Modulation of Toll-Like Receptor 2 (TLR2) and TLR4 Responses by *Aspergillus fumigatus*[▽]

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Toll-like receptor (TLR)-based signaling pathways in the host may be modulated by pathogens during the course of infection. We describe a novel immunomodulatory mechanism in which *Aspergillus fumigatus* conidia induce attenuation of TLR2- and TLR4-mediated interleukin (IL)-6 and IL-1 β proinflammatory responses in human mononuclear cells with suppression of IL-1 β mRNA transcription. Background TLR2 and TLR4 mRNA transcription was not influenced. *A. fumigatus* conidia induced TLR2 internalization and uptake into the phagosome with a resultant decrease in surface receptor expression. *A. fumigatus* hyphae, on the other hand, selectively downregulated the TLR4-mediated response. These novel immunosuppressive effects may facilitate the invasiveness of *A. fumigatus*.

Opportunistic fungal infections remain a significant cause of mortality and morbidity in immunocompromised patients. *Aspergillus fumigatus* is one of the most common infective molds worldwide (7). Acquisition of invasive aspergillosis results from inhalation of airborne conidia, which, in the absence of competent immune containment, is followed by germination, development of hyphae, and invasive disease.

Mononuclear phagocytes constitute an important component of host defense against *A. fumigatus* and also are the precursors of tissue macrophages involved in immune surveillance against invasive pulmonary aspergillosis (29, 31). Their ability to detect specific pathogen-associated molecular patterns of *Aspergillus* conidia involves expression of pathogen recognition receptors (PRRs), and the main families of these receptors are the Toll-like receptors (TLRs) and the lectin receptors. TLR2 and TLR4 have been reported to mediate recognition of various cell wall components of *Aspergillus* (2, 18, 19, 40), and dectin-1, a C-type lectin receptor, is the major PRR involved in the recognition of β -glucans (21, 29, 32). Although infection experiments with TLR-deficient mice have provided evidence for involvement of TLR2 and TLR4 in the host defense against *A. fumigatus* (1, 2), the relative importance of both these receptors and other TLRs remains to be determined. In addition, the inflammatory response triggered through TLR2 and TLR4 strongly depends on the *Aspergillus* morphotype. For example, while conidium recognition was mediated by both TLR2 and TLR4, TLR4-mediated signaling

was lost in recognition of hyphae (24). This inability to recognize and fight the most dangerous morphotype could represent an immune evasion mechanism (23). On the other hand, very little is known about whether fungal pathogens can also modulate a TLR-mediated host defense by decreasing the capacity of host cells to respond to TLR2 and TLR4 ligands.

The aim of the present study was to study the capacity of *A. fumigatus* to modulate the host immune response triggered by TLRs. The effects of *Aspergillus* modulation of TLR2 and TLR4 activation were specifically examined, given that these receptors mediate the major pathways implicated in antifungal host defense.

MATERIALS AND METHODS

Reagents. The TLR2 ligand tripalmitoyl-S-glycerol-Cys-(Lys)4 (Pam3Cys) was purchased from EMC Microcollections (Tübingen, Germany). The TLR4 ligand *Escherichia coli* lipopolysaccharide (LPS) (*E. coli* serotype O55:B5) was purchased from Sigma Chemical Co. (St. Louis, MO). An extra LPS purification step was performed as previously described (13). Cytochalasin B was purchased from BIOMOL International and dissolved at a concentration of 5 mg/ml in dimethyl sulfoxide. Laminarin, a specific inhibitor of dectin-1, and mannan, a competitive inhibitor of the mannose receptor (MR) pathway (38), were kindly provided by David Williams (University of Tennessee). *Bartonella* LPS (anti-TLR4) was obtained as previously described (27). 3-(1-Methyl-1H-indol-3-yl-methylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide (Calbiochem) was used for pharmacological inhibition of the dectin-1-associated adaptor molecule Syk.

