Myeloid differentiation factor 88 (MyD88) is required for murine resistance to *Candida albicans* and is critically involved in *Candida*-induced production of cytokines

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ABSTRACT. We have studied the role of myeloid differentiation factor 88 (MyD88), the universal Toll-like receptor (TLR) adaptor protein, in murine defenses against *Candida albicans*. MyD88-deficient mice, experimentally infected *in vivo*, had a very significant impaired survival, and a higher tissue fungal burden when compared with control mice. The recruitment of neutrophils to the site of infection was also significantly diminished in MyD88$^{-/-}$ mice. *In vitro* production of proinflammatory cytokines such as TNF-α, IFN-γ and IL-12p70, by antigen-stimulated splenocytes from mice intravenously infected with the low-virulence *C. albicans* PCA2 strain, could not be detected in MyD88$^{-/-}$ mice. This default of production of Th1 cytokines in MyD88-deficient mice correlated with a greatly diminished frequency of IFN-γ-producing CD4 + T lymphocytes. Also, the frequency of IFN-γ-producing CD8 + T lymphocytes was lower in MyD88$^{-/-}$ mice than in control mice. Although *C. albicans*-specific antibody titers in PCA2-infected mice appeared more quickly in MyD88$^{-/-}$ mice than in control mice, the MyD88$^{-/-}$ group was not able to maintain the *Candida*-specific IgM nor IgG titers at the third week of infection. The complexity of antigens recognized by sera from MyD88$^{-/-}$ mice was quite similar to that from infected control mice. Taken together, these data show that MyD88$^{-/-}$ mice are extremely susceptible to *C. albicans* infections, suggesting that MyD88-dependent signaling pathways are essential for both the innate and adaptive immune responses to *C. albicans*.

Keywords: TLR, MyD88, *Candida albicans*, murine candidiasis, TNF-α, IL-12p70, IFN-γ, antibodies

INTRODUCTION

The dimorphic fungus *Candida albicans* is both a commensal and an opportunistic pathogen in humans. Depending on the underlying host defect, this microorganism is able to cause a variety of infections that range from mucosal candidiasis to life-threatening, invasive infections. The frequency of the latter has increased in the last decades as a result of an expanding immunocompromised population [1, 2].

Experimental data and clinical evidence have indicated that both innate and adaptive immunity regulate the resistance to *C. albicans* infections [3]. The coordination of the innate and adaptive arms of antifungal defense may also be important; it has been demonstrated that Th cell reactivity plays a central role in regulating immune responses to *C. albicans*, Th1 reactivity being responsible for resistance and Th2 response being associated with susceptibility [4-6]. Interestingly, although protective immunity to *C. albicans* is mediated by Th1 cells, some Th2 cytokines are required for the maintenance of the antifungal immune protection [7, 8]; regulatory T cells (Treg), activated by IL-10-producing dendritic cells, are also involved in the induction of memory protective immunity by negative regulation of antifungal Th1 reactivity [9]. Recently a role for antibodies in the generation of memory antifungal immunity has been established [10].

Toll-like receptors (TLRs) are a family of evolutionarily conserved transmembrane proteins that function as sensors of infection and induce the activation of innate immune responses. Ten mammalian TLRs have been described so far, and TLR ligands include molecular products derived from bacteria, protozoa, viruses and fungi, and also several putative endogenous ligands. TLRs are ex-

pressed primarily on macrophages and dendritic cells and control the activation of these antigen-presenting cells. Signal transduction through TLRs results in the up-regulation of MHC class II, costimulatory molecules, cytokines and antimicrobial responses [11, 12]. TLR signaling pathways arise from the intracytoplasmic Toll/IL1-receptor (TIR) domain, which is conserved among all TLRs. Recent evidence has demonstrated that TIR domain-containing adaptors, such as MyD88, TIRAP, and TRIF, modulate TLR signaling pathways. MyD88 is essential for induction of inflammatory cytokines triggered by all TLRs [13]. The identification of TLRs as a family of receptors involved in controlling dendritic cell activation and maduration has focused attention on these receptors as possible regulators of adaptive immune responses [14].

Members of TLR family contribute to the generation of host immunity to fungal species in a manner that is dependent on fungal species, morphotype of fungal cells and site of infection [3, 15, 16]. This variability may account for the conflicting data reported by different groups. There is evidence indicating that TLR2, the receptor for phospholipomannan (a unique glycolipid present in the cell surface of *C. albicans* that stimulates TNF-α production by macrophages [17]), and TLR4, the receptor for bacterial lipopolysaccharide, participate in murine defenses against *C. albicans* infections [16]. TLR2-deficient mice show an increased susceptibility to infection, and macrophages from TLR2$^{-/-}$ mice have an impaired production of TNF-α, macrophage inhibitory protein-2 (MIP-2) and recruitment of neutrophils in response to *C. albicans* [18]; however, TLR2 is dispensable for acquired host immune resistance to *C. albicans* in a murine model of disseminated candidiasis [19]. TLR4-defective C3H/HeJ mice are more susceptible to *C. albicans* infection, although neither production of TNF-α and other proinflammatory cytokines, nor candidacidal ability of neutrophils and macrophages were affected; only the production of chemokines, such as cytokine-induced neutrophil chemoattractant (KC) and MIP-2, were decreased and accompanied by impaired neutrophil recruitment [20]. However, different results concerning the role of TLR2 and TLR4 in response to *C. albicans* have been reported, as deficient mice did not show increased susceptibility to infection, and production *in vivo* of TNF-α was impaired in TLR4$^{-/-}$ mice, but increased or normal in TLR2$^{-/-}$ mice [15, 21]. In addition, murine macrophages deficient in MyD88 showed a decreased *in vitro* cytokine production in response to *C. albicans* but not in response to *A. fumigatus* [22], whereas in another study, MyD88$^{-/-}$ mice showed a decrease in *in vivo* cytokine production in response to both *C. albicans* and *A. fumigatus* [15]. All these results underline the complexity of host-parasite relationships during fungal infections, indicating that several TLRs may simultaneously play a role in protecting mice, and that probably several ligands of the fungus are also recognized.

