The Role of Toll-like Receptor (TLR) 2 and TLR4 in the Host Defense against Disseminated Candidiasis

**Mihai G. Netea, Chantal A. A. Van der Graaf,
Alieke G. Vonk, Ineke Verschueren,
Jos W. M. Van der Meer, and Bart Jan Kullberg**

*Department of Medicine, Division of General Internal Medicine,
University Medical Center St. Radboud, Nijmegen, The Netherlands*

Toll-like receptors (TLRs) represent the main class of pattern-recognition receptors involved in sensing pathogenic microorganisms. The aim of the present study was to assess the role of TLR4 in the defense against *Candida albicans* infection. The outgrowth of *C. albicans* was 10-fold higher in TLR4-defective C3H/HeJ mice, compared with that in control C3H/HeN mice ($P < .05$). Production of tumor necrosis factor (TNF) and interleukin (IL)–1α and IL-1β by mouse macrophages in response to *C. albicans* stimulation was not affected by TLR4, and the candidacidal capacities of the neutrophils and macrophages of C3H/HeJ mice were normal. In contrast, production of the CXC chemokines KC and macrophage inhibitory protein–2 was 40%–60% lower by the macrophages of C3H/HeJ mice ($P < .05$), which resulted in a 40% decrease in neutrophil recruitment to the site of infection. *Candida*-induced TNF and IL-1β production by human peripheral blood mononuclear cells was significantly inhibited by blocking anti-TLR2 antibodies in vitro. In conclusion, TLR4-defective C3H/HeJ mice are more susceptible to *C. albicans* infection, and this is associated with impaired chemokine expression and neutrophil recruitment.

Acute disseminated candidiasis is a severe infection that occurs mainly in immunocompromised hosts. The mortality rate associated with disseminated candidiasis has changed little, despite the availability of new antifungal drugs, and the incidence of the disease has increased during recent years [1, 2]. Despite the importance of *Candida albicans* in human disease, little is known about the mechanisms through which *Candida* is recognized by the cells and triggers the host defense. Recent data have suggested an important role of the Toll-like receptors (TLRs) for the innate immunity to pathogens. Initially identified in *Drosophila* as a gene required for ontogenesis, Spaetzle/Toll interactions have been shown to control the expression of the antifungal peptide gene drosomycin and to be crucial for the antifungal response of the insect [3]. Because of the homology between the intracellular domains of the *Drosophila* Toll and the mammalian interleukin (IL)–1 receptor type I, it has been hypothesized that homologs of Toll may also be present in mammalian cells and involved in antimicrobial resistance. Indeed, studies performed during recent years have demonstrated that TLRs are the most important class of pattern-recognition receptors in mammals: TLR2 for peptidoglycans [4–6], lipoarabinomannan [7], and bacterial lipoproteins [8]; TLR4 for lipopolysaccharide (LPS) [9] and lipoteichoic acid [5]; TLR5 for flagellin [10]; and TLR9 for bacterial DNA [11].

In contrast, little information is available on the role of TLRs in the host defense against fungal pathogens in humans, despite the fact that the initial description of Toll in *Drosophila* as controlling antifungal mechanisms would suggest the possibility that TLRs play a role in the mammalian antifungal defense. It has been shown that TLR2 is involved in the recognition of zymosan, a cell-wall particle of the yeast *Saccharomyces,* which leads to NF-κB activation and tumor necrosis factor (TNF) production [12]. In contrast, a role for TLR4, but not TLR2, was found in the induction of proinflammatory cytokines by *Aspergillus fumigatus* [13], whereas TLR4 has been suggested to mediate intracellular signaling, but not TNF production, after stimulation of cells with *Cryptococcus neoformans* glucuronoxylomannan [14]. No data are available on the role of TLRs for the recognition of the important human pathogen *C. albicans.*

In the present study, the role of TLRs for the host defense against *C. albicans* was investigated. To that end, we assessed the susceptibility to disseminated candidiasis of C3H/HeJ mice, which are known to have a defective TLR4 because of a point mutation [9]. We also investigated the host defense mechanisms responsible for antifungal defense in these mice: cytokine and chemokine production, neutrophil recruitment to the site of infection, phagocytosis, and killing of the microorganisms. The possible differential role of TLR2 and TLR4 for mediating the induction of cytokines and chemokines by *C. albicans* also was assessed by blocking these receptors on the surface of human peripheral blood mononuclear cells (PBMC) with specific antagonistic antibodies, before stimulation with *C. albicans* was performed.

Materials and Methods

Animals. C3H/HeJ and C3H/HeN mice (20–25 g, 6–8 weeks old) were obtained from Jackson Laboratories. The mice were fed sterilized laboratory chow (Hope Farms) and water ad libitum.

Reagents. *Escherichia coli* LPS (serotype 055:B5) was obtained from Sigma Chemical. The mouse anti–human monoclonal anti-TLR4 HTA125 antibody was a kind gift of Dr. Kensuke Miyake (Saga Medical School, Saga, Japan). The mouse anti–human monoclonal anti-TLR2 antibody was kindly provided by Dr. Douglas Golenbock (Boston University, Boston). The mouse anti–human monoclonal anti-CD14 WT14 antibody was a kind gift of Dr. Wil Tax (University Medical Center, Nijmegen, The Netherlands). A mouse IgG control antibody was used as a control (Sigma).

