutant mice. Both MyD88–/– and TLR2–/– animals showed decreased TNF-α, IL-12p40 and/or IFN-γ expression in various organs during infection. No difference in susceptibility to experimental cryptococcosis was found between C3H/HeJ mice and C3H/HeN controls. In conclusion, our data indicate that TLR2 and MyD88, but not TLR4, critically contribute to anti-cryptococcal defenses through the induction of increased TNF-α, IL-12 and IFN-γ expression.

Introduction

Cryptococcus (Filobasidiella) neoformans is an encapsulated yeast, which is a frequent cause of severe disease in patients with impaired cell-mediated immunity. In these patients, dissemination to the central nervous system often occurs as a result of reactivation of latent foci in the lung [1]. The disease has emerged as a major cause of morbidity and mortality among patients with AIDS, particularly in developing countries. Despite the availability of new antifungal drugs, permanent neurological damage is a frequent occurrence [1]. Poorly encapsulated or unencapsulated forms of C. neoformans are ubiquitously present in the environment and are often acquired by humans via the respiratory tract [2]. Phagocytosis of either encapsulated or unencapsulated yeasts is accompanied by the production of proinflammatory cytokines and chemokines [3, 4]. Control of fungal outgrowth in the lung is associated with enhanced macrophage function and increased inducible nitric oxide synthetase expression [5]. Thus, innate immunity provides a first, effective line of defense by activating inflammation and fungicidal mechanisms. In

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Abbreviations: GBS: Group B streptococci · TLR: Toll like receptor · MyD88: Myeloid differentiation factor 88 · TIR domain: Intracellular domain of the Toll/IL-1 receptor · GXM: Glucuronoxylomannan · LTA: Lipoteichoic acid

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addition, innate immunity is essential for the initiation of adaptive T cell-mediated responses. Early production of TNF-α, IL-12 and IFN-γ is associated with the development of strong type 1 T helper cell (Th1) responses and clearance of infection [6]. In contrast, weak or delayed inflammatory reactions are associated with ineffective Th2 responses and chronic infection [7]. In humans, disseminated cryptococcal pneumonia can develop after anti-TNF-α administration for treatment of rheumatoid arthritis, confirming the protective role of TNF-α [8].

Little is known of the receptors through which cryptococci are recognized by host cells and trigger innate immune responses. Moreover, the downstream pathways leading to the production and release of TNF-α and other proinflammatory cytokines are unclear. Recent data have suggested an essential role of Toll-like receptors (TLR) in the recognition of microbial pathogens, including fungi [9–11]. These receptors have been characterized as a family of evolutionary conserved type I transmembrane proteins displaying leucin-rich extracellular domains. The cytoplasmic portion of TLR is homologous to the intracellular signaling domain of IL-1R or IL-18R (the so-called TIR domain). Intracellular pathways are activated by TLR through homologous interactions of their TIR domains with those of adaptor proteins, the best characterized of which is myeloid differentiation factor 88 (MyD88). Subsequent activation of NF-κB and MAPK leads to cytokine secretion and the surface expression of co-stimulatory and MHC molecules. By these mechanisms, TLR participate in the recognition of fungal pathogens depending on the fungal species, morphotype and site of infection [10, 11]. Thus, TLR2 is activated by a number of ligands, including bacterial lipoproteins and soluble peptidoglycan [9, 12, 13]. Different TLR are activated by different ligands in a highly specific way. For example, TLR4, in association with MD2 and CD14, recognizes the LPS component of the cell wall of gram-negative bacteria, while TLR2 is activated by a number of ligands, including bacterial lipoproteins and soluble peptidoglycan [9, 12, 13]. Different TLR appear to be involved in the recognition of fungal pathogens depending on the fungal species, morphotype and site of infection [10, 11]. Thus, TLR2 is required for TNF-α production by mouse macrophages in response to Candida albicans yeasts or hyphae [14, 15] and to Aspergillus fumigatus conidia or hyphae [16]. Moreover, Aspergillus conidia, but not hyphae, stimulate host cells through TLR4 [16]. In vivo, there appears to be a complex situation whereby multiple TLR are involved in host responses to Candida and Aspergillus [11]. Very little is known about the role of TLR/MyD88 pathways in anti-cryptococcal defenses. Glucuronoxylomanan (GXM), the main capsular component and virulence factor of cryptococci, was shown to activate NF-κB by a TLR4-dependent pathway in phagocytes, although TNF-α production was not induced [17]. It was suggested that this may represent a mechanism of immune dysregulation whereby the organism incompletely activates cascades leading to TNF-α production [17]. Recently, the role of MyD88, TLR2, TLR4 and CD14 was analyzed in experimental murine cryptococcosis models [18]. MyD88-deficient, but not TLR2-, TLR4- or CD14-deficient mice, had higher numbers of CFU in the lungs after intranasal challenge. Since MyD88 serves as an adaptor protein not only for TLR, but also for IL-1R and IL-18R signaling, it is presently unclear whether TLR are involved in host defenses against cryptococcosis. Moreover, the mechanisms underlying the decreased ability of MyD88-deficient animals to restrict in vitro growth could not be elucidated [18]. Therefore, two important questions remain unanswered: (1) are TLR involved in anti-cryptococcal defenses?; (2) is MyD88 required for the production of proinflammatory cytokines known to be critical for such defenses? In the present study we have investigated the role of TLR2, TLR4 and MyD88 in experimental cryptococcosis. It was found that TLR2 and MyD88, but not TLR4, contribute to anti-cryptococcal defenses through the induction of TNF-α, IL-12, and IFN-γ.