The discovery of TLRs has opened new perspectives to study host defenses against microbial infections. In this work, we studied the role of MyD88 in the protective response against yeasts and hyphae of *C. albicans* by using gene-targeted mice lacking MyD88 expression (MyD88$^{-/-}$). We report here the influence of MyD88 gene deletion on (i) susceptibility *in vivo* to experimental fungal infection, (ii) neutrophil recruitment to the site of infection, (iii) the production of Th1 cytokines: IFN-γ, IL-12 and TNF-α, (iv) the development of a Th1 response, and (v) the development of the acquired humoral response.

MATERIALS AND METHODS

Mice

MyD88$^{-/-}$ mice (C57BL/6 background) were kindly provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan) [23]; wild-type C57BL/6 mice (Harlan Ibérica, Barcelona, Spain) were used as controls. Mice of both sexes, between 7 and 12 weeks old were used for experiments.

Yeast strains and infections

Cells of *C. albicans* strain ATCC 26555 were grown in YPD medium (1% Difco yeast extract, 2% peptone, 2% glucose), at 28 ^{0}C up to the late exponential growth phase ($A_{600\ nm}$ 0.6-1), collected and washed with pyrogen-free water. Cells were resuspended in water, and maintained for 3 h at 28 ^{0}C with shaking, and afterwards at 4 ^{0}C for 24 or 48 h (starved yeast cells), as previously described [24, 25]. The suspension was finally washed and diluted in pyrogen-free, phosphate-buffered saline (PBS; Gibco, Barcelona, Spain) to the appropriate cell concentration before injection. Mice were challenged intravenously with 10^6 *C. albicans* yeasts in a volume of 0.2 mL, and survival was checked daily for 20 days. Cells of *C. albicans* PCA2, a low-virulence, non-germinative strain [26], were obtained as described for the high-virulence strain, and diluted in PBS to the appropriate cell concentration before intravenous injection. To assess the tissue outgrowth of the microorganism, three and six days after the infection, two mice from each group were killed and the kidneys removed aseptically, weighed, and homogenized in 1 mL of PBS; dilutions of the homogenates were plated on Sabouraud dextrose agar. The colony-forming units (CFU) were counted after 24 h of incubation at 37 ^{0}C, and expressed as CFU per gram of tissue.

Experiments were performed under conditions designed to minimize endotoxin contamination: endotoxin-free water and PBS were used, and fungal culture media were passed through a detoxi-gel endotoxin-removing gel (Pierce, Rockford, IL, USA) and tested for the absence of endotoxin by the E-toxate assay (Sigma, Madrid, Spain).

In vitro *cytokine production by splenocytes*

Six days after mice infection with *C. albicans* PCA2 cells, total spleen cells were obtained by collagenase D treatment of the spleens, washed once with complete cell culture medium (RPMI 1640 medium supplemented with 5% heat-inactivated FBS and 1% penicillin-streptomycin, Gibco, Barcelona, Spain), and plated at a density of 10^7 cells in 1 mL of medium per well in a 24-well tissue culture plate, in the presence of 2.5 μg per mL of Amphotericin B (Gibco, Barcelona, Spain). Cells were challenged with the indicated stimuli for 48 h. Supernatants were then harvested and tested by commercial ELISA kits for TNF-α, IL-12p70 (eBioscience, San Diego, CA, USA) and IFN-γ (R&D Systems, Minneapolis, MN, USA).

The stimuli used were LPS from *Escherichia coli* O111:B4 (Sigma, Madrid, Spain), and two heat-inactivated *C. albicans* ATCC 26555 forms, yeast and hypha, obtained as reported elsewhere [24, 25]. Briefly,

starved yeast cells were inoculated (200 µg [dry weight] of cells per mL) in a minimal synthetic medium and incubated for 3 h at 28 ^{0}C to obtain yeasts or at 37 ^{0}C to obtain hyphae. For heat inactivation, yeasts and hyphae were resuspended in water and treated at 100 ^{0}C for 1 h. After inactivation, fungal cells were extensively washed in PBS and brought to the desired cell density in complete cell culture medium.

Recruitment of neutrophils

To investigate the recruitment of neutrophils at the site of infection, groups of five mice were injected intraperitoneally with 10^7 heat-killed *C. albicans* ATCC 26555 yeast cells. After 4 h, peritoneal cells were collected and washed once with PBS. The percentage of neutrophils was determined by labeling cells with FITC-conjugated anti-mouse Ly-6G antibody (clone RB6-8C5; eBioscience, San Diego, CA, USA), and analysed by flow cytometry, using an EPICS XL-MCL flow cytometer (Coulter Beckman).