C. albicans infection model. *C. albicans* UC 820, a strain well-described elsewhere [15], has been used in all experiments. A nonlethal experimental model of disseminated candidiasis was used in which C3H/HeJ and C3H/HeN mice were injected intravenously with *C. albicans* (1×10^5 cfu/mouse) in a 100-μL volume of sterile pyrogen-free PBS. Subgroups of 5 animals were killed on day 1 or 7 of infection. To assess the tissue outgrowth of the microorganisms on these days, the liver and kidneys of the killed animals were removed aseptically, weighed, and homogenized in sterile saline in a tissue grinder. The number of viable *Candida* cells in the tissues was determined by plating serial dilutions on Sabouraud dextrose agar plates, as described elsewhere [16]. The colony-forming units were counted after 24 h of incubation at 37°C and expressed as log colony-forming units per gram of tissue.

In vitro cytokine production. Groups of 5 C3H/HeJ and C3H/HeN mice were killed, and resident peritoneal macrophages were harvested by injecting 4 mL of sterile PBS that contained 0.38% sodium citrate [17]. After centrifugation and washing, the cells were resuspended in RPMI 1640 medium that contained 1 mM pyruvate, 2 mM L-glutamine, 100 μg/mL gentamicin, and 2% fresh mouse plasma (culture medium). Cells were cultured in 96-well microtiter plates (Greiner) at 10^5 cells/well, in a final volume of 200 μL. The cells were stimulated with either control medium or 1×10^7 cfu/mL heat-killed (1 h at 100°C) *C. albicans*. After 24 h of incubation at 37°C, the plates were centrifuged (500 g for 10 min), and the supernatant was collected and stored at -80°C until cytokine assays were performed.

Recruitment of neutrophils. To investigate the recruitment of polymorphonuclear leukocytes (PMNL) at the site of *Candida* infection, groups of 5 C3H/HeJ and C3H/HeN mice were injected intraperitoneally (ip) with 10^7 cfu of heat-killed *C. albicans*, in a volume of 100 μL. In pilot experiments, the maximum neutrophil infiltration after ip injection of heat-killed *Candida* was found after 4 h. After 4 h, peritoneal cells from C3H/HeJ and C3H/HeN mice were collected in sterile saline that contained 0.38% sodium citrate, and the total cell number was counted in a hemacytometer. The percentage and the absolute numbers of neutrophils were determined in Giemsa-stained cytocentrifuge preparations.

Phagocytosis and killing of C. albicans *by macrophages and neutrophils.* Either resident peritoneal macrophages or exudate peritoneal PMNL were obtained, and phagocytosis and killing were performed according to a modification of a method described elsewhere [17]. Exudate peritoneal phagocytes from groups of 5 C3H/HeJ and C3H/HeN mice were elicited by an ip injection of 10%

proteose peptone. Cells were collected in separate sterile tubes by washing the peritoneal cavity with 4 mL of ice-cold PBS that contained 50 U/mL heparin for 4 (65% ± 11% PMNL) or 72 h (84% ± 6% macrophages) after injection. Phagocytes were centrifuged (for 10 min at 2250 g), counted in a Bürker counting chamber, and resuspended in RPMI 1640 Dutch modification (with 20 mM Hepes and without L-glutamine; ICN Biomedicals) supplemented with 5% heat-inactivated fetal calf serum, 1% gentamicin, 1% L-glutamine, and 1% pyruvate (henceforth, RPMI-dm). The processes of phagocytosis and intracellular killing were studied in an adherent monolayer of phagocytes. To create a monolayer of phagocytes, 5×10^5 cells in 100 μL of RPMI-dm were dispensed into the wells of a 96-well flat-bottom plate (Costar) and incubated at 37°C in air and 5% CO$_2$. Granulocytes were allowed to adhere for 0.5 h and macrophages for 2 h before the monolayers were gently washed with culture medium to remove nonadherent cells. The percentage of adherence was calculated as $(1 - [$ number of nonadherent cells/$5 \times 10^5]) \times 100$. Subsequently, the cells were incubated with 1×10^4 cfu *C. albicans,* which were opsonized for 45 min at 24°C in modified Eagle's medium (MEM; Gibco Life Technologies) that contained 2.5% fresh mouse serum (effector:target ratio, 40:1). After 15 min, supernatants were aspirated, and monolayers were gently washed with MEM to remove noningested microorganisms. The supernatant and well washings that contained the noningested *Candida* blastoconidia were combined and plated in serial dilutions on Sabouraud agar plates. The percentage of phagocytized microorganisms was defined as $[1 - ($number of uningested cfu/cfu at the start of incubation$)] \times 100$.

Killing of *C. albicans* by phagocytes was assessed in the same monolayers. After removal of the nonphagocytized *Candida* blastoconidia, 200 μL of culture medium, consisting of Sabouraud in MEM (50% vol/vol), was added to the monolayers. After 3 h of incubation at 37°C in air and 5% CO$_2$, the wells were scraped gently with a plastic paddle and washed with 200 μL distilled H$_2$O to achieve lysis of phagocytes. This procedure was repeated 3 times, after which the pooled washes were adjusted to a final volume of 1 mL with distilled water. Microscopic examination of the culture plates showed that there was a complete removal of phagocytes. To quantify the number of viable intracellular *Candida* blastoconidia, 10-fold dilutions of each sample were spread on Sabouraud agar plates and incubated at 37°C for 24 h. The percentage of yeast killed by the phagocytes was determined as follows: $[1 - ($cfu after incubation/number of phagocytized cfu$)] \times 100$. Phagocyte-free incubations of blastoconidia were included as a control for yeast viability.