Results

2.1 MyD88 is required for in vitro TNF-α responses to C. neoformans

In initial experiments, we tested resident peritoneal macrophages from wild-type (WT) mice (C57BL/6) for their ability to produce TNF-α after stimulation with cryptococci. Since the capsule is an important virulence factor, the encapsulated strains H99 (serotype A) and B-3501 (serotype D), as well as an unencapsulated B-3501-derived mutant (CAP67), were used as stimuli. Heat-killed group B streptococci (GBS) were used as a control, since they were found to induce strong MyD88-dependent responses [19]. TNF-α was undetectable in supernatants from unstimulated cells (not shown). However, all of the cryptococcal preparations produced detectable TNF-α elevations, although the levels attained were considerably lower than those induced by GBS, used as a positive control (Fig. 1A). Since the highly virulent H99 strain induced weaker responses than either B-3501 or CAP67, (Fig. 1A), only the latter two strains were used in further experiments.

To investigate the role of TLR2 and MyD88, macrophages from MyD88- or TLR2-deficient mice were stimulated with live B-3501 or CAP67. Using either stimulus, TNF-α levels were significantly decreased in MyD88- or TLR2-deficient peritoneal macrophages, relative to WT controls (Fig. 1B). There was a tendency for TNF-α responses to be more severely affected by
MyD88 deficiency, relative to TLR2 deficiency. As expected, lipoteichoic acid (LTA), a known TLR2-dependent stimulus [20], produced significant TNF-α elevations in WT, but not in TLR2- or MyD88-deficient cells (Fig. 1B). In contrast, the TLR2-independent GBS control could stimulate TLR2-, but not MyD88-deficient cells.

In further experiments, the role of TLR4 was investigated using macrophages from LPS hyporesponder mice (C3H/HeJ), which have a loss of function mutation in TLR4 [13]. Fig. 1C shows that no differences were found in peritoneal macrophages from C3H/HeJ and C3H/HeN control mice, concerning the ability to produce TNF-α in response to live CAP67 yeasts. Collectively, these data indicated that MyD88 and TLR2, but not TLR4, are involved in in vitro TNF-α responses to cryptococci.

In addition to TNF-α, IL-12 and IFN-γ production is considered essential for effective anti-cryptococcal defenses, particularly for the polarization of adaptive responses toward a protective Th1 type. Therefore, in further experiments, we measured IL-12p40, IL-12p70 and IFN-γ levels in supernatants from peritoneal macrophages stimulated with live CAP67 or B-3501 yeasts. However, no significant elevations in the levels of these cytokines were detected in WT C57BL/6 cells after cryptococcal stimulation (data not shown).

