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Immunomodulatory Effects of Voriconazole on Monocytes Challenged with *Aspergillus fumigatus*: Differential Role of Toll-Like Receptors[▽]

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Voriconazole (VRC) has activity against *Aspergillus fumigatus*, the most frequent cause of invasive aspergillosis in immunocompromised patients. The combination of VRC and *A. fumigatus* hyphae induced a more pronounced profile of expression of genes encoding inflammatory molecules in human monocytes than *Aspergillus* alone did. Herein, we provide further evidence of the potential mechanism underlying this immunomodulatory effect of VRC on human monocytes in response to *A. fumigatus* hyphae. A significant additive antifungal effect was shown when VRC was combined with monocytes against *A. fumigatus* hyphae. Both *A. fumigatus* hyphae and VRC induced pronounced profiles of mRNA and protein expression of Toll-like receptor 2 (TLR2) as well as tumor necrosis factor alpha (TNF-α) in THP-1 monocytic cells compared to untreated cells. The VRC-induced increase was greater than that induced by hyphae. The combination of VRC and hyphae increased mRNA and protein expression of TLR2 and TNF-α to even higher levels than did either VRC or hyphae alone. In contrast, TLR4 expression, both at the mRNA and protein levels, was not increased by either VRC or hyphae or their combination. In addition, significantly more NF-κB was translocated to the nuclei of THP-1 cells treated with VRC than untreated cells. While VRC induced more NF-κB than hyphae did, treatment with the combination of the two factors induced the greatest NF-κB expression. The pronounced profile of TLR2 signaling, TNF-α expression, and NF-κB activation in the presence of VRC suggests an immunomodulatory effect leading to a more efficient response to *A. fumigatus*.

Invasive aspergillosis (IA) is a major threat in immunocompromised patients (1, 35, 36). The initial host defense against IA comes predominantly from the phagocytic cell population of the innate immune system, of which monocytes (MNC) are an important component (26). Phagocytes are activated by a set of pattern recognition receptors that recognize pathogen-associated molecular patterns of the fungus and trigger expression of cytokines, which modulate the host antifungal defense system (5, 24). More specifically, membrane-bound Toll-like receptors (TLRs) have been shown to recognize cell surface components of *Aspergillus fumigatus* and initiate a series of cellular events leading to NF-κB activation and nuclear translocation. This sets off the production of proinflammatory cytokines, chemokines, and immunoregulatory molecules for the recruitment of additional Th1 and Th2 cell populations (33). In particular, Toll-like receptor 2 (TLR2) and TLR4 have been shown to be variably involved in activation of the innate immune response after challenge of macrophages as well as neutrophils with *A. fumigatus* (2, 5, 20).

Voriconazole (VRC) is an antifungal triazole active against medically important fungi causing infections in immunocompromised patients (11). In particular, it has been found to be efficacious against IA, improving the overall survival of the patients (15). VRC either alone or combined with phagocytes has variable effects in inhibiting fungal growth (19, 31). We hypothesized that VRC also has direct immunomodulatory effects. We found that in combination with VRC, *A. fumigatus* hyphae induce a more pronounced profile of gene expression in the THP-1 human monocytic cell line than the hyphae alone, potentially leading to a more efficient host resistance to this fungus (30), which supports this hypothesis.

In order to understand the possible mechanisms underlying these immunomodulatory properties, we studied the interaction of VRC and MNC in response to *A. fumigatus* by evaluation of hyphal damage as well as mRNA and protein expression patterns of TLR2, TLR4, and tumor necrosis factor alpha (TNF-α) and nuclear translocation of NF-κB.