IFN-γ secretion assay

Three days after the mice infection with *C. albicans* PCA2 cells, total spleen cells were obtained by collagenase D treatment of the spleens as described above and plated at a density of 10^7 cells in 1 mL of medium per well in a 24-well tissue culture plate, in the presence of 2.5 µg per mL of amphotericin B (Gibco, Barcelona, Spain). Cells were challenged with *C. albicans* ATCC 26555 yeast or hypha for 18 h, and analyzed for IFN-γ secretion using the mouse IFN-γ secretion assay-cell enrichment and detection kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The cells were also incubated with a saturating amount of FITC-conjugated CD8 mAb (53-6.7, Pharmingen, Belgium) and PE-Cy5-conjugated CD3 mAb (145-2C11, Pharmingen, Belgium). After washing twice with PBS, the stained cells were analysed on an EPICS XL-MCL flow cytometer (Coulter Beckman).

Semi-quantitative determination of Candida *-specific antibodies by ELISA*

Serum obtained by retro-orbital bleeding of experimental animals was assayed by ELISA to determine *Candida*-specific antibody levels. Microtiter plates were coated with 50 µL of 60 mM sodium carbonate (pH 9.6) containing soluble cell extracts (1 µg of protein per well), obtained from yeasts or hyphae by boiling cells in PBS supplemented with 1% SDS and 1% DTT. After overnight incubation at 4 ^{0}C, the wells were washed with 0.05% (v/v) Tween 20 in PBS (washing buffer) and blocked with 50 µL of PBS supplemented with 1% BSA, for 1 h at room temperature. Three washes with washing buffer were followed by the addition of 50 µl per well of serum samples diluted in PBS with 0.01% of Tween 20 and 0.5% BSA and further incubation for 2 h at 37 ^{0}C. After washing, goat anti-mouse IgG horseradish peroxidase-conjugated (Zymed, San Francisco, CA, USA) or goat anti-mouse IgM horseradish peroxidase-conjugated (Sigma, Madrid, Spain) were added to the wells, incubated for 1 h, and the color intensity was determined at 450 nm following the addition of substrate mixture.

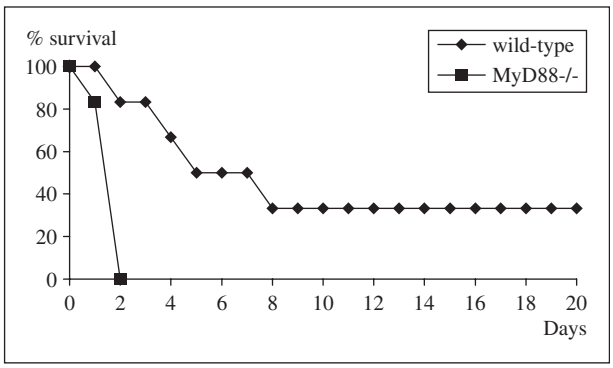


Figure 1
Survival of animals infected with *C. albicans*.
Wild-type mice and MyD88$^{-/-}$ mice were infected intravenously with 10^6 yeasts per mouse, and observed for 20 days. Data showed are from one representative assay of two, using six mice for each group. Log rank $P < 0.05$.

Western blot

Soluble cell extracts obtained from yeasts or hyphae by boiling cells in PBS supplemented with 1% SDS and 1% DTT, were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Buckinghamshire, England). Blots were incubated with a 1/20 dilution of infected or control mice sera. Reactive bands were developed using goat anti-mouse IgG horseradish peroxidase-conjugated (1/1 000 dilution; Zymed, San Francisco, CA, USA), and hydrogen peroxide and 4-chloro-1-naphthol as the chromogenic reagent.

Statistical analysis

Survival curves were analyzed by Kaplan-Meyer log rank test. Student's two-tailed *t* -test was used to compare cytokine production and neutrophil recruitment. Data are expressed as mean ± SD. Significance was accepted at the $P < 0.05$ level.

RESULTS

Susceptibility of MyD88-deficient mice to disseminated experimental candidiasis

The role of MyD88 in host defenses against *C. albicans* was assessed by monitoring the survival curves of MyD88$^{-/-}$ and control mice infected with *C. albicans* yeast cells *(Figure 1)*. Intravenous inoculations were performed with 10^6 *C. albicans* ATCC 26555 yeast cells per mouse, a dose selected following preliminary studies evaluating the mortality rates in C57BL/6 mice, mortality having been followed for 20 days. All of the MyD88$^{-/-}$ mice died within two days while only 66.67% of the wild-type animals had died at day eight and no further deaths occurred until day 20. The mortality of MyD88$^{-/-}$ mice was significantly higher when compared with control mice ($P = 0.0054$, log rank test).

Role of MyD88 in the recruitment of neutrophils

To investigate the recruitment of neutrophils to the site of a *C. albicans* infection, groups of MyD88$^{-/-}$ and