MyD88 or TLR2 deficiency affects host resistance to C. neoformans infection

Next, we investigated whether the above-described in vitro defects in TNF-α production were associated with altered susceptibility to cryptococcal infection. To this end, MyD88- or TLR2-deficient mice were infected with a sublethal dose (2.5 × 10⁴ CFU) of the highly virulent H99 strain and survival of the animals was monitored daily. Similarly infected C57BL/6 mice served as WT controls. As shown in Fig. 2A, under these conditions, all of the WT controls survived. In contrast, all of the MyD88−/− and 62% of the TLR2−/− animals succumbed within 60 days. Mice were observed for additional 30 days, but further deaths were not recorded (not shown). A few days before death, signs of neurological involvement (staggered gait, lethargy and unresponsiveness) were noted in both MyD88-deficient and TLR2-deficient mice. Lethality of the latter mice was significantly different from that of either MyD88-deficient or WT animals by Kaplan Meyer analysis (Fig. 2A). Thus, TLR2−/− mice showed increased susceptibility to infection, but they were not quite as susceptible as MyD88−/− mice.

In further experiments MyD88- or TLR2-deficient mice were challenged with a higher cryptococcal inoculum (1 × 10⁵ CFU). Under these conditions, all of the WT animals died within 55 days. Both MyD88 and TLR2 KO mice had shorter survival times than WT controls (p<0.004, and p<0.04, respectively), confirming their increased susceptibility to cryptococcal infection (Fig. 2B). Next, we determined whether TLR4 had a role in host defense against cryptococcosis using C3H/HeJ LPS hyporesponder mice. C3H/HeJ and C3H/HeN (WT) mice were challenged with both a low (2.0 × 10⁴ CFU) and a high (8.5 × 10⁴ CFU) dose of the
H99 strain. Using the low dose, 25% of TLR4 mutant and 12.5% percent of the WT controls died (Fig. 2C). These differences were not statistically significant. No significant differences in survival were detected also with the higher dose (Fig. 2D). These data indicate that TLR4 does not have an obligatory role in host defenses against *C. neoformans*.

To determine whether the increased susceptibility to cryptococcosis of MyD88- and TLR2-deficient mice was the result of an impaired ability to control *in vivo* yeast replication, groups of five mice were sacrificed at different times after low dose infection and CFU were measured in the brain, lungs and spleen (Fig. 3). In WT animals, cryptococci were detected in the spleen and the lungs at 3 days after i.p. inoculation and disseminated to
the brain and lungs by 7 days after challenge. Fungal burden increased until day 18 in the brain and until day 32 in the spleen and lungs, to become stable thereafter. In contrast, the number of CFU steadily increased in the organs of MyD88−/− animals throughout the observation period. As a result, significantly higher colony counts were observed in the organs of MyD88-deficient animals at all times after challenge, relative to WT controls. TLR2-deficient animals showed significantly higher colony counts at all times in the brain, but only at selected time points in the lungs (day 46) and in the spleen (days 18 and 46), relative to WT controls. These data indicated that both MyD88 and TLR2 deficiency are associated with a decreased ability to control cryptococcal growth. The effects of TLR2 deficiency appeared less pronounced than those of MyD88 deficiency, especially in the lungs and spleen.

Effects of MyD88 or TLR2 deficiency on cytokine expression during C. neoformans infection

TNF-α, IL-12p40, and IFN-γ mRNA expression was measured in the organ homogenates obtained from C. neoformans-infected mice using a sensitive real time RT PCR assay (Fig. 4). Results were expressed as n-fold increases in expression relative to uninfected mice. Four to 29-fold increases in TNF-α mRNA expression were detected in the brain and lungs of infected WT mice.

Fig. 4. Kinetics of TNF-α, IL-12p40 and IFN-γ mRNA expression in brain, lungs and spleen of WT, MyD88- and TLR2-deficient mice infected as described in Fig. 2. Total RNA was extracted from organ homogenates at the indicated times after infection and expression of cytokines was evaluated by real-time RT-PCR. Data represent n-fold increases in mRNA expression relative to uninfected animals. Data are expressed as means ± SEM of four independent observations, each conducted on a different animal. *, p<0.05 vs. WT mice by one-way analysis of variance and the Student’s-Keuls-Newman test.
between day 7 and 46 post-infection. In contrast, slight (3- to 5-fold) increases were detected in the spleen. Significantly lower TNF-α expression was detected in both MyD88- and TLR2-deficient mice, relative to WT controls, at all times in the brain, but only at one time point (day 7 post-infection) in the lungs. IL-12p40 mRNA levels moderately increased in the lungs and brain of WT mice, beginning at days 7 and 18, respectively, after challenge. A significant increase in IL-12p40 mRNA levels was detected in the spleen at day 7 only. IL-12p40 expression was significantly lower in either MyD88- or TLR2-deficient mice, relative to WT controls, in the lungs and spleen, but not in the brain. IFN-γ mRNA increased consistently in infected mice, relative to uninfected ones, beginning at day 7 (brain and lungs) or 18 (spleen) and remained elevated thereafter.