***A. fumigatus*.** Strain V05-27, a previously characterized clinical isolate of *A. fumigatus* (24), was grown on Sabouraud glucose agar supplemented with chloramphenicol for 4 to 7 days at 35°C. Abundant conidia were produced under these conditions. Conidia were harvested by gently scraping the surfaces of slants and suspending the conidia in phosphate-buffered saline (PBS) with 0.05% Tween 80. To remove hyphae and debris, the conidial suspension was filtered through four layers of sterile gauze. To obtain hyphal fragments, conidia were added to 5 ml of yeast nitrogen base (Difco Laboratories) at a final concentration of 10⁸ microorganisms/ml. After 18 h of incubation at 37°C, the tubes were centrifuged at 1,550 $\times$ g for 10 min, and the pellet, containing mycelia almost exclusively, was

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washed twice in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} and resuspended in PBS. Aliquots of the conidial suspension and mycelium were heat killed for 60 min at 56°C . Nonviable conidia and hyphal fragments were centrifuged at $4,000 \times g$ for 15 min, resuspended, and vortexed vigorously. Finally, both suspensions were washed three times with Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} to remove released *A. fumigatus* components and then kept frozen at -80°C until they were used. Viability was checked by culturing in Sabouraud glucose broth, and no growth was observed after heat treatment. The *Aspergillus* materials were prepared in an LPS-free fashion. For the experiments, 10^7 microorganisms/ml of heat-killed *A. fumigatus* conidia or hyphae and 10^6 microorganisms/ml of live *A. fumigatus* conidia were used for priming, unless otherwise indicated.

Stimulation assays. Venous blood was drawn into EDTA tubes from healthy volunteers after informed consent was obtained. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). Cells were washed twice in saline and counted, and the concentration was adjusted to 5×10^6 cells/ml. A 100- μl volume of the PBMC preparation, suspended in culture medium (RPMI 1640 DM; ICN Biomedicals, Costa Mesa, CA) supplemented with 10 $\mu\text{g}/\text{ml}$ gentamicin, 10 mM L-glutamine, and 10 mM pyruvate, was added to flat-bottom 96-well plates (Greiner, The Netherlands).

PBMC were preincubated with *A. fumigatus* conidia, hyphae, or RPMI 1640 (as an unprimed control). After 24 h of incubation at 37°C , the culture supernatants were replaced with fresh culture medium containing a secondary stimulus consisting of either the TLR2 ligand (10 $\mu\text{g}/\text{ml}$ Pam3Cys) or the TLR4 ligand (1 ng/ml LPS). The supernatants were collected after 24 h of incubation at 37°C and stored at -20°C until the cytokine assay was performed. To block phagocytosis, PBMC were preincubated with 1 $\mu\text{g}/\text{ml}$ of cytochalasin B for 30 min at 37°C before stimulation with *A. fumigatus* components as described above. For receptor-blocking experiments, mannan (200 $\mu\text{g}/\text{ml}$), laminarin (200 $\mu\text{g}/\text{ml}$), or *Bartonella* LPS (160 ng/ml) fractions were preincubated with PBMC for 1 h at 37°C prior to stimulation with *Aspergillus*. Dectin-1-mediated intracellular pathways were inhibited by preincubation of cells with a Syk inhibitor (50 nmol/liter) for 30 min before incubation with *A. fumigatus*. The experiments were performed in the absence or presence of inhibitors using PBMC from the same volunteers.

Differentiation of monocyte-derived macrophages (MDM) was performed as previously described (10). In brief, monocytes were isolated by adherence following PBMC isolation, washed, and resuspended in freshly prepared RPMI 1640 containing 10% pooled human serum supplemented with pyruvate, L-glutamine, and gentamicin. The cells were maintained in a humidified atmosphere (5% CO_2) at 37°C for 6 days to permit differentiation, and the medium was refreshed after 3 days. The procedure used for stimulation assays involving MDM was similar to the procedure used for PBMC.