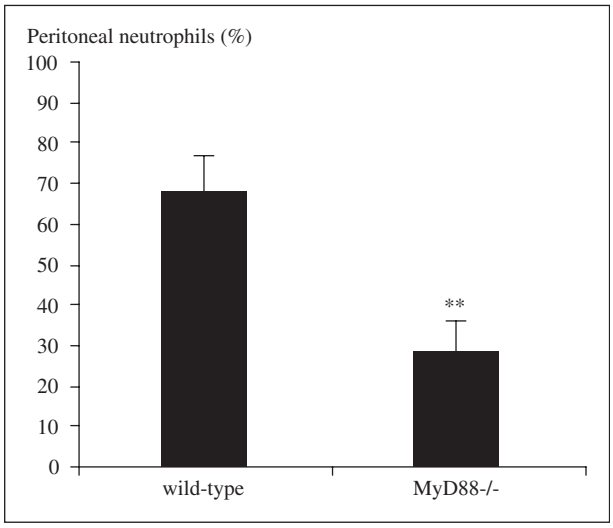


Figure 2
Recruitment of neutrophils in MyD88$^{-/-}$ mice in response to *C. albicans*.
C57BL/6 and MyD88$^{-/-}$ mice were injected intraperitoneally with 10^7 heat-killed *C. albicans* yeasts per mouse. Four hours later, the percentage of neutrophils present in the peritoneal cavity was quantified by labelling with FITC-labeled anti-Ly-6G antibody (clone RB6-8C5) and analysed by flow cytometry. Data represent means ± SD of five mice, from one representative experiment of two. (**) $P < 0.01$ with respect to the stimulated wild-type mice.

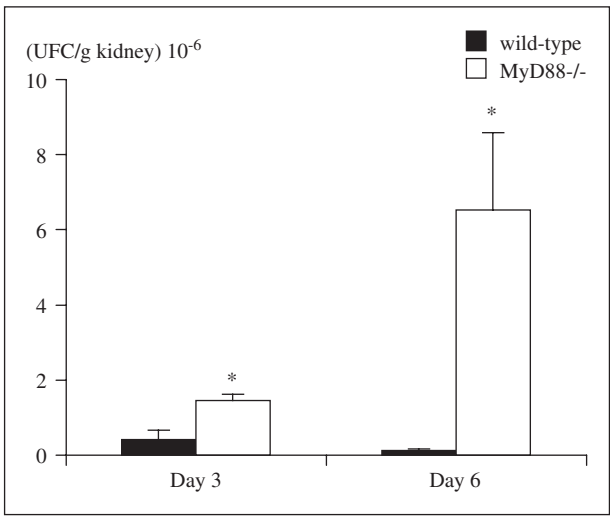


Figure 3
Recovery of *C. albicans* PCA2 from kidneys of infected MyD88$^{-/-}$ mice.
C57BL/6 and MyD88$^{-/-}$ mice were injected intravenously with 400 000 *C. albicans* PCA2 cells per mouse, and two mice from each group were killed on days three and six postinfection, to assess the outgrowth of the yeasts in the kidneys. Data represent means ± SD, from one representative experiment of two. (*) $P < 0.05$ with respect to the wild-type mice.

C57BL/6 mice were injected intraperitoneally with heat-killed *C. albicans* yeasts, and peritoneal cells were harvested 4 h later. The percentage of neutrophils was determined by flow cytometry using the RB6-8C5 monoclonal antibody. RB6-8C5 is directed against Ly-6G, previously known as Gr-1, an antigen on the surface of murine granulocytes whose expression increases with cell maturity and is absent in precursor cells. As shown in *Figure 2*, there was significantly fewer neutrophils in the peritoneal cavity of MyD88$^{-/-}$ than in the cavity of control mice (58.45% inhibition; $P = 0.000049$).

Renal invasion of C. albicans *in MyD88$^{-/-}$ mice with experimentally induced disseminated candidiasis*

To further investigate the role of MyD88 in disseminated candidiasis, mice were infected with the low-virulence PCA2 strain by the intravenous route at a rate 400 000 yeast cells per mouse, and mice were killed at random on days three and six, and CFU per gram of kidney were determined *(Figure 3)*. The fungal burden was significantly higher in MyD88-deficient mice, compared with control mice, on day three ($P = 0.0111$), and also on day six ($P = 0.032$). Interestingly, control mice were able to reduce renal involvement by the low-virulence strain on day six as compared with day three, whereas MyD88-deficient mice had a fungal growth in the kidneys which was significantly more advanced at day six than at day three, indicating that these mice were not able to resist invasion of renal tissue.

Cytokine production by splenocytes from MyD88$^{-/-}$ infected mice in response to C. albicans

To assess the antifungal production of cytokines upon primary infection in MyD88$^{-/-}$ mice in comparison with wild-type mice, animals were infected i.v. with the non-germinative, low-virulence strain PCA2 (400 000 yeasts per mouse), and the *in vitro* levels of TNF-α, IFN-γ, and IL-12p70 production by *Candida*-stimulated splenocytes were assessed six days after infection *(Figure 4)*. Wells containing splenocytes stimulated by LPS, a known TLR4 agonist, and unstimulated cells served as controls. No cytokine could be detected in MyD88$^{-/-}$ mice in contrast to wild-type mice in response to LPS, yeast and hypha.

Role of MyD88 in the development of the Th1 response in mice

To define the role of MyD88 in the development of T-helper type 1 anticandidal response, we determined the frequency of IFN-γ-producing CD4$^+$ T lymphocytes in mice infected with *C. albicans*. As IFN-γ may be also secreted by CD8$^+$ T cells and CD8 could be involved in antifungal protection, we also determined the percentage of IFN-γ-producing CD8$^+$ T lymphocytes in the same mice. To this purpose, mice were infected i.v. with the low-virulence strain PCA2 (400 000 yeasts per mouse). Three days after the infection, total spleen cells were challenged *in vitro* with *C. albicans* ATCC 26555 yeast or hypha for 18 h, and analyzed for IFN-γ secretion using the mouse IFN-γ secretion assay-cell enrichment and detection kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The cells were simultaneously labelled with FITC-conjugated CD8 mAb and PE-Cy5-conjugated CD3 mAb. As shown in *Figure 5*, the frequency of IFN-γ-producing CD4 T lymphocytes (CD3$^+$ CD8$^-$) in *C. albicans*-infected mice was significantly reduced in MyD88$^{-/-}$ mice compared with C57BL/6. This result indicates that MyD88 is essential for the development of Th1 adaptive immunity to *C. albicans*. Also the frequency of IFN-γ-producing CD8 + T lymphocytes was diminished in MyD88-deficient mice as compared with wild-type mice, which is in agreement with the lack of detection of the cytokine *in vitro* (see above).