Brain IFN-γ mRNA expression was significantly decreased in TLR2- and MyD88-deficient animals relative to WT ones (Fig. 4). In the lung, however, IFN-γ expression was decreased only at day 7 after challenge in immune deficient mice. Collectively, these data indicated that MyD88 or TLR2 deficiency is associated with decreased type 1 cytokine expression during infection with C. neoformans. However, cytokine expression was not completely abrogated, indicating the existence of MyD88-independent mechanisms, especially in the spleen. Moreover, there was no difference in liver TNF-α, IL-12p40 or IFN-γ expression between infected MyD88−/− or TLR2−/− and WT mice, at all tested times (3, 7 and 32 days post-infection; data not shown). Collectively, these data indicated a crucial role of MyD88 and TLR2 in maximal type 1 cytokine responses during cryptococcal infection and a considerable degree of organ specific heterogeneity in the “MyD88 dependency” of such responses.

Discussion

Infection with C. neoformans is common in humans. Disease rarely occurs in immunocompetent individuals, but can develop under conditions associated with immune deficiency as a result of reactivation of latent foci. To test the requirement for TLR2, TLR4 and the TLR-associated adaptor protein MyD88 in control of cryptococcosis, we developed a murine model of persistent infection mimicking, to some extent, the latency status found in immunocompetent humans. In this model, a small number of virulent yeasts spreads from the inoculation site to different organs and grows locally to reach a stable plateau (Fig. 3). Under these conditions, MyD88 deficiency was associated with uncontrolled fungal proliferation in the spleen, lungs and brain while control mice remained asymptomatic. TLR2-deficient animals were also highly susceptible to infection, as shown by increased lethality and fungal load, especially in the brain. The phenotype of these animals, however, was less severe than that of MyD88−/− mice, as evidenced by increased survival and a tendency towards lower fungal load. This difference may be explained by assuming that, in addition to TLR2, other MyD88-dependent receptors, including IL-1R, IL-1R or other TLR, contribute to anti-cryptococcal defenses. While the effects of IL-1R deficiency have not been studied, a moderately increased susceptibility to cryptococcosis was observed in IL-1R-deficient mice [21]. Further studies are, therefore, required to assess the role of IL-1R and additional TLR in cryptococcal infection.

The important role described here for MyD88 in anti-cryptococcal defenses is in agreement with observations conducted with other fungal and bacterial pathogens [19, 22]. For example, in a recent study, MyD88 was required for both primary and secondary responses to Aspergillus or Candida, as shown in different animal models [11]. Our data on the central role of MyD88 in anti-cryptococcal defenses are in agreement with those of a recent study, which appeared after our study was completed [18]. However, in that report, the mechanism underlying the increased susceptibility of MyD88-deficient mice could not be elucidated. Specifically, decreased host resistance could not be associated with defective proinflammatory cytokine production in the organs of infected immune deficient animals [18]. This is not surprising since in the model used by these authors no cytokine elevations could be detected in control wild type animals in response to cryptococcal infection. In contrast, we selected in vivo and in vitro systems in which cytokine responses to cryptococci could be clearly evidenced under control conditions and found that either MyD88 or TLR2 deficiency are associated with decreased type 1 cytokine production.

Using peritoneal macrophages, we observed that MyD88 and TLR2, but not TLR4, are required for the induction of optimal levels of TNF-α, an inflammatory mediator with a crucial role in the induction of other type 1 cytokines, including IL-12 and IFN-γ [6], and of protective anti-cryptococcal responses [8, 23]. Moreover, both MyD88- and TLR2-deficient animals showed reduced TNF-α, IL-12 and IFN-γ mRNA levels in different organs at various times during infection. It is well established that IL-12 and INF-γ are absolutely required to shift the balance of adaptive responses in favor of a protective type I, versus detrimental type II, Th cell responses [24, 25]. Moreover, the phenotype of IL-12- or IFN-γ-deficient mice, which is characterized by markedly increased susceptibility to infection [21, 24], is similar to the one described here for MyD88- or TLR2-deficient mice. Collectively these data indicate that decreased production of TNF-α, IL-12 and IFN-γ at least...
in part underlies the profound loss of resistance to cryptococcosis observed here in MyD88<sup>−/−</sup> and TLR2<sup>−/−</sup> mice. Interestingly, although MyD88 was required in the present study for the production of optimal levels of TNF-α, IL-12 and IFN-γ, significant MyD88-independent expression of these mediators could be demonstrated. In fact, cytokine production was reduced, but not completely abrogated, in various organs of MyD88-deficient mice. Moreover, strikingly, no differences were noted between MyD88 null and control mice in cytokine production in the liver. Therefore, it is likely that different cell types, which are heterogeneous in terms of MyD88 requirement for cytokine induction, participate in innate responses during cryptococcosis.