MATERIALS AND METHODS

Cell culture. The THP-1 monocytic cell line (ATCC TIB202; American Type Culture Collection [ATCC], Manassas, VA) was grown in a humidified CO_2 incubator at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (FCM) (Gibco BRL, Life Technologies Ltd., Paisley, Scotland). The THP-1 cell line has been previously used as a readily available source of physiologically robust monocytes/macrophages as evidenced by expression of cytokines, differentiation, and phagocytosis (3, 18). A 6-hour incubation was used for expression of cytokines as described previously (30). THP-1 cells were adjusted to a concentration of 1×10^6 cells/ml and placed into the wells of 12-well culture plates. The cells were incubated with 10 ng/ml phorbol myristate acetate at 37°C for 6 h. Cells were then washed once with Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (Gibco BRL) and incubated with FCM at 37°C for 22 h prior to incubation with hyphae at an effector cell/hyphae (E/T) ratio of 10:1 either alone or with 0.5 μg/ml VRC at 37°C with 5% CO_2 for 6 h.

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Preparation of human monocyte-enriched mononuclear leukocytes. MNC were obtained from blood samples from healthy adult volunteers and separated by centrifugation over Ficoll as previously described in detail (27). The cells were washed and resuspended in HBSS without Ca^{2+} and Mg^{2+}. MNC were adjusted to a concentration of 1×10^6 cells/ml with FCM. Monocyte-enriched cell populations were obtained from mononuclear leukocytes by adherence on plastic surfaces in the wells of 12-well plates during incubation in a humidified CO_2 incubator at 37°C for 2 h (27). After this incubation, the cells were washed with warm HBSS without Ca^{2+} and Mg^{2+} and incubated in fresh FCM at 37°C for 22 h prior to any treatment in order to avoid cell activation due to handling. Cell viability of MNC was checked by trypan blue staining. The viability of the cells was >95% at the end of each experiment.

Drug preparation. VRC (Pfizer Inc., Groton, Conn.), a lyophilized powder, was reconstituted in dimethyl sulfoxide, diluted with sterile water at a concentration of 1 mg/ml, and stored at −30°C. According to the manufacturer (Pfizer), the compound was endotoxin free. The working concentration of VRC tested was either 0.1 or 0.5 μg/ml. For the functional assays, we chose to work with 0.1 μg/ml VRC as a slightly subinhibitory concentration, since the MIC_{50} of VRC for various *A. fumigatus* clinical strains is 0.25 μg/ml (10). The higher concentration of VRC (0.5 μg/ml) is the MIC determined for the *A. fumigatus* strain AF4215, and thus, it was used in the gene expression and NF-κB nuclear detection experiments. Furthermore, both concentrations tested are easily achievable in the sera and tissues of most patients with IA receiving VRC (32).

Fungal growth conditions. A well-characterized *A. fumigatus* isolate (strain AF4215, deposited in the ATCC as MYA 1163) recovered from a cancer patient with invasive pulmonary aspergillosis was used in these studies. The isolate was preserved on potato dextrose agar (PDA) (Merck Darmstadt, Germany) slants frozen at −24°C. *A. fumigatus* conidia were cultured on PDA plates at 37°C for 2 days, harvested, and suspended in HBSS without Ca^{2+} and Mg^{2+} (Biochrom KG, Berlin, Germany) (27). For hyphal growth, 5×10^4 conidia per ml were suspended in yeast nitrogen base broth (YNB) (Scharlau Chemie S.A., Spain) supplemented with 2% glucose and incubated at 37°C for 12 h.

Hyphal damage assay. Hyphal damage induced by MNC and by THP-1 cells was assessed by a method modified from the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]2H-tetrazolium-5-carboxanilide (XTT) assay (23). *A. fumigatus* hyphae were generated as described above. Once the hyphal network was established, YNB was replaced by FCM. VRC at 0.1 or 0.5 μg/ml and MNC or THP-1 cells at an E/T ratio of 10:1 or the combination of VRC and effector cells were added at corresponding wells. After incubation at 37°C with 5% CO_2 for 6 h (THP-1) or 20 h (MNC), cells were lysed by two washes in 200 μl of H_2O. Then, 150 μl of phosphate-buffered saline solution was added containing 0.25 mg/ml XTT and 40 μg/ml coenzyme Q_0 (both from Sigma). After incubation at 37°C and 5% CO_2 for 1 h, 100 μl of XTT was transferred to a new plate, and the optical density (OD) was assessed spectrophotometrically (Anthos 2000, Austria) at a wavelength of 450 nm using a 690-nm reference filter. Antihyphal activity was calculated according to the formula: percent hyphal damage = $(1 - X/C)100$, where X is the OD of experimental wells and C is the OD of control wells with hyphae only.