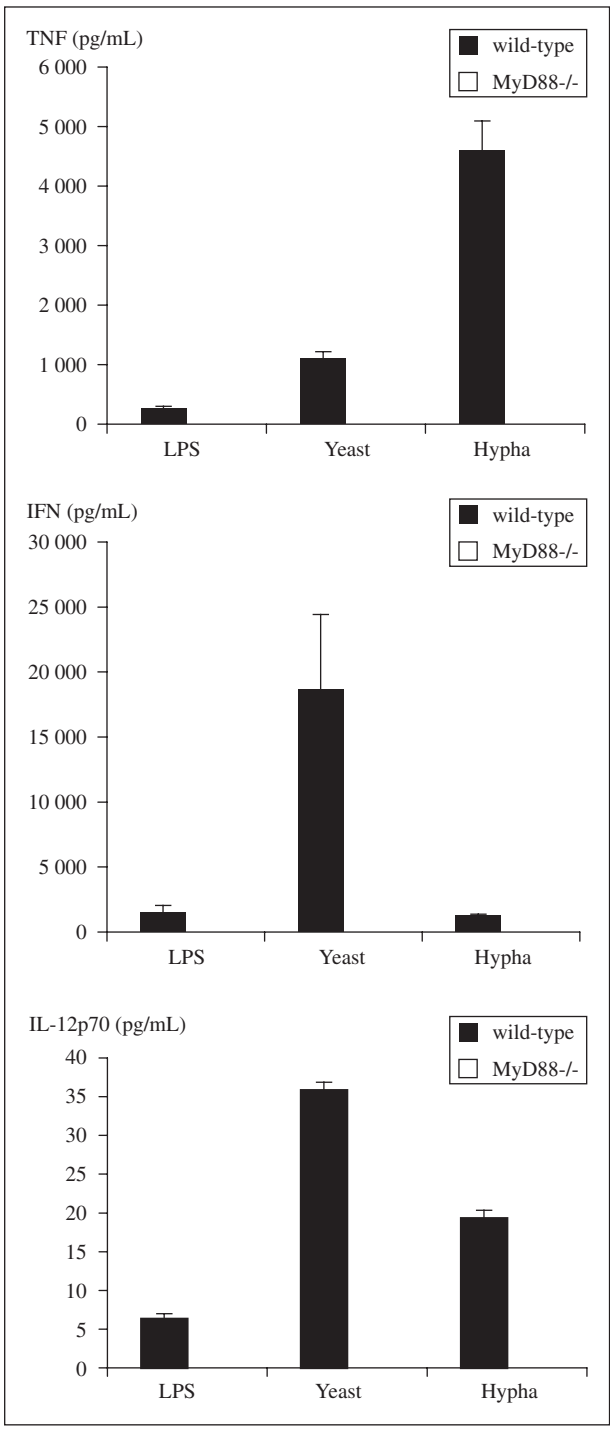


Figure 4
TNF-α, IFN-γ and IL-12p70 production by splenocytes in response to *C. albicans*.
MyD88$^{-/-}$ and wild-type mice were infected i.v. with 400 000 PCA2 cells. Six days later, splenocytes were isolated, and challenged for 48 h with heat-inactivated *C. albicans* PCA2 yeasts (30 µg [dry weight] of cells/mL), *C. albicans* ATCC 26555 hyphae (30 µg [dry weight] of cells/mL) or LPS (500 ng per mL). Concentrations of TNF-α, IFN-γ and IL-12p70 in the culture supernatants were measured by ELISA. Means of duplicates from one representative experiment of two are shown.

Candida *-specific antibodies in MyD88$^{-/-}$ infected mice*

C. albicans-specific antibody titers in PCA2-infected mice were measured to assess the ability of MyD88$^{-/-}$ mice to mount a humoral response against the fungal pathogen. Sera were collected from infected mice on days seven, 14 and 21 after infection, and *C. albicans*-specific IgM and IgG levels were measured by ELISA *(Figure 6A)*. We used microtitre plates coated with soluble cell extracts obtained from yeasts or hyphae. On day seven, a slight higher mean IgM titer was found in wild type mice, but on day 14 both IgM and IgG titers were clearly higher in the MyD88$^{-/-}$ group. However, on day 21, the wild type group showed increased titers of both IgM and IgG, whereas the MyD88$^{-/-}$ group was not able to maintain the *Candida*-specific antibody titers. The effect of MyD88 deficiency on the spectrum of *C. albicans*-specific antigens recognized by the humoral response was assessed by Western blot analysis, using the same cell extracts obtained from yeasts or hyphae *(Figure 6B)*. The complexity of antigens recognized by sera from MyD88$^{-/-}$ mice was quite similar to that from infected control mice. The number of bands and the intensity of the staining was actually greater with sera from MyD88$^{-/-}$ mice than with those from wild type littermates at day 14, but on day 21 the staining was similar.