Nitric oxide (NO) production. Resident peritoneal macrophages were collected as described above. Cells were counted and adjusted to 5×10^6/mL and stimulated with heat-killed *C. albicans* (10^7 cfu/mL) and recombinant human IFN-γ (100 U/mL) for 24 h at 37°C. Nitrite concentrations in the supernatants were determined by the Griess reaction [18].

Superoxide production. PMNL of TLR4-defective C3H/HeJ and control C3H/HeN mice were collected 4 h after intraperitoneal injection of proteose peptone, as described above. Luminol-enhanced chemiluminescence of proteose-peptone–elicited PMNL was measured on a Victor 1420 multilabel counter (Wallac) at 20°C by use of white 96-well microplates (Costar), as described elsewhere [19]. Each well contained 2×10^5 cells, 50 μM luminol, 4.5 U/mL horseradish peroxidase (HRP), and 50 ng/mL phorbol 12-myristate 13-

acetate (PMA) in 200 μL of Hanks's balanced salt solution without phenol red (Gibco Life Technologies) supplemented with 0.25% human albumin (Behringwerke). Reactions were started by adding *Candida* blastospores plus PMA. Each experiment was performed in duplicate. HRP was added to the system in order to overcome extracellular peroxidase deficiency. In previous experiments, we found that the addition of extra peroxidase did not affect superoxide production (measured as reduction of cytochrome c) of human neutrophils stimulated with PMA but increased luminol-enhanced chemiluminescence 3–4-fold. Hence, only the detection of superoxide is enhanced in the presence of extra peroxidase. The chemiluminescence was expressed as the total amount of superoxide produced during the assay period by integrating the area under the curve (in mV) per PMNL.

The role of TLR2, TLR4, and CD14 for the induction of cytokines and chemokines by C. albicans *in human PBMC.* Venous blood was drawn from a cubital vein of 7 healthy volunteers into 3 10-mL EDTA tubes (Monoject). Isolation of PBMC was performed as described elsewhere [20], with minor modifications. The PBMC fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech AB). PBMC were washed twice in saline and suspended in culture medium (RPMI 1640 medium supplemented with gentamicin [10 μg/mL], 10 mM L-glutamine, and 10 mM pyruvate). The cells were counted in a Coulter counter (Coulter Electronics), and the number was adjusted to 5×10^6 cells/mL; 5×10^5 PBMC in a 100-mL volume were incubated in 96-wells plates with 100 mL of either culture medium or heat-killed (30 min at 100°C) *C. albicans* (ATCC 10231; final concentration 10^5–10^7 cfu/mL). In blocking studies, PBMC were preincubated (1 h at 37°C) with the various monoclonal antibodies (anti-TLR4, anti-TLR2, and anti-CD14; 20 μg/mL) before the stimulation with heat-killed *C. albicans.* All 3 antibodies used (anti-TLR2, anti-TLR4, and anti-CD14) were mouse anti–human IgG antibodies, and we used an mouse IgG isotype in all control wells. After 4 or 24 h incubation at 37°C, the supernatants were collected and stored at −70°C until assay.

Cytokine assays. Murine IL-1α, IL-1β, and TNF-α were determined by specific RIAs (detection limit, 20 pg/mL), as described elsewhere [21]. Murine KC and macrophage inhibitory protein (MIP)−2 concentrations were measured by commercial ELISA kits, used according to the instructions of the manufacturer (R&D Systems). Human TNF-α and IL-1β concentrations were determined by specific RIAs, as described elsewhere [20]. Human IL-8 was measured by a commercial ELISA kit (Pelikine Compact; CLB), according to the instructions of the manufacturer.

Statistical analysis. The differences between groups were analyzed by the Mann-Whitney U test and, where appropriate, by Kruskal-Wallis analysis of variance test. The level of significance between groups was set at $P < .05$. All experiments were performed at least twice, and the data are presented as cumulative results of all experiments performed.

Results

C. albicans *infection model.* The kidneys are the target organ for *C. albicans* outgrowth in murine candidiasis, and the TLR4-deficient C3H/HeJ mice were found to have a 10-fold increased

load of *C. albicans* in the kidneys on days 1 and 7 postinfection ($P < .05$), compared with C3H/HeN control mice, which have an intact TLR4 (figure 1). A similar tendency, although not significant, was apparent for the outgrowth of *C. albicans* in the liver of the C3H/HeJ mice (data not shown).

In vitro cytokine production. To investigate the role of TLR4 in the stimulation of cytokines and chemokines by *C. albicans,* we stimulated in vitro peritoneal macrophages of C3H/HeJ and control C3H/HeN mice with heat-killed *Candida* blastospores. Cytokine production from unstimulated C3H/HeJ and C3H/HeN macrophages was below the detection limit for all cytokines studied (data not shown). *Candida*-stimulated production of TNF and IL-1α was similar in macrophages isolated from C3H/HeJ and C3H/HeN mice (figure 2A). In contrast, the synthesis of the chemokines KC and MIP-2 was significantly lower in C3H/HeJ macrophages stimulated with *Candida,* compared with controls (figure 2B). These data suggest that TLR4 plays an important role in the stimulation of the chemokines KC and MIP-2, but not of the proinflammatory cytokines TNF, IL-1α, and IL-1β, by *C. albicans.*

Recruitment of PMNL. To investigate the recruitment of neutrophils to the site of a *C. albicans* infection, groups of C3H/HeJ and C3H/HeN mice were infected intraperitoneally with 10^7 cfu *C. albicans,* and exudate peritoneal neutrophils were harvested and counted 4 h later. As shown in figure 3, there was significantly less infiltration of neutrophils in the peritoneal cavity of C3H/HeJ mice than in that of C3H/HeN mice (39% reduction; $P < .05$).