Cytokine measurements. Interleukin-6 (IL-6) and IL-1 β concentrations were measured by using commercial sandwich enzyme-linked immunosorbent assay kits (Pelikine Compact, CLB, Amsterdam, The Netherlands) according to the manufacturer's instructions. Human tumor necrosis factor alpha (TNF- α) concentrations were determined by a specific enzyme-linked immunosorbent assay as described elsewhere (12).

Quantitative PCR. To determine TLR2 and TLR4 mRNA expression, RNA was extracted from PBMC primed with *A. fumigatus* conidia or hyphae for 24 h. For IL-1 β and TNF- α mRNA expression, PBMC were primed with *A. fumigatus* and then stimulated with Pam3Cys, LPS, or RPMI 1640 as described above for 4 h. RNA was extracted from 10^7 PBMC by using 1 ml TRIzol reagent (Sigma, St. Louis, MO). Subsequently, 200 μl chloroform and 500 μl 2-propanol (Merck, Darmstadt, Germany) were used to separate the RNA from DNA and proteins. Finally, after the preparation was washed with 75% ethanol, the RNA was dissolved in 50 μl of diethylpyrocarbonate water.

To obtain cDNA, we reverse transcribed 1 μg DNase-treated total RNA with oligo(dT) primers (0.01 $\mu\text{g}/\text{ml}$) in a reverse transcription-PCR mixture (total volume, 20 μl). Quantitative real-time PCR to monitor DNA synthesis was performed using a Bio-Rad iCycler and SYBR green. The following primers were used (5'-3'): GAATCCTCAATCAGGCTTCTCT (forward) and GCCCTGAGGGAATGGAGTTTA (reverse) for TLR2, GGCATGCCTGTGCTGAGTT (forward) and CTGCTACAACAGATACATAACAAGCACACT (reverse) for TLR4, GCCCTAAACAGATGAAGTGCTC (forward) and GAACCAGCATCTCCTCAG (reverse) for IL-1 β , TGGCCAGGCAGTCAGA (forward) and GGTTTGCTACAACATGGGCTACA (reverse) for TNF- α , and ATGAGTATGCCTGCCGTGTG (forward) and CCAAATGCGGCATCTTCAAAC (reverse) for β_2 microglobulin (Biolegio, The Netherlands). Quantification of the PCR signals for each sample was performed by comparing the cycle threshold values in duplicate for the gene of interest with the cycle threshold values for the β_2 microglobulin housekeeping gene. The mean relative mRNA expression was

calculated using the Pfaffl method. The results are expressed below as the ratio of the amount of increase to the mRNA level for unprimed cells.

TLR2 and TLR4 expression as determined by flow cytometry. From PBMC, CD14 $^{+}$ monocytes were isolated by positive selection with anti-CD14 microbeads according to the manufacturer's instructions (Miltenyi Biotec, Germany). Following incubation with *A. fumigatus* conidia or hyphae or an RPMI 1640 control for 24 h, cells were cooled to 4°C , washed two times in 0.5% bovine serum albumin-PBS, and incubated for 15 min with the following monoclonal antibodies (MAbs): anti-TLR2-fluorescein isothiocyanate and anti-TLR4-phycoerythrin (eBioscience, San Diego, CA). The cells were washed two more times and then analyzed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Phagocytosis of *Aspergillus* conidia and TLR2 internalization. Blankophor staining (30) was used to differentiate phagocytosed *Aspergillus* conidia from bound conidia. *A. fumigatus* conidia were incubated with monocytes obtained via adherence from PBMC for 30 min at 37°C . To permit phagocytosis, monocytes were washed at 4°C , and Blankophor stain was added. The fresh mount was then visualized using a Leica fluorescence microscope. To visualize internalization of surface TLR2, monocytes were allowed to adhere to fibronectin, and the cells were preincubated with an Alexa 488-labeled TLR2-specific MAb (eBioscience, San Diego, CA) or its isotype control. *A. fumigatus* conidia were added, and phagocytosis was assessed after 30 min of incubation at 37°C . After this incubation the samples were washed, fixed in 4% paraformaldehyde, and visualized using an Olympus FV1000 confocal microscope.