DISCUSSION

By using MyD88$^{-/-}$ mice, we have shown that MyD88 deficiency is associated with an overall increased susceptibility of a mouse host to systemic *C. albicans* infection. The survival rate was lower and the kidney fungal burden was higher in MyD88$^{-/-}$ mice than in C57BL/6 control mice. The recruitment of neutrophils into the peritoneal cavity of MyD88$^{-/-}$ mice was also significantly lower than in control mice, and this may represent an important mechanism for the decreased resistance to disseminated candidiasis as described for TNF-α lymphotoxin-alpha double knockout mice [27]. Moreover, recently Marr *et al.* [22] have shown that macrophages derived from MyD88$^{-/-}$ mice demonstrated impaired *in vitro* phagocytosis and intracellular killing of *C. albicans* compared to wild-type macrophages. Bellocchio *et al.* [15] found the same result using neutrophils from MyD88$^{-/-}$ mice. Therefore, phagocytes, such as neutrophils and macrophages, that are crucial for clearing the pathogen via phagocytosis, are less attracted to the site of infection and furthermore have impaired ability to phagocytose and kill *C. albicans*.

It is well documented that MyD88-knockout mice do not respond to TLR4 ligands in terms of macrophage production of inflammatory cytokines, and that all the responses to TLR2 ligands are abolished in MyD88$^{-/-}$ mice [13]. Resting macrophages from MyD88$^{-/-}$ mice were not able to secrete *in vitro* TNF-α in response to yeasts or hyphae of *C. albicans* [22]; and *in vivo*, MyD88$^{-/-}$ *C. albicans*-infected mice showed greatly impaired levels of this cytokine in the kidneys compared with wild-type mice [15]. In this work, we have studied the *in vitro* secretion of TNF-α, IFN-γ and IL-12p70 by splenocytes from *C. albicans*-infected mice, in response to both morphotypes of *C. albicans*, yeast and hypha. These three cytokines are required for resistance to *C. albicans* infection [3], and none of them were detected in supernatants of MyD88$^{-/-}$ cells stimulated by yeasts or hyphae of *C. albicans*. Wild-type mice produced more IL-12p70 upon exposure to yeasts, than upon exposure to hyphae, which is in accordance with previous results showing that IL-12 production by human blood monocytes, and mice and human dendritic cells