Phagocytosis and killing of C. albicans. Phagocytosis of *C. albicans* by PMNL of C3H/HeJ mice was similar to that by PMNL
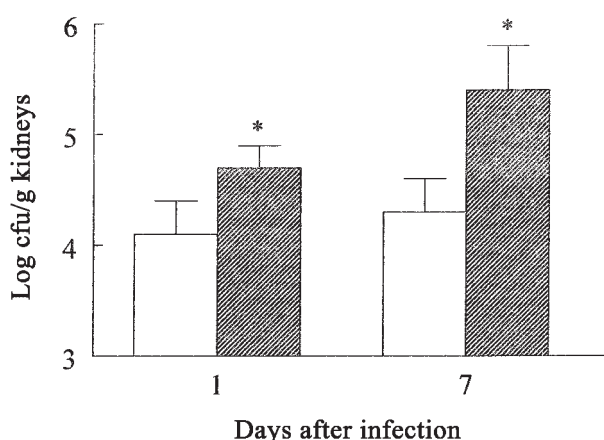

Figure 1. Increased outgrowth of *Candida albicans* in Toll-like receptor 4 (TLR4)–defective C3H/HeJ mice. Control C3H/HeN *(white bars)* and TLR4-defective C3H/HeJ *(hatched bars)* mice were infected intravenously with 1×10^5 cfu of *C. albicans,* and subgroups of animals were killed on days 1 and 7 postinfection, to assess the outgrowth of the yeasts in the organs. Data represent means $\pm$ SE of 10 mice. *$P < .05$, Mann-Whitney U test.
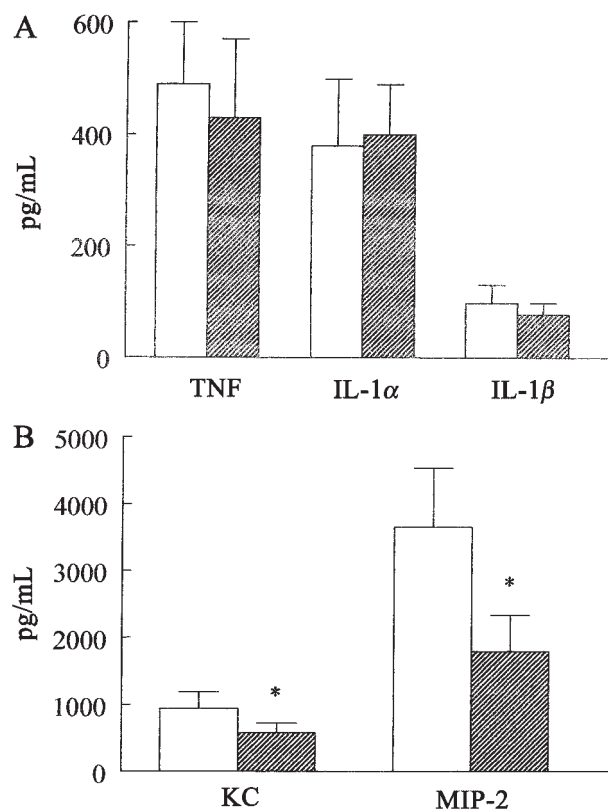
A

B

Figure 2. Production of cytokines and chemokines by macrophages of Toll-like receptor 4 (TLR4)–defective C3H/HeJ mice. Murine peritoneal macrophages were harvested from C3H/HeN mice *(white bars)* and C3H/HeJ mice *(hatched bars)* and stimulated with 10^7 cfu/mL heat-killed *Candida albicans*. Cytokines *(A)* or chemokines *(B)* were measured 24 h later. Data represent means ± SE of 10 mice. *$P < .05$, Mann-Whitney *U* test. IL, interleukin; MIP, macrophage inhibitory protein; TNF, tumor necrosis factor.

of C3H/HeN mice (34% vs. 38% phagocytized in 15 min; $P > .05$). In addition, neutrophils of C3H/HeJ and C3H/HeN mice were equally potent to kill the phagocytized *Candida* blastospores (95% ± 3% vs. 94% ± 4% in 4 h; $P > .05$). This was accompanied by similar production of both superoxide and NO production by PMNL and macrophages, respectively, isolated from C3H/HeJ and C3H/HeN mice (table 1).

The role of TLR2, TLR4, and CD14 for the induction of cytokines and chemokines by C. albicans *in PBMC.* Preincubation of PBMC for 1 h with anti-TLR4 antibodies did not influence the production of TNF, IL-1β, and IL-8 after stimulation with 10^7 *C. albicans* blastospores (figure 4), whereas the anti-TLR4 antibodies significantly down-regulated the production of LPS-stimulated TNF (figure 4, *inset*). In contrast to the lack of effect of TLR4 on *Candida*-induced cytokines, blockade of TLR2 by a neutralizing antibody resulted in a significant reduction of the production of TNF and IL-1β but not of the CXC chemokine IL-8 (figure 4). To investigate the role of CD14 in the interaction between *C.*

albicans and host cells, we preincubated PBMC with a neutralizing anti-CD14 antibody. Anti-CD14 antibody did not influence *Candida*-induced production of TNF (anti-CD14, 2800 ± 460 vs. control, 3380 ± 580 pg/mL; $P > .05$) or IL-1β (1570 ± 330 vs. 1740 ± 430 pg/mL; $P > .05$). The anti-CD14 antibody almost completely (80%–90%) inhibited the induction of TNF and IL-1β production by LPS ($P < .05$; data not shown). Similar data were obtained when 10^5 or 10^6 *Candida* cfu/mL were used (data not shown). When PBMC were stimulated with *C. albicans* for 4 instead of 24 h, IL-1β and IL-8 production was very low, whereas synthesis of TNF was 40%–60% inhibited by the anti-TLR2 antibody ($P < .05$) but not by the anti-TLR4 or anti-CD14 antibodies (data not shown).

