Aspergillus fumigatus Evades Immune Recognition during Germination through Loss of Toll-Like Receptor-4–Mediated Signal Transduction

Mihai G. Netea,1,3 Adilia Warris,2,4 Jos W. M. Van der Meer,1,3 Matthew J. Fenton,4 Trees J. G. Verver-Janssen,1,3 Liesbeth E. H. Jacobs,1,3 Tonje Andresen,4 Paul E. Verweij,2,3 and Bart Jan Kullberg 1,3

Departments of 1Medicine and 2Medical Microbiology, University Medical Center St. Radboud, and 3Nijmegen University Center for Infectious Diseases, Nijmegen, The Netherlands; 4Pulmonary Center, Boston University School of Medicine, Boston, Massachusetts

Peritoneal macrophages from Toll-like receptor (TLR) 4–deficient ScCr mice produced less tumor necrosis factor, interleukin (IL)–1α, and IL-1β than did macrophages of control mice, when stimulated with conidia, but not with hyphae, of Aspergillus fumigatus, a finding suggesting that TLR4-mediated signals are lost during germination. This hypothesis was confirmed by use of a TLR4-specific fibroblast reporter cell line (3E10) that responded to the conidia, but not to the hyphae, of A. fumigatus. In contrast, macrophages from TLR2-knockout mice had a decreased production of proinflammatory cytokines in response to both Aspergillus conidia and Aspergillus hyphae, and these results were confirmed in 3E10 cells transfected with human TLR2. In addition, Aspergillus hyphae, but not Aspergillus conidia, stimulated production of IL-10 through TLR2-dependent mechanisms. In conclusion, TLR4-mediated proinflammatory signals, but not TLR2-induced anti-inflammatory signals, are lost on Aspergillus germination to hyphae. Therefore, phenotypic switching during germination may be an important escape mechanism of A. fumigatus that results in counteracting the host defense.

Invasive aspergillosis is a life-threatening disease that occurs predominantly in immunocompromised patients. As the number of immunocompromised patients has increased, Aspergillus fumigatus has become the second most common opportunistic fungal infection [1]. Despite the availability of new antifungal drugs, the number of deaths due to invasive aspergillosis has increased steadily in the last decades [2], and, therefore, development of additional therapies directed toward the augmentation of host defense mechanisms is urgently needed. A better understanding of the mechanisms responsible for defense against invasive Aspergillus infection is required to develop strategies aimed at boosting the antifungal actions of the immune system.

In nature, A. fumigatus survives as a saprophyte, and its ability to adapt to a wide variability of conditions accounts for its worldwide distribution. To cause infection in an organism, the fungus has to escape or resist the immune system, and germination of hyphal forms is an important mechanism for initiating and establishing an infection [3]. The exact molecular mechanisms used by Aspergillus to evade the host defense during germination are yet unknown. Toll-like receptors (TLRs) are pattern-recognition receptors believed to play a central role in innate immunity to pathogens. Toll was initially identified in Drosophila as a gene required for ontogenesis and antifungal resistance [4]; it has been suggested that homologues of Toll play an important role in innate immunity to pathogens. Toll was initially identified in Drosophila as a gene required for ontogenesis and antifungal resistance [4]; it has been suggested that homologues of Toll play an important role in innate immunity to pathogens. In particular, TLR4 and TLR9 have been shown to play crucial roles in the recognition of pathogen-associated molecular patterns such as lipopolysaccharide (LPS) [6] and lipoteichoic acid [7];
TLR2 for peptidoglycans [7, 8], lipoarabinomannan [9], and bacterial lipoproteins [10]; TLR5 for flagellin [11]; and TLR9 for bacterial DNA [12]. In a recent study, it was suggested that TLR4 is important for the stimulation of monocytes by A. fumigatus hyphae [13], whereas both TLR2 and TLR4 are required for the host defense against Candida albicans [14]. Bearing in mind the differences in the structural components of the cell walls of Aspergillus conidia and Aspergillus hyphae [15], we hypothesized both that TLR stimulation by conidia and that by hyphae are different and that germination may offer Aspergillus the ability to evade recognition by TLRs.