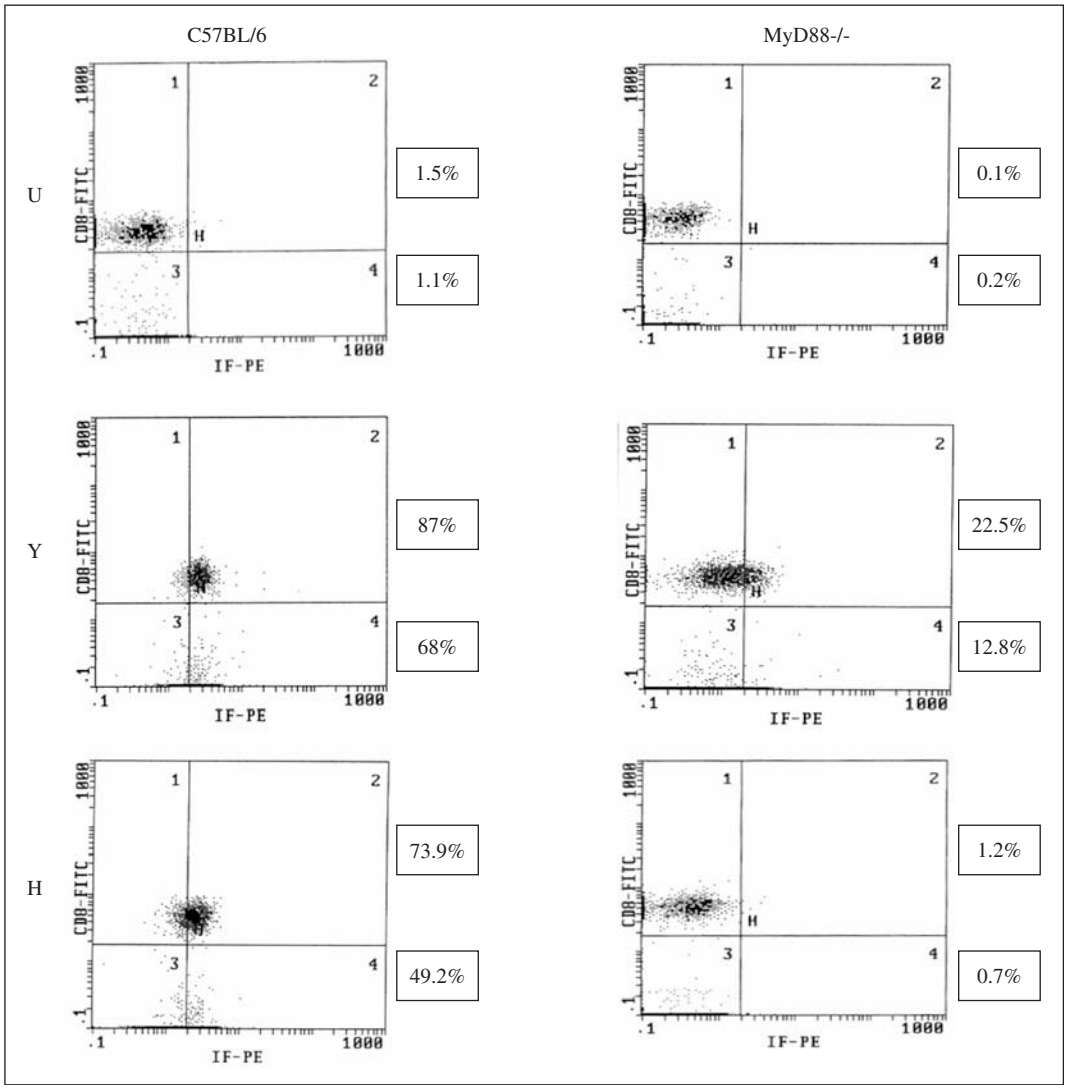


Figure 5

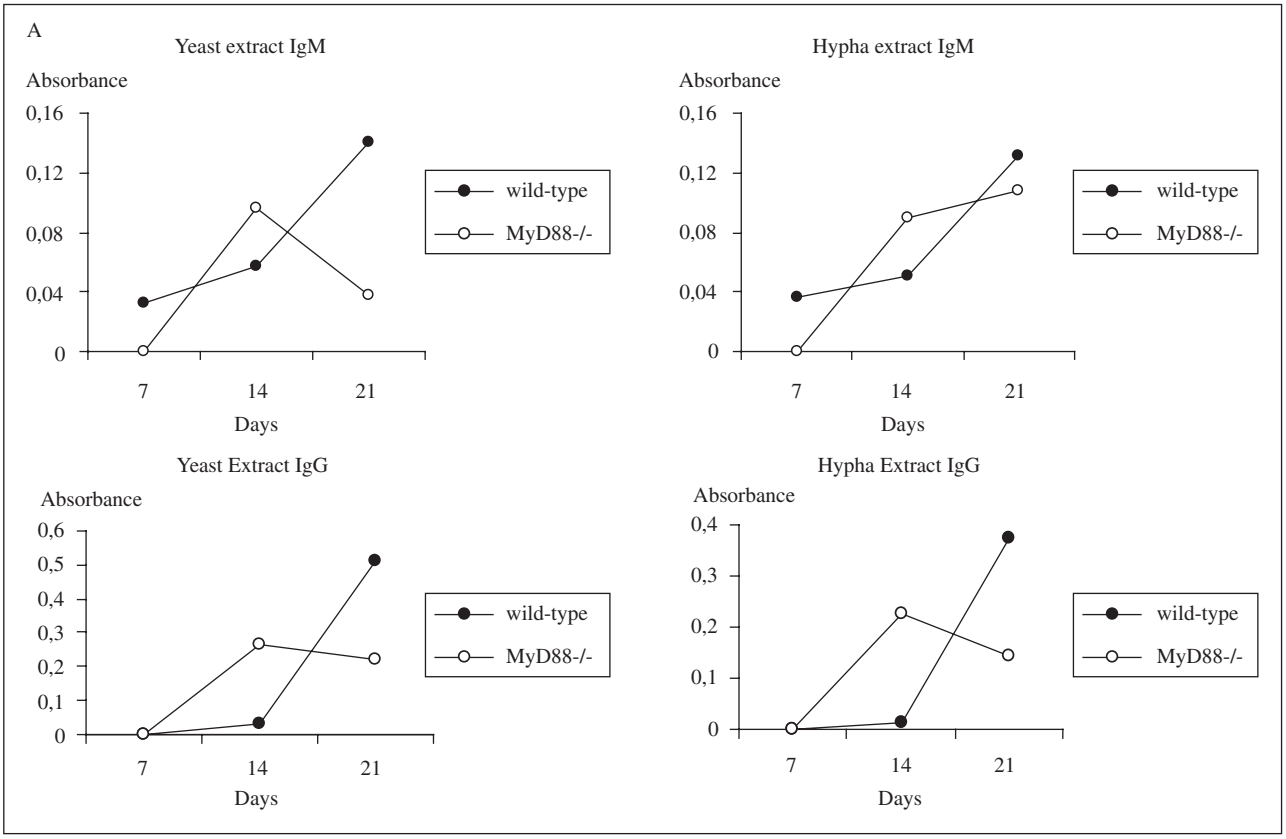
Detection and analysis of IFN-γ-secreting T cells in response to *C. albicans.*

MyD88$^{-/-}$ and C57BL/6 mice were infected i.v. with 400 000 PCA2 cells per mouse. Three days after the infection, total spleen cells were unstimulated (U) or challenged *in vitro* with heat-inactivated *C. albicans* ATCC 26555 yeasts (Y) or hyphae (H) for 18 h, as in *Figure 4*. Secretion of IFN-γ was examined using the mouse IFN-γ secretion assay detection kit (Miltenyi Biotec) as described in Materials and Methods (IF-PE). IFN-γ secretion was analyzed on electronically gated CD3^{+} cells by flow cytometry. Percentages reflect IFN-γ-secreting CD8^{+} cells and IFN-γ-secreting CD4 cells (CD3^{+} CD8^{-}). Similar results were obtained in two independent experiments.

basically occurs in response to the yeast form of *C. albicans* [28-31]. Interestingly the lower production of IL-12p70 in response to hyphae correlates with the lower production of IFN-γ against this morphotype. Our results indicate that early in infection, production of proinflammatory cytokines, including Th1 cytokines (IL-12p70 and IFN-γ) is abolished in MyD88-deficient mice.