Statistical analysis. Results from at least three sets of experiments were pooled and analyzed using SPSS 16.0 statistical software. The data are expressed below as means $\pm$ standard errors of the means, and the Wilcoxon signed-rank test was used to compare differences between groups (unless otherwise stated). The level of significance was defined as a P value of <0.05 .

RESULTS

***A. fumigatus* conidia and hyphae differentially modulate the host proinflammatory cytokine response induced by TLR2 and TLR4.** Following preincubation of PBMC with heat-killed *A. fumigatus* conidia and hyphae, a dose-dependent cytokine response was observed following Pam3Cys (TLR2) or LPS (TLR4) stimulation, as indicated by IL-6 production. A differential and inverse dose-dependent cytokine response was observed following preincubation with *A. fumigatus* hyphae and subsequent TLR2 stimulation (Fig. 1a and 1b). As 10^7 microorganisms/ml of *A. fumigatus* conidia and hyphae resulted in the strongest responses during most TLR stimulations, subsequent experiments were performed using 10^7 microorganisms/ml of conidia and hyphae.

Overall, preincubation of PBMC with heat-killed *A. fumigatus* conidia significantly attenuated IL-6 production in response to TLR2 and TLR4 stimulation (Fig. 1c and 1d). Similarly, IL-1 β production induced by TLR2 was also strongly inhibited by *A. fumigatus* conidia. In contrast, the TNF- α response was not influenced after preincubation of cells with heat-killed *A. fumigatus* conidia.

On the other hand, preincubation with heat-killed *A. fumigatus* hyphae had a limited immunomodulatory effect. Preincubation with *Aspergillus* hyphae attenuated the TLR4-induced IL-6 and TNF- α production but increased the TLR2-induced TNF- α production (Fig. 1e and 1f). TLR4 stimulation with LPS did not induce detectable levels of secreted IL-1 β in cells preincubated with either RPMI 1640 or *Aspergillus*. This failure to release IL-1 β into the environment is a known phenomenon due to initiation of macrophage differentiation (22, 41).

There was no increase in lactate dehydrogenase levels in the samples containing the *Aspergillus* fractions compared to con-

trols, indicating that cellular toxicity did not have a role in these effects (data not shown).

Live *Aspergillus* conidia had effects (Fig. 1g and 1h) similar to those of heat-killed conidia, with attenuation of TLR2- and TLR4-induced IL-6 responses. However, in contrast to heat-killed conidia, live conidia decreased TLR4-mediated TNF- α production. This response was similar to that observed with heat-killed hyphae (Fig. 1f). This differential TNF- α response following stimulation with heat-killed conidia and with live conidia may be explained by the hyphal development from the live conidia during the course of the experiment.

The modulatory effects of *A. fumigatus* conidia on MDM were similar to those on PBMC. Following preincubation with conidia, the IL-6 production in response to TLR2 and TLR4 stimulation was attenuated (Fig. 1i and 1j).

IL-1 β and TNF- α mRNA expression. IL-1 β mRNA expression was utilized as a surrogate to assess IL-1 β and IL-6 transcription as this process is known to be tightly regulated (9). Parallel with the attenuation of secreted IL-1 β by PBMC, *A. fumigatus* conidia markedly reduced IL-1 β mRNA transcription induced by TLR2 or TLR4 ligands (ratios of 0.34 and 0.42 relative to unprimed controls, respectively) (Fig. 2). Notably, PBMC incubated with conidia alone also displayed attenuation of IL-1 β mRNA transcription. This strengthened the evidence for the immunomodulatory potential of *Aspergillus* conidia. *Aspergillus* hyphae also inhibited TLR4-induced IL-1 β mRNA transcription, while TLR2-induced IL-1 β mRNA transcription was not affected. Cytokine mRNA expression was consistent with trends observed for measured secreted cytokines.

As observed for the cytokine stimulation pattern, the TNF- α mRNA expression was distinct from IL-1 β mRNA expression (Fig. 2). *Aspergillus* hyphae slightly amplified TNF- α mRNA expression induced by TLR2 ligation, which eventually translated into elevated TNF- α levels detectable in the supernatants (Fig. 1b).