Discussion

The results of the present study demonstrate that the absence of intact TLR4-mediated signal transduction in C3H/HeJ mice leads to an increased *C. albicans* outgrowth in the kidneys. This effect of TLR4 is not exerted at the level of proinflammatory cytokine production or antifungal killing mechanisms. Rather, the recruitment of neutrophils to the site of infection is impaired in C3H/HeJ mice, and this correlates with a defective production of the neutrophil chemokines KC and MIP-2. In contrast to the synthesis of the CXC chemokines, the production of the proinflammatory cytokines TNF and IL-1β by *C. albicans* is induced through signals mediated by TLR2.

Our finding that TLR4-deficient C3H/HeJ mice have an increased susceptibility to disseminated candidiasis is in agreement with studies showing that TLR4 is involved in the recognition
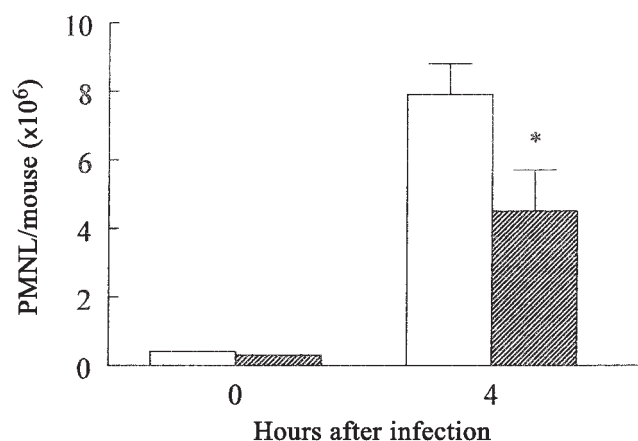

Figure 3. Recruitment of polymorphonuclear leukocytes (PMNL) in Toll-like receptor 4 (TLR4)–defective C3H/HeJ mice. C3H/HeN *(white bars)* and C3H/HeJ *(hatched bars)* mice were injected intraperitoneally with 10^7 cfu of heat-killed *Candida* per mouse. Four hours later, fewer PMNL infiltrate the peritoneal cavity of TLR4-deficient C3H/HeJ mice than of C3H/HeN controls. Data represent means ± SE of 10 mice. *$P < .05$, Mann-Whitney *U* test.

Table 1. The role of Toll-like receptor 4 (TLR4) for stimulation of nitric oxide (NO) and superoxide (O_2) by *Candida albicans*.

Product	C3H/HeN	C3H/HeJ
NO_2, μg/mL	112 ± 21	107 ± 11
O_2, mV/PMNL	0.19 ± 0.04	0.21 ± 0.07

NOTE. Resident peritoneal macrophages were harvested from C3H/HeN mice and C3H/HeJ mice and stimulated with a combination of 10^7 cfu/mL heat-killed *C. albicans* and recombinant interferon-γ (100 U/mL) for 24 h to measure nitrite concentrations in the supernatants. Polymorphonuclear leukocytes (PMNL) of TLR4-deficient and normal mice were collected 4 h after intraperitoneal injection of proteose peptone and stimulated with 10^7 cfu/mL of *Candida* plus phorbol 12-myristate 13-acetate, to assess their capacity to release reactive oxygen species. Data represent means $\pm$ SE of 10 mice.

and host defense to *A. fumigatus* and *C. neoformans,* 2 other important fungal pathogens [13, 14]. The increased susceptibility of C3H/HeJ mice to disseminated candidiasis was manifested as an increased outgrowth of the microorganism in the kidneys, which are the target organ of disseminated candidiasis in mice. It is important to emphasize that C3H/HeJ mice have a defective TLR4 because of a point mutation, and even more accentuated differences, compared with control mice, may be expected in mice with a null mutation, such as ScCr mice or knockout TLR4$^{-/-}$ mice.

The increased fungal load in the organs of the TLR4-defective C3H/HeJ mice was associated with a blunted production of CXC chemokines KC and MIP-2, which resulted in a reduced recruitment of neutrophils to the infection site and led to overwhelming multiplication of the yeasts. This is in line with earlier data showing that translocation of NF-κB to the nucleus, which is required for the transcription of KC and MIP-2 genes [22, 23], is under control of TLR4. The implication of defective chemokine synthesis and neutrophil recruitment in the increased susceptibility of TLR4-deficient mice during *Candida* infection is highly similar to the mechanism that leads to *E. coli* urinary tract infection in TLR4-deficient mice, which also display decreased chemokine production and lower neutrophil infiltration in the urinary tract after infection with *E. coli* [24].