To correlate these findings concerning the innate antifungal response with the acquired immunity to *C. albicans*, we measured the frequency of IFN-γ-producing CD4^{+} T lymphocytes in mice systemically infected with the low-virulence strain PCA2. In wild-type mice, the infection with this low-virulence agerminative strain of *C. albicans* induced substantial Th1 acquired protection to reinfection with highly virulent yeast cells [32-34]. Our results showed a greatly diminished frequency of IFN-γ-producing CD4^{+} T cells in MyD88$^{-/-}$ mice as compared to control mice. Similar results have been described in MyD88$^{-/-}$ mice intragastrically infected with high-virulence *C. albicans* cells [15]. Interestingly, the frequency of IFN-γ-producing cells (CD4 or CD8) was always higher when the *in vitro* stimulation was performed with yeasts than when the stimulation was performed with hyphae, which is in accordance with the levels of the cytokine measured by ELISA.

In this work we have studied the role of MyD88 in the development of the acquired humoral response against *C. albicans* during invasive infection. We found that on day 14, both IgM and IgG titers were clearly higher in the MyD88$^{-/-}$ group. This is probably due to a greater number of *C. albicans* cells present in tissues of more susceptible mice. However, on day 21 the titers of both IgM and IgG increased in the wild type group whereas the MyD88$^{-/-}$ group was not able to maintain the *Candida*-specific antibody titers, although the complexity of antigens recognized by MyD88$^{-/-}$ mice was quite similar to that in infected control mice. The decrease in antibody titers in MyD88$^{-/-}$ mice may be due to the lack of help (IFN-γ secretion by the Th cell initiating contact) for the antigen-primed B cells. We show in our model that the acquired



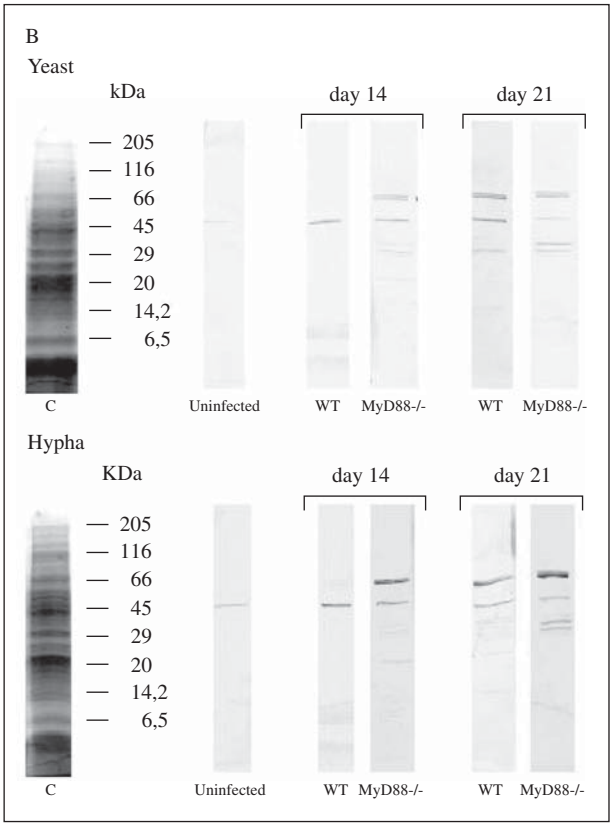


Figure 6

Effect of MyD88-deficiency on the humoral immune response in *C. albicans*-infected mice.
MyD88$^{-/-}$ and C57BL/6 mice were infected i.v. with 210 000 PCA2 cells per mouse, and blood samples were obtained on days seven, 14 and 21 after the infection. (A) *C. albicans*-specific IgM and IgG in sera of mice was assayed by ELISA, using microtiter plates coated with soluble cell extracts obtained from yeasts (yeast extract) or hyphae (hypha extract). (B) *C. albicans*-specific IgG in sera of uninfected and infected mice was assayed by Western blot of soluble cell extracts obtained from yeasts (yeast) or hyphae (hypha); C shows cell extracts stained with Coomassie brilliant blue to visualise proteins. The position of molecular mass markers is indicated. Each serum sample was a pool from four randomly selected mice.

humoral response can occur in the absence of MyD88, although the intensity of the response is slightly diminished probably due to the impaired Th1 response, and therefore there is no evidence indicating whether these antibodies are protective. Although it is accepted that the humoral response is not critical to host defense against *C. albicans* during primary systemic infection, administration of antibodies against certain *C. albicans* antigens confers protection against infection in naive mice [35-37]. The mechanisms by which specific antibodies mediate protection against fungi are not well characterized in all cases [36]. Mycograb (*Neu* Tec Pharma plc) is a human recombinant antibody against fungal heat shock protein 90 (HSP90), which has been designed as a new class of antifungal drug. Mycograb does not have an Fc component, and its antifungal activity is not dependent on Fc-mediated recruitment of leukocytes or complement, but to its ability to bind and inhibit HSP90 [38]. Recently Montagnoli *et al.*, [10] found that B-cell-deficient mice were resistant to the primary systemic infection, but their resistance to reinfection was severely impaired, despite the occurrence of activated Th1 cells. This correlated with the failure to generate IL-10-producing dendritic cells and regulatory $CD4^+$ $CD25^+$ T cells, suggesting a role for antibodies in the generation of memory antifungal immunity.

In summary, we show that MyD88-deficient mice have high mortality after *C. albicans* infection, and that there is abrogation of proinflammatory cytokines secretion, and a profound defect in the activation of *Candida*-specific Th1 cells, however they are capable of mounting a specific antibody response to *C. albicans*.

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