In a previous study it was found that GXM, the major capsular component and virulence factor of C. neoformans, activates TLR4 leading to NF-κB translocation to the nucleus but not TNF-α production [17]. We have found that lethality and fungal burden are similar in TLR4-defective and in control mice, indicating that TLR4-dependent pathways do not play an obligatory role in the induction of anti-cryptococcal responses. This is at variance with observations conducted using other fungal pathogens, such as Candida albicans and Aspergillus fumigatus. In fact, in an aspergillosis models, TLR4-dependent pathways were essential for in vivo control of fungal growth [11]. Moreover, in a candidiasis model the absence of a functional TLR4 resulted in tenfold increased fungal burden [26], while TLR2 deficiency resulted in resistance to infection through the abrogation of TLR2-dependent IL-10 and T-regulatory cell induction [27]. Since TLR2 deficiency had a detrimental role and TLR4 had no effect in our cryptococcosis model, there appears to be a high degree of species- or genus specificity in the effects of TLR activation by different fungal pathogens. It will be of interest to further identify species-specific fungal products responsible for TLR activation and to characterize their activities.

Our data concerning the important role of TLR2 in anti-cryptococcal responses are partially at variance with those of Yauch et al. [18] who found variable effects of TLR2 deficiency. In their study, TLR2<sup>−/−</sup> mice died earlier after intranasal, but not intravenous, infection relative to WT animals and, in the former model, increased lethality of TLR2-deficient mice could not be related to increased fungal burden [18]. We believe that the discrepancies within the Yauch et al. study, as well as the differences with our study, can be explained by differences in the severity of experimentally induced infection, as measured by survival time of control WT animals. Studies from our laboratory have indicated that the severity of challenge, and the associated control lethality, deeply influences the effects of immune deficiencies in a number of experimental models ([19], and authors unpublished results). For example, in the present study, markedly decreased survival of TLR2<sup>−/−</sup> animals was evident using a low dose challenge that resulted in sublethal infection in TLR2-sufficient mice (Fig. 2A). Using a higher dose, however, differences in lethality between TLR2-deficient and sufficient animals became less evident (Fig. 2B). Survival times of control WT mice were much shorter in the intravenous infection model of Yauch et al. relative to those of either their intranasal model or any of our models [18]. Therefore differences in control lethality, rather than inoculation route, may account for the observed discrepancies on the role of TLR2 [18]. It seems logical to use relatively low infecting doses to study the effect of immune deficiencies, both to mimic naturally occurring infections, as discussed at the beginning of this section, and to avoid the possible occurrence of immune suppression associated with overwhelming cryptococcosis and the consequent release of GXM, which has known immunosuppressive activities [17].

In conclusion, the present study indicates that both MyD88 and TLR2 are required for optimal host responses to C. neoformans. These data may be useful in the development of immune-based alternative strategies to control cryptococcosis.

Materials and methods

Experimental animals

MyD88<sup>−/−</sup> and TLR2<sup>−/−</sup> mice, derived as previously described [12, 28] and backcrossed for five generations with C57BL/6, were obtained from Dr. Shizuo Akira (Osaka University, Japan) through Dr. Douglas Golenbock (University of Massachusetts, MA). Control WT C57BL/6 mice were purchased from Charles River Italia (Calco, Lecco, Italy). C3H/HeLPS-hyposensitive mice, which have a loss of function mutation in TLR4, and control C3H/HeN mice, were also purchased from Charles River. The mice (6-8-week-old) were housed at the Department of Pathology and Experimental Microbiology of the University of Messina. All in vivo studies were performed in agreement with the European Union guidelines of animal care and were approved by the relevant national committees.