Taken together, these results indicate that *Aspergillus* conidia alter TLR2- and TLR4-mediated signaling, resulting in downregulation of cytokine production, and this is corroborated by mRNA measurements. *Aspergillus* hyphae also induced attenuation of TLR4-mediated signaling, although there was a tendency toward an increase in the TLR2 response.

Role of *Aspergillus* conidia uptake for the immunomodulatory effects. In order to assess whether phagocytosis of conidia was involved in the modulation of TLR-induced activation, cytochalasin B, an inhibitor of actin polymerization, was added to PBMC prior to priming with conidia or hyphae. Cytochalasin B reversed the inhibition of TLR2-induced IL-6 levels (Fig. 3a) and IL-1 β levels (data not shown). Interestingly, the effects of *Aspergillus* on TLR4-induced IL-6 were not influenced by cytochalasin B; i.e., these effects were independent of phagocytosis (Fig. 3b).

FIG. 2. IL-1 β and TNF- α mRNA expression in PBMC preincubated with *A. fumigatus* components for 24 h, followed by RPMI 1640, TLR2 ligand, or TLR4 ligand stimulation for 4 h. IL-1 β mRNA expression was suppressed by *Aspergillus* conidia in the absence (RPMI 1640) and in the presence (Pam3Cys and LPS) of secondary stimulation. The data are the cumulative results of three sets of experiments and expressed as the means $\pm$ standard errors of the means. *, $P < 0.05$ relative to the ratio of the fold increase in the mRNA levels of unprimed cells. Asp, *Aspergillus*.

Role of other PRRs in immunomodulation induced by *A. fumigatus*. In order to assess the role of the various PRRs involved in the recognition of *A. fumigatus*, we blocked the respective receptors prior to *Aspergillus* incubation using specific inhibitors. This was followed by TLR2 or TLR4 ligand stimulation as described above. IL-6 production was used as a representative read-out parameter.

Blockage of the MR by mannan had no effect on IL-6 at-

FIG. 1. Effects of preincubation of PBMC (a to h) or MDM (i and j) with heat-killed *Aspergillus* conidia (a to d, i, and j), hyphae (e and f), or live conidia (g and h) on the host TLR2 (Pam₃Cys)- and TLR4 (LPS)-mediated proinflammatory response, compared to results obtained with the corresponding unprimed controls (RPMI 1640). (a and b) Dose-dependent IL-6 response to TLR2 agonist and TLR4 agonist. Live *Aspergillus* conidia (g and h) resulted in IL-6 attenuation similar to that obtained with heat-killed components, but the TLR4-induced TNF- α response was attenuated with germination and hyphal development (as anticipated with heat-killed hyphae [f]). The data are the cumulative results of at least three sets of experiments and are means and standard errors of the means. *, $P < 0.05$ for a comparison with corresponding unprimed PBMC control. Asp, *Aspergillus*; HK, heat killed.

FIG. 3. IL-6 production after TLR2 or TLR4 stimulation by PBMC preincubated with *A. fumigatus* conidia or hyphae in the presence or absence (Control) of cytochalasin B (CytoB) compared to the results for corresponding unprimed PBMC (RPMI 1640). Inhibiting phagocytosis reversed TLR2-induced IL-6 attenuation (a) but not TLR4-induced IL-6 attenuation (b). The data are the cumulative results of three sets of experiments and are means and standard errors of the means. *, $P < 0.05$ compared to the corresponding unprimed PBMC following secondary stimulation.

tenuation by *Aspergillus* conidia, but it did have an effect on hyphal modulation of LPS-induced IL-6 expression (Fig. 4a and 4b). No effects of dectin-1 blockage with laminarin (Fig. 4c and 4d) or of TLR4 blockage with *Bartonella* LPS (Fig. 4e and 4f) were observed for *Aspergillus*-induced modulation of TLR responses. Chemical blockage of the dectin-1 adaptor molecule Syk using a specific chemical inhibitor did not have an effect on immunomodulation (data not shown).