**MATERIALS AND METHODS**

**Animals.** TLR2−/− mice on a C57BL/6J background were provided by Shizuo Akira (Osaka University). Control C57BL/6J mice were obtained from Jackson Laboratories. C57BL/ScCr mice that were naturally TLR4 deficient [6] and control C57BL/10J mice were obtained from colonies bred in the local animal facility. Six-to-eight-week-old mice weighing 20–25 g were used for experiments. The mice were fed sterilized laboratory chow (Hope Farms) and water ad libitum. The experiments were approved by the Ethics Committee on Animal Experiments of Nijmegen University.

*A. fumigatus isolate.* The strain V05-27, a clinical isolate of *A. fumigatus*, was grown on Sabouraud glucose agar supplemented with chloramphenicol, for 4–7 days at 35°C. Abundant conidia were produced under these conditions. Conidia were harvested by gently scraping the surface of the slants and suspending them in PBS with 0.05% Tween 80. To remove hyphae and debris, the conidial suspension was filtered through 4 layers of sterile gauze. To yield hyphal fragments, conidia were resuspended in PBS, and both the conidia or hyphae were subsequently heat-killed for 60 min at 56°C. Nonviable conidia and hyphal fragments were centrifuged and were resuspended vigorously. Finally, both suspensions were washed 3 times with HBSS without Ca++ and Mg++ and were kept frozen at −80°C, until use. The lack of viability was always checked by culture in Sabouraud glucose broth, in which no growth was observed after the heat treatment. The *Aspergillus* materials were prepared in an LPS-free fashion and were checked by Limulus assay. No detectable LPS concentrations were found in any of the preparations.

**Reagents and cell lines.** *Escherichia coli* LPS (serotype O55:B5) was obtained from Sigma. Synthetic lipopeptide Pam3Cys (S-2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-[R]-Cys-[S]-Ser-Lys4-OH trihydrochloride) was obtained from EMC Microcollections. Mouse anti-human monoclonal anti-TLR4 HTA125 antibody was a gift of Kensuke Miyake (Saga Medical School). Mouse anti-human monoclonal anti-TLR2 antibody was provided by Douglas Golenbock (University of Massachusetts Medical School). Mouse IgG control antibody (Sigma) was used as a control. The TLR2-deficient CHO/CD14 cell line (clone 3E10), which expresses endogenous hamster TLR4, a nuclear factor-κB (NF-κB)—dependent reporter plasmid drives the expression of surface CD25, as a result of NF-κB translocation [16, 17]. The 3E10/TLR2 cell line that expresses Flag-tagged TLR2 was engineered by stable transfection of the 3E10 reporter cell line with an expression plasmid encoding for human TLR2 in the pFLAG-cytomegalovirus-1 vector, as described elsewhere [16]. Surface CD25 was assessed by flow cytometry, as described below.

**In vitro cytokine production by mouse macrophages.** Groups of 5 mice were killed, and resident peritoneal macrophages were harvested by injecting 4 mL of sterile PBS containing 0.38% sodium citrate [18]. After centrifugation and washing, the cells were resuspended in RPMI 1640 containing 1 mmol/L pyruvate, 2 mmol/L L-glutamine, 100 μg/mL gentamicin, and 2% fresh mouse plasma (culture medium). Cells were cultured in 96-well microtiter plates (Greiner) at 105 cells/well, in a final volume of 200 μL. The cells were stimulated with either control medium or heat-killed conidia or hyphae of *A. fumigatus* (10° microorganisms/mL unless otherwise indicated), which has been shown in previous experiments to induce optimal cytokine release. In pilot experiments, we were not able to find any differences between cytokine production induced by live organisms or that induced by killed conidia or hyphae of *A. fumigatus* (10° microorganisms/mL unless indicated), which has been shown in previous experiments to induce optimal cytokine release. In pilot experiments, we were not able to find any differences between cytokine production induced by live organisms or that induced by killed organisms, when short incubation times (4 h) were used (unpublished data). However, optimal cytokine induction requires longer incubation periods, which result in overgrowth of the microorganisms with lysis of the cultured cells, and we have therefore used killed microorganisms in the subsequent experiments. After incubation for 24 h at 37°C, the plates were centrifuged at 500 g for 10 min, and the supernatant was collected and was stored at −80°C, until cytokine assays were performed.