In vitro stimulation of peritoneal cells

For in vitro stimulation of resident peritoneal macrophages, three C. neoformans strains were used: (1) the highly virulent serotype A strain H99 (ATCC 208821); (2) serotype D strain B-3501, kindly provided by Dr. James Kronstad (University of British Columbia, Vancouver, BC); (3) CAP67, a stable acapsular mutant derived from strain B-3501, kindly provided by Dr. Jacobson (McGuire Veterans Affairs Medical Center, Richmond, USA) [29]. The latter two strains were used since they are well-characterized reference strains and sequence information of the B-3501 genome is available (http://wwwsequence.stanford.edu/group/C.neoformans/index.html).
To stimulate macrophages, yeast cells were grown to the exponential phase in chemically defined medium [30, 31], washed and resuspended in nonpyrogenic PBS (0.01 M phosphate, 0.15 M NaCl, pH 7.4) to the desired concentrations. Endotoxin contamination in all of the preparations used was <0.1 EU, as detected by a sensitive Limulus amebocyte lysate assay kit (Associates of Cape Cod, Cod, PBI, Milan, Italy).

Resident mouse peritoneal macrophages were isolated from the peritoneal cavity by washing with ice-cold PBS, sedimented by centrifugation and resuspended in RPMI 1640 (Invitrogen Life Technologies, San Giuliano Milanese, Italy) supplemented with 5% heat-inactivated fetal calf serum, 50 U of penicillin/ml, and 50 μg/ml of streptomycin. Cells were then seeded in 96-well dishes at a density of 2×10⁵/well and incubated at 37°C in a 5% humidified CO₂ environment. After 24 h, nonadherent cells were removed by washing with medium. Adherent cells were stimulated for the indicated times with live cryptococci (5×10⁶ cells/ml). Heat-killed GBS, obtained as previously described [32], were used as a positive control. LTA of Staphylococcus aureus [33], was kindly provided by Dr. Thomas Hartung (University of Konstanz, Konstanz, Germany). S. minnesota R595 ultra pure LPS was obtained from List Biological (Campbell, CA, distributed by Vinci-Biochem, Firenze, Italy).

Peritoneal cell culture supernatants were collected 22 h after the addition of stimuli and assayed for TNF-α, IL-12p40, and IFN-γ using murine specific ELISA kits, according to the manufacturers instructions (mouse TNF-α module set, Bender MedSystem, Vienna, Austria; Quantikine M mouse IL-12p70 and IL-12p70, R&D Systems, Minneapolis, MN; murine IFN-γ reagent set, Euroclone, Wetherby, UK). The lower detection limits of these assays were 16, 12, 8 and 31 pg/ml, respectively.

Murine cryptococcosis model

The highly virulent serotype A C. neoformans strain H99 was used to establish a model of experimental cryptococcosis. Yeast cells were grown to the mid-log exponential phase at 30°C in chemically defined medium under agitation with an orbital shaker. Yeast cells were then washed in PBS, counted on a hemacytometer, and resuspended in sterile nonpyrogenic PBS. For challenge, C57BL/6, TLR2–/– and MyD88 –/– mice were injected i.p. with 2.5×10⁴ or 8.5×10⁴ C. neoformans cells, corresponding to sublethal and fully lethal doses in WT animals, respectively. C3H/HeJ and C3H/HeN mice were correspond to sublethal and fully lethal doses in WT mice, respectively. Differences in cytokine levels and organ CFU were assessed by one-way analysis of variance and the Student’s-Keuls-Newman test. When p values of less than 0.05 were obtained, differences were considered statistically significant. Survival data were analyzed with Kaplan-Meier survival plots followed by the log rank test (JMP Software; SAS Institute, Cary, NC) on an Apple Macintosh computer.

Data expression and statistical significance

Cytokine levels and log CFU were expressed as means ± the standard error of the mean (SEM) of five determinations, each conducted on a different animal. Differences in cytokine levels and organ CFU were assessed by one-way analysis of variance and the Student’s-Keuls-Newman test. When p values of less than 0.05 were obtained, differences were considered statistically significant. Survival data were analyzed with Kaplan-Meier survival plots followed by the log rank test (JMP Software; SAS Institute, Cary, NC) on an Apple Macintosh computer.

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