Although TLR4 is involved in host defense to *C. albicans* infection through modulation of chemokine synthesis and neutrophil recruitment, the normal TNF and IL-1 production by TLR4-deficient macrophages implies that other TLRs mediate stimulation of proinflammatory cytokines by *Candida.* Underhill et al. [12] have shown a crucial role of TLR2/TLR6 heterodimers for the recognition of zymosan, a cell wall component of *Saccharomyces cerevisiae,* after internalization of the particles into phagosomes. Indeed, blocking TLR2 on human mononuclear cells with a specific anti-TLR2 antibody led to a 60%–75% inhibition of TNF and IL-1β production induced by *C. albicans.* This indicates that the induction of proinflammatory cytokines by *Candida* species is partially, but not exclusively, mediated by TLR2. Blocking

TLR4 did not influence the production of proinflammatory cytokines, which is in line with the in vivo data obtained in C3H/HeJ mice. Of interest, the production of IL-8, a CXC chemokine considered to be a human homolog of murine KC and MIP-2, was not influenced by blocking either TLR2 or TLR4, which suggests species specificity for the effect of TLRs. This may imply a different role of TLR4 in the defense against *C. albicans* in humans, compared with experimental models of disseminated
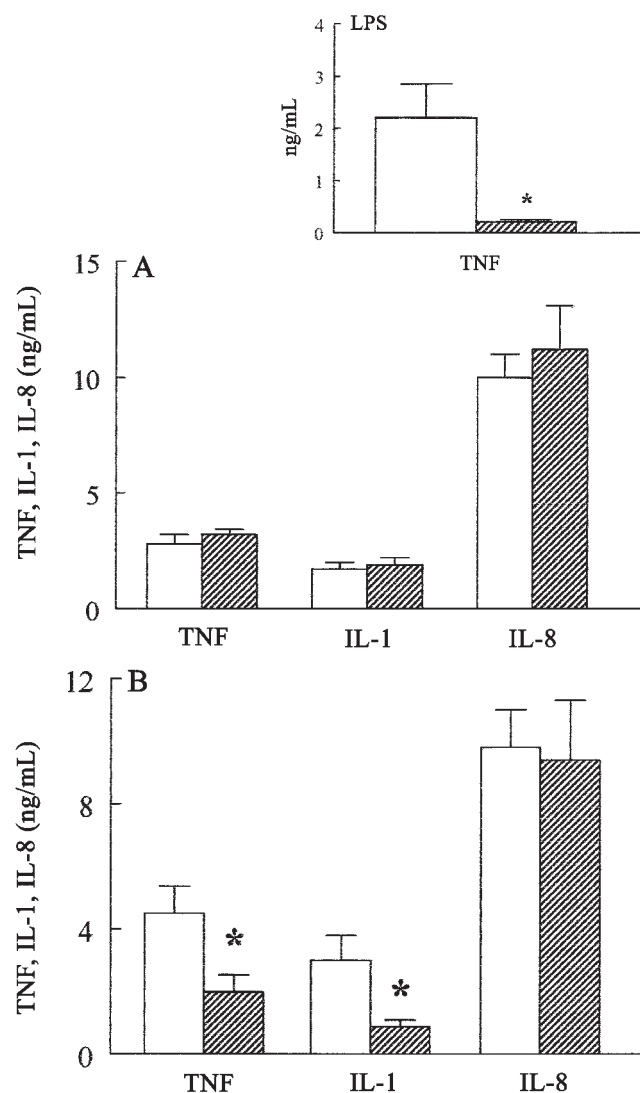

Figure 4. The effect of Toll-like receptor (TLR) 4 and TLR2 on stimulation of cytokines in human peripheral blood mononuclear cells (PBMC) by *Candida albicans*. Human PBMC were stimulated with 10^7 cfu/mL heat-killed *C. albicans* or 10 ng/mL lipopolysaccharide *(inset),* in the absence *(white bars)* or presence *(hatched bars)* of anti-TLR4 *(A)* or anti-TLR2 *(B)* antibodies (20 μg/mL). Tumor necrosis factor (TNF), interleukin (IL)–1β, and IL-8 were measured 24 h later. Cytokine production in unstimulated cells was below the detection limit. Data represent means $\pm$ SE of 7 volunteers. *$P < .05$, Mann-Whitney U test. LPS, lipopolysaccharide.

candidiasis in mice. Differences in TLR2-mediated NO synthesis and mycobacterial killing between murine and human macrophages have been demonstrated recently [25], pointing to significant functional dissimilarities between human and murine TLRs.

The use of TLR2 and TLR4 by *C. albicans* to differentially induce production of proinflammatory cytokines and chemokines implies that a single microorganism can selectively involve different TLRs to activate distinct host defense mechanisms. Although it has been proposed that TLRs transmit a "common danger signal" that leads to the synthesis of inflammatory mediators in response to the presence of pathogen-associated molecular patterns (PAMPs), recent reports have suggested not only that distinct TLRs can discriminate between different PAMPs from the same microorganism but also that the signals induced by the various TLRs may differ in terms of which set of genes is activated. In this context, similar to *C. albicans,* which uses both TLR2 and TLR4 for signaling, *Neisseria meningitidis* stimulates TLR4 through its LPS component and TLR2 through membrane-associated proteins [26, 27], and *M. tuberculosis* uses both TLR2 and TLR4 to stimulate cytokine production [7]. Even more challenging is the concept that various TLRs activated by the same microorganism can activate different pathways of the host defense, which implies differences between the intracellular signal pathways triggered by the TLRs. Indeed, recent data have suggested differences in the intracellular protein complexes involving MyD88, TIRAP, Tollip, and p85 formed on binding to TLR4/TLR4 and TLR2/TLR6 dimers [28]. This results in differential activation of gene sets: stimulation of TLR2 by *Porhyromonas gingivalis* LPS leads to expression of the genes for TNF, IL-12p70, MIP-2, and IP-10 but not to expression of the genes for IFN-γ, IL-12p40, and MCP-5, whereas stimulation of TLR4 by *E. coli* LPS induces the expression of all these genes [29]. In addition, the TLR2 agonists lipoarabinomannan and phosphatidylinositol are able to induce TNF and NO, but not IL-1β, whereas the TLR4 agonist *E. coli* LPS induces all 3 products [30]. Therefore, TLR2 and TLR4 can induce different intracellular signals, and this appears to be also the case for *C. albicans* stimulation.