Taken together, the results indicate that cytokine production following TLR2 and TLR4 stimulation is downregulated by *Aspergillus* conidia, an immunomodulatory process that is phagocytosis dependent via the TLR2-mediated pathway and largely independent of other PRRs, such as TLR4, MR, and

dectin-1. *A. fumigatus* hyphae induced downregulation of TLR4-mediated signaling, which was MR dependent.

Modulation of TLR2 and TLR4 transcription and expression by *A. fumigatus*. One possible mechanism through which *Aspergillus* may modulate cytokine responses is by directly influencing TLR2 and TLR4 expression. We first assessed whether the amount of TLR mRNA transcription in PBMC was modulated after incubation with *A. fumigatus* conidia and hyphae. In the presence of *A. fumigatus* conidia, there was minimal reduction in TLR2 and TLR4 mRNA transcription compared to that in unprimed cells (Fig. 5). Likewise, in PBMC primed with hyphae, neither TLR2 expression nor TLR4 mRNA expression was significantly altered.

FIG. 4. IL-6 production after TLR2 or TLR4 stimulation by PBMC preincubated with *A. fumigatus* conidia or hyphae in the presence or absence (Control) of the specified inhibitor compared to those for the corresponding unprimed PBMC (RPMI 1640). The inhibitors used were mannan (which blocks MR), laminarin (which blocks dectin-1), and *Bartonella* LPS (TLR4 antagonist). TLR4-mediated attenuation of IL-6 by *A. fumigatus* hyphae was reversed using mannan (b). Modulatory effects were not reversed by blocking the dectin-1 receptor or TLR4 (c to f). The data are the cumulative results of three sets of experiments and expressed as the means $\pm$ standard errors of the means. *, $P < 0.05$ compared to the respective unprimed PBMC. Bart, *Bartonella*.

Flow cytometry was performed with positively selected CD14⁺ monocytes incubated with *A. fumigatus* conidia and hyphae. This methodology was employed because of binding of conidia to monocytes, which affected scatter characteristics and possibly altered CD14 expression upon exposure to *Aspergillus* (5). After stimulation with *A. fumigatus* conidia, surface TLR2 expression on the monocytes was significantly reduced compared to TLR4 expression (Table 1).

These data, together with the minimally influenced mRNA expression, suggest that *A. fumigatus* conidia induced depletion

of surface TLR2 expression on PBMC by inducing receptor engagement and internalization rather than by inhibiting TLR transcription.

Internalization of TLR2 following phagocytosis of *Aspergillus* conidia. An alternative explanation to the decreased surface TLR2 expression described above could be that there was adherence of *Aspergillus* conidia to surface TLR2 binding sites, which blocked access to anti-TLR2 MAb (Table 1). Blankophor staining was utilized to distinguish between ingested and adherent conidia. As shown in Fig. 6a and 6b, following incu-

FIG. 5. Relative TLR2 and TLR4 mRNA expression in PBMC following incubation with *Aspergillus* conidia and hyphae. The data are the cumulative results of three sets of experiments and expressed as the means $\pm$ standard errors of the means. The values are not statistically significant. Asp, *Aspergillus*.

bation of *Aspergillus* conidia with monocytes, all phagocytosed conidia remained unstained, in contrast to conidia in the external milieu. This indicates that interference of TLR2-bound conidia on the cell surface with an anti-TLR2 MAb is very unlikely. In addition, using confocal microscopy, we demonstrated that there was internalization of surface TLR2 into the phagosome together with the *A. fumigatus* conidia (Fig. 6c to f).