RNA isolation and reverse transcriptase PCR analysis. After the incubation of THP-1 cells with VRC and/or *A. fumigatus* hyphae described above, total RNA was extracted from THP-1 cells of each experimental condition using the nucleospin total RNA isolation kit (Macherey-Nagel GmbH, Germany) according to the manufacturer's instructions. Total RNA (0.5 μg) was reverse transcribed by RT/Platinum *Taq* mix (Invitrogen) and then amplified by 30 cycles using TLR2 (5′-TCC TCC AAT CAG GCT TCT CTG TCT T-3′ and 5′-GGA ACT GCG AGA TAC TGA TT-3′, 637 bp), TLR4 (5′-TTT CCA GCA ACA AGA TTC AA-3′ and 5′-CAT CAT TGG TGT GTC GGT CCT-3′, 1,371 bp) and TNF-α (5′-CTT GTT CCT CAG CCT CTT CT-3′ and 5′-ACT CGG CAA AGT CGA GAT AG-3′, 578 bp) primers (TIB MOLBIOL, Dahlem, Germany). A negative control containing RNA instead of cDNA was included to rule out genomic contamination. All PCR products were separated on 1.5% agarose gels. The amounts of DNA on agarose gels were quantified using the UviDoc software program (Cambridge, United Kingdom). Three independent reverse transcriptase PCR experiments were performed in order to confirm the sensitivity of the method.

Western blot analysis. The cells (5×10^6 cells per experimental condition) were washed once with 0.5 ml of a buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, and 10 mM KCl. The cells were then lysed in the above buffer supplemented with 1% Nonidet P-40, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml pepstatin A, and 2 μg/ml leupeptin. The protein concentrations of lysates were determined by the Bradford protein assay (7). Twenty micrograms of total cytoplasmic protein from each condition was suspended in 2× Laemmli buffer under reducing conditions (0.125 M Tris [pH 6.8], 4% sodium dodecyl sulfate [SDS], 20% glycerol, 10% 2-mercaptoethanol, 2% bromophenol blue), boiled for 5 min, separated on 10% SDS-polyacrylamide gels using 0.025 M Tris (pH 8.3), 0.192 M glycine, and 0.1% SDS as the running buffer for 2 h at 60 mA, and transferred onto polyvinylidene difluoride membranes (Whatman Inc., United Kingdom). Membranes were blocked overnight at 4°C and incubated with the primary antibodies (mouse anti-human TLR2 and TLR4; AbD Serotec GmbH, Germany) for 2 h. Horseradish peroxidase (HRP)-conjugated horse anti-mouse immunoglobulin G (Cell Signaling Technology Inc., MA) was used as the secondary antibody at 1:2,000 dilution. After the membranes were washed, they were developed by using the chemiluminescent substrate LumiGLO (Cell Signaling Technology Inc.). Three independent experiments were performed.

Preparation of nuclear extracts and NF-κB p65 binding assay. After the incubation of THP-1 cells with VRC and/or *A. fumigatus* hyphae, nuclear extracts were prepared, and NF-κB p65 (RelA) activation was detected using the TransAm NF-κB kit (Active Motif, Belgium) according to the manufacturer's instructions. Briefly, 10^7 THP-1 cells per experimental condition were washed with ice cold phosphate-buffered saline containing phosphatase inhibitors (125 mM NaF, 250 mM β-glycerophosphate, 250 mM *para*-nitrophenyl phosphate, 25 mM $NaVO_3$). After centrifugation at 500 rpm and 4°C, the cell pellets were resuspended in 0.5 ml hypotonic solution containing 20 mM HEPES (pH 7.5), 5 mM NaF, 10 μM Na_2MoO_4, and 0.1 mM EDTA, incubated on ice for 15 min, and lysed by adding Nonidet P-40 to a final concentration of 0.5%. Nuclei were pelleted (14,000 × *g* for 30 seconds at 4°C) and resuspended in 50 μl lysis buffer containing 10 mM dithiothreitol and protease inhibitor cocktail (2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml pepstatin A, 2 μg/ml leupeptin).