**Flow cytometric analysis of 3E10 cell lines.** Cells were plated in 12-well tissue culture plates, at a density of 3 × 104 cells/well, and were incubated overnight at 37°C in a 5% CO2 atmosphere. Thereafter, cells were washed 3 times with PBS, were resuspended in medium, and were stimulated for 18 h with either conidia or hyphal preparations of *A. fumigatus*, the TLR2 agonist Pam3Cys (100 ng/mL), or the TLR4 agonist LPS (100 ng/mL). The cells were detached from the plastic by use of trypsin-EDTA and were examined by cell-cytometric analysis for the presence of surface CD25, as described elsewhere [16].

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by A. fumigatus, in human peripheral blood mononuclear cells (PBMCs). Venous blood was drawn from cubital veins of 4 healthy volunteers, into 3 10-mL EDTA tubes (Monoject). PBMCs were isolated as described elsewhere [19], 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). PBMCs were washed twice in saline and were suspended in culture medium (RPMI 1640 supplemented with 10 μg/mL gentamicin, 10 mmol/L L-glutamine, and 10 mmol/L pyruvate). The cells were counted in a Coulter counter (Coulter Electronics), and the number was adjusted to 5 × 10⁶ cells/mL. PBMCs (5 × 10⁶ cells/mL, in a 100-mL volume) were incubated in 96-well plates with 100 mL (PBMCs).

Conidia of either culture medium or heat-killed conidia or hyphae of A. fumigatus (final concentration, 10⁷ microorganisms/mL, unless otherwise indicated). In blocking studies, PBMCs were preincubated for 1 h at 37°C with monoclonal anti-human TLR4 or anti-TLR2 antibodies (20 μg/mL) before stimulation with heat-killed A. fumigatus. All antibodies used were mouse anti-human IgG antibodies, and we used a mouse IgG isotype in all control wells. After incubation for 24 h at 37°C, the supernatants were collected and were stored at −80°C, until assays were performed.

Cytokine assays. Mouse IL-1α, IL-1β, and TNF-α were determined by specific RIAs (detection limit, 20 pg/mL), as described elsewhere [20]. Human TNF-α concentrations were determined by specific RIAs, as described elsewhere [19]. IL-10 was measured by a commercial ELISA kit (Pelikine Compact; CLB), according to the instructions of the manufacturer.

Statistical analysis. Differences between groups were analyzed by Mann-Whitney U 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 done.

RESULTS

Proinflammatory cytokine production on challenge of TLR4- and TLR2-deficient mouse macrophages with conidia or hyphae of A. fumigatus. Incubation of macrophages with either conidia or hyphae of A. fumigatus results in a dose-dependent stimulation of TNF production, with maximal cytokine release induced by stimulation with 10⁷ microorganisms/mL, which was used for the subsequent experiments. To assess the role that TLR4 plays in the stimulation of cytokines by A. fumigatus, we stimulated peritoneal macrophages of TLR4−/− mice and control C57BL/6J mice, with heat-killed Aspergillus conidia and heat-killed Aspergillus hyphae. Synthesis of TNF, IL-1α, and IL-1β was lower in TLR2β/β macrophages stimulated with either Aspergillus conidia or Aspergillus hyphae than it was in macrophages of control mice (figure 2), a finding demonstrating that TLR2 mediates, at least in part, the stimulation of the proinflammatory cytokines by both Aspergillus conidia and Aspergillus hyphae.

Intracellular signaling induced by A. fumigatus in cell lines expressing TLR4 and/or TLR2. The hypothesis that TLR4 is involved in signal transduction upon challenge with A. fum-
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**Figure 2.** Production of proinflammatory cytokines by macrophages of Toll-like receptor (TLR) 2−/− mice. Mouse peritoneal macrophages of C57BL/6J mice (TLR4+/+) and TLR2−/− mice were stimulated with 10^7 cfu/mL of either heat-killed conidia (A) or hyphae (B) of *Aspergillus fumigatus*. Tumor necrosis factor (TNF), interleukin (IL)–1α, and IL-1β were measured after 24 h of stimulation. Data represent mean ± SE of 10 mice. *, by Mann-Whitney U test.