Because CD14 is able to function as coreceptor for both TLR2 and TLR4 [13, 31], we investigated whether CD14 is involved in the induction of cytokines by *Candida.* In contrast to the almost complete inhibition of LPS-induced cytokines, blocking CD14 had no effect on induction of TNF, IL-1β, or IL-8 by *C. albicans*. CD11b/CD18 is also a cellular receptor for *Candida* blastospores [32]. Because it has been shown recently that CD11b/CD18 acts together with the TLR4/CD14 complex to elicit a full LPS response [33], it may be possible that CD11b/CD18 plays a similar role in the *Candida*-TLR interaction.

Phagocytosis of *C. albicans* was normal in the C3H/HeJ mice, which demonstrates that TLR4 is not involved in the phagocytosis of the fungus, which is in line with the studies suggesting that the mannose receptor (MR) is the most important phagocyte receptor for *C. albicans* [34]. MR is involved in the internalization of *Candida* [35], and blockade of this step by mannoside

inhibits the cytokine production [36]. It has been shown elsewhere that internalization of zymosan through MR recruits TLR2 to macrophage phagosomes, leading to intracellular signals and cytokine production [12]. It is tempting to speculate that the same cooperation between 2 classes of pattern-recognition receptors, namely phagocytic receptors such as MR and signaling receptors such as TLRs, is responsible for the recognition of *C. albicans.*

In *Drosophila,* Spaetzle/Toll interactions have been shown to induce direct antifungal mechanisms by controlling the expression of the defensin-like gene drosomycin [3]. Recent studies have also suggested activation by TLRs of direct antimicrobial mechanisms, such as oxygen species generation [37] and NO production [25], which are the 2 main candidicidal mechanisms employed by the host [38]. We found that the candidicidal capacity of neutrophils was not impaired in TLR4-deficient C3H/HeJ mice, and this was accompanied by normal NO and superoxide production. It appears, therefore, that TLR4 is not involved in inducing these direct candidicidal host mechanisms.

In conclusion, TLR4 contributes to the host defense against disseminated *C. albicans* infection. The increased fungal load in the organs of TLR4-defective mice was associated with an impaired synthesis of CXC chemokines and a blunted neutrophil recruitment to the site of infection, which is a novel mechanism under the control of TLRs during systemic infection. Although cells of TLR4-deficient mice produce normal amounts of TNF and IL-1 after stimulation with *C. albicans,* the production of proinflammatory cytokines appears to be largely TLR2-dependent. We therefore conclude that both TLR2 and TLR4 are involved in the activation of host defense during disseminated candidiasis.

References

1. Pfaller MA, Jones RN, Doern GV, et al. International surveillance of blood stream infections due to *Candida* species in the European SENTRY Program: species distribution and antifungal susceptibility including the investigational triazole and echinocandin agents. Diagn Microbiol Infect Dis **1999**;35:19–25.
2. Edmond MB, Wallace SE, McClish DK, Pfaller MA, Jones RN, Wenzel RP. Nosocomial bloodstream infections in United States hospitals: a three-year analysis. Clin Infect Dis **1999**;29:239–44.
3. Lemaitre B, Nicolas E, Michaut L, Reichhart J-M, Hoffmann JA. The dorsoventral regulatory gene cassette Spaetzle/Toll/Cactus controls the potent antifungal response in *Drosophila* adults. Cell **1996**;86:973–83.
4. Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D. Recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J Immunol **1999**;163:1–5.

5. Takeuchi O, Hoshino K, Kawai T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity **1999**;11:443–51.

6. Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. Peptido-glycan- and lipoteichoic acid–induced cell activation is mediated by toll-like receptor 2. J Biol Chem **1999**;274:17406–9.

7. Means TK, Wang S, Lien E, Yoshimura A, Golenbock DT, Fenton MJ. Human Toll-like receptors mediate cellular activation by *Mycobacterium tuberculosis.* J Immunol **1999**;163:3920–7.

8. Hirschfeld M, Kirschning CJ, Schwandner R, et al. Inflammatory signaling by *Borrelia burgdorferi* lipoproteins is mediated by Toll-like receptor 2. J Immunol **1999**;163:2382–6.

9. Poltorak A, He X, Smirnova I, et al. Defective LPS signalling in C3H/HeJ and C57Bl/10ScCr mice: mutations in TLR4 gene. Science **1998**;282: 2085–8.

10. Hayashi F, Smith KD, Ozinsky A, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature **2001**; 410:1099–103.

11. Hemmi H, Takeuchi O, Kawai T, et al. A Toll-like receptor recognizes bacterial DNA. Nature **2000**;408:740–5.

12. Underhill DM, Ozinski A, Hajjar AM, et al. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminate between pathogens. Nature **1999**;401:811–5.