DISCUSSION

In the present study, we show that *A. fumigatus* conidia have the modulatory potential to selectively attenuate TLR2- or TLR4-induced proinflammatory IL-1 β and IL-6 cytokine production in mononuclear phagocytic cells. The modulatory effects were observed in PBMC and MDM and could be induced with both heat-killed and live conidia. The immunomodulatory effects of *Aspergillus* conidia on TLR responses are apparent at the transcriptional level due to inhibition of cytokine mRNA. Downregulation of the TLR2-mediated pathway involved internalization of the receptor together with *Aspergillus* conidia into the phagosome, resulting in decreased expression of TLR2 on the cell membrane. *Aspergillus* hyphae displayed a propensity to selectively downmodulate TLR4-mediated responses, an

FIG. 6. (a) Direct microscopic view of a monocyte with three phagocytosed *Aspergillus* conidia and an adherent conidium in the external milieu (arrow). (b) Blankophor staining of the same field, except that the externalized conidium (arrow) was fluorescent. (c and d) Light and fluorescent views of a monocyte labeled with Alexa 488-TLR2 MAb obtained by confocal microscopy, showing localization of TLR2 on cell surface. (e and f) Following phagocytosis of *Aspergillus* conidia (asterisk), concurrent internalization of TLR2 with the phagosome was observed.

effect partially mediated by the MR. The TLR2- and TLR4-mediated phenomena described above were not attributable to altered receptor mRNA transcription.

As *Aspergillus* conidia are the primary morphotype infecting the host, it is possible that the conidial form, because of its ability to decrease the host proinflammatory response via TLR suppression, provides an early advantage to the fungus that enhances its chance of survival and compromises initial attempts by the host defense to contain germination. Hyphal development further induces a selective loss of TLR4-mediated proinflammatory response capacity. However, one has to acknowledge that these effects are most likely relevant only in immunocompromised patients, as host defenses in immunocompetent individuals are usually effective against invasive *Aspergillus* infection.

Conidial and hyphal forms of *A. fumigatus* are able to differentially modulate the host response and generate distinct cytokine responses. Notably, *A. fumigatus* conidia attenuated both TLR2- and TLR4-induced responses (IL-1 β and IL-6 concentrations), but its hyphae resulted in retention or even accentuation of the TLR2-induced response (TNF- α production and mRNA concentration) while they suppressed the TLR4 activity. The decreased extracellular cytokine levels were corroborated by mRNA measurements. Diminished hypha-induced TLR4-mediated proinflammatory signaling is consistent with our earlier observation that *A. fumigatus* hyphae could induce a loss of TLR4-mediated proinflammatory signals while sparing the TLR2-mediated proinflammatory signals (24). In light of this differential response, recent data have suggested that activation of TLR2 in the absence of TLR4 signals (as observed here with *A. fumigatus* hyphae) may favor

TABLE 1. Expression of surface TLR2 and TLR4 on CD14⁺ monocytes following incubation with *A. fumigatus* conidia or hyphae or with an unprimed control (RPMI 1640)

Prepn	TLR2 expression		TLR4 expression	
	Mean fluorescence intensity ^a	P ^b	Mean fluorescence intensity ^a	P ^b
RPMI	85 $\pm$ 17		48 $\pm$ 14	
<i>A. fumigatus</i> conidia	42 $\pm$ 15	0.03 ^c	33 $\pm$ 5	0.05
<i>A. fumigatus</i> hyphae	83 $\pm$ 20	0.46	43 $\pm$ 15	0.25

^a The values are the cumulative results of three sets of experiments and are means $\pm$ standard deviations of mean geometric fluorescence intensities as determined by flow cytometric analysis.

^b P values for comparisons with the corresponding RPMI 1640 controls (Wilcoxon signed-rank test).

^c Statistically significant.

a Th2-type response and provide an advantage to the pathogen (8, 28).