**DISCUSSION**

The results of the present study have demonstrated that TLR2 and TLR4 are important for the release of both TNF and IL-10.

**Figure 3.** Role of Toll-like receptor (TLR) 4 and TLR2 for stimulation of cytokines by *Aspergillus fumigatus*, in transfected cell lines. A, CD25 expression after stimulation with TLR2 agonist (Pam3Cys), TLR4 agonist (lipopolysaccharide (LPS)), or *Aspergillus conidia* and *Aspergillus* hyphae, assessed in 3E10 cells expressing hamster TLR4 and human TLR2, in which nuclear factor-κB (NF-κB) reporter plasmid drives CD25 expression. The expression of CD25 on the surface of 3E10 cells was stimulated by *Aspergillus conidia* but not by *Aspergillus hyphae* (figure 3A). The expression of CD25 on the surface of 3E10 cells was stimulated by *Aspergillus conidia* but not by *Aspergillus hyphae* (figure 3A). TLR2-dependent cellular activation by *A. fumigatus* was further tested in 3E10 cells transfected with the cDNA for TLR2, in which CD25 expression was used as a marker of activation. The TLR2 agonist Pam3Cys stimulated expression of CD25 on the surface of 3E10/TLR2 cells, as did both *Aspergillus conidia* and *Aspergillus hyphae* (figure 3B).

The role of TLR4 and TLR2 for the stimulation of proinflammatory cytokines by *A. fumigatus*, in human cells. Preincubation of freshly isolated human PBMCs, with antibodies to human TLR4, did not influence the production of TNF after stimulation with 10^7 cfu/mL of either conidia or hyphae of *A. fumigatus* (50%–70% inhibition; *P* < .01) (figure 4B).

**TLR2, but not TLR4, mediates stimulation of IL-10 by hyphae of *A. fumigatus*.** Recent data suggest that TLR2 stimulation mediates signals that favor a bias toward a T helper (Th) 2 cytokine response [21], which leads to an increased susceptibility to *Aspergillus* infections [22, 23]. We therefore compared the capacities of *Aspergillus conidia* and *Aspergillus* hyphae to stimulate production of the anti-inflammatory cytokine IL-10 and found that hyphae induced a much higher production of IL-10 than did conidia, in both mouse peritoneal macrophages (figure 5) and human PBMCs (hyphae, 23 ± 7 pg/mL; conidia, <4 pg/mL; *P* < .01). The release of IL-10 induced by *Aspergillus* hyphae was TLR2-dependent, because the macrophages of TLR2−/− mice did not show significant IL-10 release upon stimulation (figure 5). In contrast, stimulation of IL-10 was not influenced by the absence of TLR4 in ScCr mice (44 ± 23 vs. 31 ± 12 pg/mL, in control mice; *P* > .05).

**Figure 4.** Production of proinflammatory cytokines by macrophages of Toll-like receptor (TLR) 2−/− mice. Mouse peritoneal macrophages of C57BL/6J mice (TLR4+/+) and TLR2−/− mice were stimulated with 10^7 cfu/mL of either heat-killed conidia (A) or hyphae (B) of *Aspergillus fumigatus*. Tumor necrosis factor (TNF), interleukin (IL)–1α, and IL-1β were measured after 24 h of stimulation. Data represent mean ± SE of 10 mice. *, by Mann-Whitney U test.

**Figure 5.** Role of Toll-like receptor (TLR) 4 and TLR2 for stimulation of cytokines by *Aspergillus fumigatus*, in transfected cell lines. A, CD25 expression after stimulation with TLR2 agonist (Pam3Cys), TLR4 agonist (lipopolysaccharide (LPS)), or *Aspergillus conidia* and *Aspergillus hyphae*, assessed in 3E10 cells expressing hamster TLR4, in which nuclear factor-κB (NF-κB) reporter plasmid drives CD25 expression. The expression of CD25 on the surface of 3E10 cells was stimulated by *Aspergillus conidia* but not by *Aspergillus hyphae* (figure 3A). The expression of CD25 on the surface of 3E10 cells was stimulated by *Aspergillus conidia* but not by *Aspergillus hyphae* (figure 3A). TLR2-dependent cellular activation by *A. fumigatus* was further tested in 3E10 cells transfected with the cDNA for TLR2, in which CD25 expression was used as a marker of activation. The TLR2 agonist Pam3Cys stimulated expression of CD25 on the surface of 3E10/TLR2 cells, as did both *Aspergillus conidia* and *Aspergillus hyphae* (figure 3B).
induced production of TNF and IL-1 in a dose-dependent manner, a finding that is consistent with previous data [26, 27].