13. Wang JE, Warris A, Ellingsen EA, et al. Involvement of CD14 and Toll-like receptors in activation of human monocytes by *Aspergillus fumigatus* hyphae. Infect Immun **2001**;69:2402–6.

14. Shoham S, Huang C, Chen J-M, Golenbock DT, Levitz SM. Toll-like receptor 4 mediates intracellular signaling without TNF release in response to *Cryptococcus neoformans* polysaccharide capsule. J Immunol **2001**;166:4620–6.

15. Lehrer RI, Cline MJ. Interactions of *Candida albicans* with human leukocytes and serum. J Bacteriol **1969**;98:996–1004.

16. Kullberg BJ, Van't Wout JW, Van Furth R. Role of granulocytes in enhanced host resistance to *Candida albicans* induced by recombinant interleukin-1. Infect Immun **1990**;58:3319–24.

17. Kullberg BJ, Van't Wout JW, Hoogstraten C, Van Furth R. Recombinant interferon-γ enhances resistance to acute disseminated *Candida albicans* infection in mice. J Infect Dis **1993**;168:436–43.

18. Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. J Immunol **1988**;141:2407–12.

19. Lundqvist H, Dahlgren C. Isoluminol-enhanced chemiluminescence: a sensitive method to study the release of superoxide anion from human neutrophils. Free Radic Biol Med **1996**;20:785–92.

20. Drenth JPH, Van Uum SHM, Van Deuren M, Pesman GJ, Van der Ven-Jongekrijg J, Van der Meer JWM. Endurance run increases circulating IL-6 and IL-1ra but downregulates ex vivo TNF-α and IL-1β production. J Appl Physiol **1995**;79:1497–503.

21. Netea MG, Demacker PNM, Kullberg BJ, et al. Low-density–lipoprotein receptor deficient mice are protected against lethal endotoxinemia and severe Gram-negative infections. J Clin Invest **1996**;97:1366–72.

22. Tebo JM, Datta S, Kishore R, et al. Interleukin-1–mediated stabilization of mouse KC mRNA depends on sequences in both 5′- and 3′-untranslated regions. J Biol Chem **2000**;275:12987–93.

23. Lemay S, Lebedeva TV, Singh AK. Inhibition of cytokine gene expression by sodium salicylate in a macrophage cell line through an NF-κB–independent mechanism. Clin Diagn Lab Immunol **1999**;6:567–72.

24. Frendeus B, Wachtler C, Hedlund M, et al. *Escherichia coli* P fimbriae utilize the Toll-like receptor 4 pathway for cell activation. Mol Microbiol **2001**;40:37–51.

25. Thoma-Uszynski S, Stenger S, Ochoa MT, et al. Induction of direct antimicrobial activity through mammalian Toll-like receptors. Science **2001**;291:1544–7.

26. Pridmore AC, Wyllie DH, Abdillahi F, et al. A lipopolysaccharide-deficient mutant of Neisseria meningitidis elicits attenuated cytokine release by human macrophages and signals via Toll-like receptor (TLR) 2 but not via TLR4/MD2. J Infect Dis **2001**;183:89–96.

27. Ingalls RR, Lien E, Golenbock DT. Membrane-associated proteins of a lipopolysaccharide-deficient mutant of *Neisseria meningitidis* activate the inflammatory response through Toll-like receptor 2. Infect Immun **2001**;69:2230–6.

28. Golenbock DT, Fenton MJ. Extolling the diversity of bacterial endotoxins. Nat Immunol **2001**;2:286–8.

29. Hirschfeld M, Weis JJ, Toshchakov V, et al. Signaling by Toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. Infect Immun **2001**;69:1477–82.

30. Jones BW, Means TK, Heldwein KA, et al. Different Toll-like receptor agonists induce distinct macrophage responses. J Leukoc Biol **2001**;69: 1036–44.

31. Kielian TL, Blecha F. CD14 and other recognition molecules for lipopolysaccharide: a review. Immunopharmacology **1995**;29:187–205.

32. Forsyth CB, Mathews HL. Lymphocytes utilize CD11b/CD18 for adhesion to *Candida albicans.* Cell Immunol **1996**;170:91–100.

33. Perera P-Y, Mayadas TN, Takeuchi O, et al. CD11b/CD18 acts in concert with CD14 and Toll-like receptor (TLR) 4 to elicit full lipopolysaccharide and taxol-inducible gene expression. J Immunol **2001**;166:574–81.

34. Vasquez-Torres A, Balish E. Macrophages in resistance to candidiasis. Microbiol Mol Biol Rev **1997**;61:170–92.

35. Marodi L, Korchak HM, Johnston RB Jr. Mechanisms of host defense against *Candida* species. I. Phagocytosis by monocytes and monocyte-derived macrophages. J Immunol **1991**;146:2783–9.

36. Yamamoto Y, Klein TW, Friedman H. Involvement of mannose receptor in cytokine interleukin-1β (IL-1β), IL-6, and granulocyte-macrophage colony-stimulating factor responses, but not in chemokine macrophage inflammatory protein 1β (MIP-1β), MIP-2, and KC responses, caused by attachment of *Candida albicans* to macrophages. Infect Immun **1997**;65:1077–82.

37. Aliprantis AO, Yang R-B, Mark MR, et al. Cell activation and apoptosis by bacterial lipoproteins through Toll-like receptor-2. Science **1999**; 285:736–9.

38. Fang FC. Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide–related antimicrobial activity. J Clin Invest **1997**;12:2818–25.