Nonetheless, while it might be tempting to speculate that the observations described above are means of an immune escape mechanism employed by the pathogen to evade the host innate defense, these data may need to be interpreted in the context of the other modulatory consequences of *Aspergillus* infection (5). For instance, we also noted a trend toward increased TNF- α production induced by *A. fumigatus* hyphae via TLR2, which may be a compensatory response to the TLR4-associated attenuation. The immunomodulatory effects on cytokine production by *A. fumigatus* conidia are also not generalized but are significantly more profound for IL-1 β and IL-6, while TNF- α production remains largely unaffected. It appears that TNF- α synthesis may be compensated for by alternative mechanisms, such as induction through lectin receptors like dectin-1 or MRs (20, 34). Our results also suggest that there are differences in the pathways leading to TNF- α and IL-1 β transcription and that these pathways are differentially regulated by the corresponding *Aspergillus* morphotypes. The concept that *Aspergillus* conidia and hyphae induce differential immune responses has recently been suggested (5, 31). Cortez et al. showed that conidia can induce differential expression of genes implicated in host defense and immunomodulation (5). The diminished IL-6 and IL-1 β response reported in our study may be biologically significant as these cytokines orchestrate the host proinflammatory antimicrobial response, including neutrophil recruitment (14, 39). In addition, altered levels of these proinflammatory cytokines affect the Th1-Th2 equilibrium, which may result in an attenuated host phagocytic and cytotoxic response (33).

We demonstrated that the inhibition of TLR2-mediated responses by *Aspergillus* conidia was due to induction of TLR2 engagement and internalization rather than to mediation via other PRRs, like MR, TLR4, or dectin-1. This conclusion is supported by recent studies which described TLR2 involvement in phagocytosis of *A. fumigatus* conidia, as well as internalization and recruitment of TLR2 in phagosomes (17, 36). However, contrary to the notion that phagocytosis is often coupled with elaboration of a proinflammatory cytokine response, as seen in *Candida* and gram-positive bacteria (37), our findings suggest that in the case of *A. fumigatus* conidia, phagocytosis may result in surface receptor depletion and consequently decreased receptor-mediated signaling. We also noted that use of laminarin (a dectin-1 receptor-specific inhibitor) did not reverse the inhibitory effect induced by conidial phagocytosis involving the TLR2 pathway. Our finding counters prior observations that the dectin-1 receptor is intrinsically involved in phagocytosis of *A. fumigatus* conidia and supports the notion that a dectin-1-independent mode of phagocytosis exists based on our experiments using heat-killed conidia (11, 17).

Our observations have also indicated that the MR, another C-type lectin receptor, has a possible immunomodulatory role. Despite its reported ability to recognize fungi like *Candida albicans*, *Cryptococcus neoformans*, and *Pneumocystis*, little is known about the role of MR as a PRR for *A. fumigatus* (3, 6, 26). This is intriguing given that terminal mannose, fucose, and *N*-acetylglucosamine are established ligands for MR (35) and such mannose-based moieties are a major component of the *Aspergillus* cell wall (15). Although MR has been implicated in

the phagocytosis of fungi, it did not seem to play a significant role in the inhibitory effects on TLR2 signals that are mediated through phagocytosis of *Aspergillus* conidia (16, 42). However, we show that mannan reversed TLR4-mediated attenuation of IL-6 production following preincubation with *A. fumigatus* hyphae. It is thus very likely that a ligand of the MR on the *Aspergillus* cell wall is involved in the modulatory capability of the fungus. This is supported by emerging data suggesting that MR has an immunoregulatory role in curtailing the proinflammatory response (4, 43, 44).

A. fumigatus remains the most important filamentous fungal pathogen with significant attributable morbidity and mortality in at-risk patient cohorts (7, 25). We have attempted to show how *A. fumigatus* may alter the host response, in particular, the TLR-mediated host defense, during the course of infection. The observations reported here demonstrate how a pathogen may intrinsically induce downregulation of selective host proinflammatory responses to its advantage. However, the absence of invasive infection in healthy individuals, despite the ubiquitous presence of *Aspergillus*, attests to the effective antifungal mechanisms usually present in a healthy host. In addition to the modulatory effects induced by the pathogen reported above, further immune deficiencies in a compromised host provide the "second hit," leading to disease development. Although the latter factor undoubtedly is the major determining factor in acquisition of invasive disease, it is believed that the intrinsic immune modulatory potential of *A. fumigatus* contributes to the net deficit in the proinflammatory response that skews the Th1-Th2 equilibrium toward an advantage for the pathogen.

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