The experiments with macrophages from TLR2−/− mice and those with blocking anti-TLR2 antibodies in human PBMCs demonstrate that TLR2 is important for stimulation of cytokines by both Aspergillus conidia and Aspergillus hyphae. Our findings are in line with those of Mambula et al. [28], who showed that TLR2 is the main TLR receptor used by both Aspergillus conidia and Aspergillus hyphae, and they are similar to results showing the involvement of TLR2 in the stimulation of cytokines by fungal components from Candida albicans [14] and zymosan from Saccharomyces cerevisiae [29]. In this respect, TLR2 is a crucial pattern-recognition receptor in the context of innate immunity, which recognizes not only fungal components but also peptidoglycans [7, 8], lipoarabinomannan [9], and bacterial lipoproteins [10]. Whether recognition of A. fumigatus by TLR2 involves heterodimerization with TLR6 or even with another putative TLR, is not known.

The most interesting finding of the present study is the observation that, upon stimulation with conidia of A. fumigatus but not with hyphae of A. fumigatus, macrophages from TLR4-deficient ScCr mice respond with a decreased production of proinflammatory cytokines. Similarly, hamster 3E10 cells expressing TLR4 but not expressing TLR2 were activated after challenge with conidia of A. fumigatus but not after challenge with hyphae of A. fumigatus. Our data are slightly different from those of Mambula et al. [28], who observed TLR4-mediated TNF production with swollen conidia, but not with resting conidia, in mice. The explanation for these differences may lie in the fact that we tested our preparation in ScCr mice that had a null mutation in the TLR4 gene, whereas they used C3H/HeJ mice that had a dysfunctional TLR4 due to a missense mutation. These data imply that mouse and hamster TLR4 can recognize A. fumigatus conidia, but this capacity is lost on

1 induced by A. fumigatus. Most interestingly, TLR4 induces signals only upon stimulation with the conidial form of the fungus, whereas the phenotypic switch to hyphae leads to loss of TLR4-mediated signaling. The stimulation of TLR2 by Aspergillus hyphae, in the absence of TLR4 signals, results in the enhanced release of IL-10, a finding that may represent an important escape mechanism used by Aspergillus during germination.

A. fumigatus is a pathogenic fungus in which germination from conidia to a hyphal form serves as an escape mechanism from the host defense [3]. Innate immunity plays a central role in the defense against invasive aspergillosis, with tissue macrophages being involved in the ingestion and killing of Aspergillus conidia and neutrophils, by use of oxidative mechanisms to attack hyphae germinating from conidia that escape macrophage surveillance [24]. For proper antifungal activities of both macrophages and neutrophils, stimulation by proinflammatory cytokines, such as TNF and IL-1, is crucial, whereas blocking these mediators in experimental models of aspergillosis is deleterious [25]. In contrast, anti-inflammatory cytokines, such as IL-4 and IL-10, inhibit the cellular mechanisms important for antifungal defense [22, 23]. Little is known about the molecular mechanisms leading to production of proinflammatory and anti-inflammatory cytokines, by A. fumigatus. In the present study, Aspergillus conidia and Aspergillus hyphae
germination to hyphae. It has been shown that TLR4 agonists are more potent than TLR2 agonists, in terms of TNF and IL-1 production, and TLR4 agonists induce release of proinflammatory products such as IL-12, interferon (IFN)–γ, IFN-inducible protein 10, and inducible nitric oxide synthase, which are not stimulated by TLR2 [30, 31]. That TLR2 mediates release of IL-12p40, whereas TLR4 mediates production of IL-12p70 and IFN-γ [30], suggests that TLR2 signals tend to drive the immune response toward a Th2 phenotype, whereas TLR4 drives the development of a Th1 phenotype [21, 32]. This finding also suggests that hyphae of *Aspergillus fumigatus* may be less effective in stimulation of Th1 cytokines than are conidia and is similar to the data reported for the yeast and hyphal forms of *C. albicans* [33, 34]. A strong proinflammatory Th1 cytokine response is, in turn, responsible for a stronger activation of neutrophils for the killing of hyphae [35] and for host survival in experimental models [36, 37]. The loss of TLR4-mediated signals by germination to hyphae may therefore represent an important mechanism through which *A. fumigatus* escapes the antifungal mechanisms.

Interestingly, no inhibitory effects of an anti-human TLR4 antibody on *Aspergillus*-induced TNF and IL-1, by PBMCs, could be observed. Because the antibody displayed potent inhibitory effects on LPS-induced cytokines, there are 2 possible explanations. First, the epitope recognized by the anti-TLR4 HTA125 antibody may be located at a site of the TLR4 molecule that mediates LPS binding but not binding of *A. fumigatus*. This hypothesis is sustained by the data of Wang et al. [13], who showed that a synthetic analogue of *Rhodobacter capsulatum* lipid A blocks the stimulation of TLR4 by LPS but not by *Aspergillus* hyphae. Second, there may be functional differences between the mouse or hamster TLR4 and the human TLR4. Our data contrast with those of Wang et al. [13], who showed a slight but significant inhibitory effect of anti-TLR4 antibodies after stimulation of human cells with *Aspergillus* hyphae. However, anti-TLR4 antibodies inhibited only 30%–40% of TNF stimulation in their study, a finding implying that other receptors are more important than TLR4.

In contrast to the induction of proinflammatory cytokines through TLR4, TLR2 signals result in a strong anti-inflammatory response [21]. The stimulation of TLR2 by *Aspergillus* hyphae, in the absence of TLR4 signals, resulted in the release of IL-10, which is known to impair the cellular immune responses necessary for the host defense against *Aspergillus* [22]. This finding is in line with other studies that also show preferential induction of IL-10 by *Aspergillus* hyphae [38], whereas the conidia mainly induce a proinflammatory cytokine profile [27]. The stimulation of IL-10 by *Aspergillus* hyphae was totally TLR2-dependent. The switch from a proinflammatory to an anti-inflammatory cytokine profile during germination of *Aspergillus* conidia to *Aspergillus* hyphae, by induction of TLR2-mediated IL-10 production, may therefore represent an important immunosuppressive mechanism used by the fungus to evade host defense, as was recently shown to be used by *Yersinia enterocolitica* [39], and this mechanism is similar to the recently described selectivity of the TLR system to the host-specific modifications of LPS from *Pseudomonas aeruginosa*: Whereas TLR4 does not recognize the pentaacyl LPS from environmental strains of *P. aeruginosa*, which are able to infect the host, it is able to recognize the hexaacyl LPS expressed by colonizing strains of *Pseudomonas* isolated from patients with cystic fibrosis. The latter recognition by TLR4 results in more robust proinflammatory signals [40].

The cell wall components of *A. fumigatus* that stimulate cytokine production by mononuclear cells are not known. There are multiple candidate molecules—such as galactomannan, α(1,3)-glucans, β(1,3)/(1,4)-glucans, β(1,3)/(1,6)-glucans, and chitin—as well as cell wall proteins [15]. In *C. albicans*, mannanproteins are responsible for stimulation of proinflammatory cytokines [41], whereas a β(1,6)-glucan component appears to be responsible for chemokine induction [42]. Whether molecules responsible for the different patterns of TLR4-dependent activation by the cell walls of *Aspergillus* conidia and *Aspergillus* hyphae are located in the polysaccharide or protein fractions remains to be elucidated.

In conclusion, we have demonstrated that TLRs are important for stimulation of proinflammatory cytokines by *A. fumigatus*. Whereas both *Aspergillus* conidia and *Aspergillus* hyphae stimulate cytokines through TLR2, only the conidia are capable of stimulating the cells via TLR4. The loss of TLR4-mediated signals during germination results in strong stimulation of IL-10, a finding suggesting that an escape mechanism of *A. fumigatus* is applied by the microorganism to counteract the host defense.

References
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