The protein concentration was measured by the Bradford assay. Two micrograms of total nuclear protein per experimental condition was incubated at room temperature for 1 h with 30 μl of binding buffer, and the NF-κB consensus oligonucleotide (5′-GGGACTTTCC-3′) was immobilized at the bottom of a 96-well plate. After the wells were washed three times with this wash buffer, DNA-protein complex was bound to primary NF-κB antibody (1:1,000 dilution) and to a secondary HRP-conjugated antibody (1:1,000 dilution) for 1 h each at room temperature. The DNA-protein complex was detected colorimetrically by reading the absorbance of the blue color development on a spectrophotometer set at 450 nm with a reference wavelength of 620 nm. Jurkat nuclear extract (2.5 μg) was added to additional wells as a positive control for NF-κB activation. Twenty picomoles of competitor oligonucleotide was added to each well in another set of control wells in order to monitor the specificity of the assay. Three independent experiments were performed. Data are expressed in OD units.

Statistical analysis. Quadruplicate wells were used for each condition per experiment to assess the damage induced by MNC ($n = 5$) on *A. fumigatus* hyphae. The averages of the replicate wells of each experiment were then used in the data analysis to calculate the mean ± standard error (SE) for all the donors at the same conditions. The statistical program Instat (GraphPad, Inc., San Diego, CA) was used. Parametric analysis of variance (ANOVA) with Dunnett's test was used for statistical comparisons between treated and untreated cells in all the experiments including molecular studies. A P value of <0.05 was considered statistically significant.

RESULTS

Hyphal damage induced by VRC and MNC or by THP-1 cells. An additive effect on hyphal damage was observed when MNC and VRC were incubated together with *A. fumigatus* hyphae for 20 h compared to the effect on hyphal damage when *A. fumigatus* was incubated with MNC or VRC alone ($P < 0.01$; Fig. 1). After a shorter coincubation time of 6 h, VRC at 0.1 μg/ml did not show any combinational effect with MNC against *A. fumigatus* (data not shown).

Prior to performing a targeted analysis of TLR2 and TLR4 macrophage receptors in THP-1 cells, we studied the antifungal activity of these cells. We observed similar antifungal activities between MNC isolated from healthy donors and the THP-1 monocytic cell line, alone or when combined with VRC at 0.5 μg/ml following a 6-h challenge with hyphae (Fig. 2A and B).

TLR2 and TLR4 mRNA expression in THP-1 cells incubated with VRC and *A. fumigatus* hyphae. Intracellular extracts of THP-1 cells treated with VRC or hyphae contained in-

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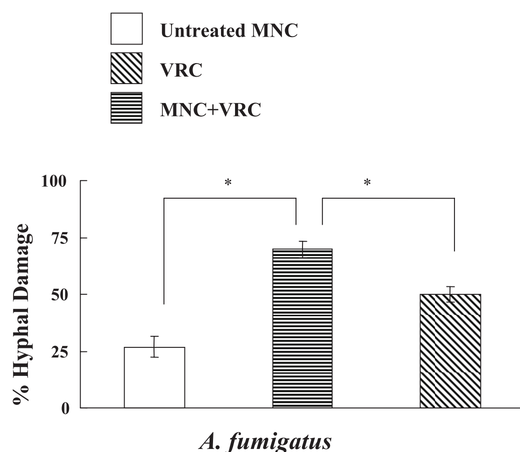

FIG. 1. Percentage of hyphal damage induced by MNC in the presence or absence of VRC. Hyphae (5×10^4/ml) were incubated at an E/T ratio of 10:1 with untreated MNC, VRC at 0.1 μg/ml, and MNC plus VRC for 20 h. The bars represent means ± SEs (error bars) for data derived from five experiments. Combinational activities of VRC with MNC were evaluated by repeated measures of ANOVA by Dunnett's posttest for multiple comparisons. Values that were significantly different from each other ($P < 0.01$) are indicated by the bars and asterisks above the bars in the bar graph.

creased amounts of TLR2 mRNA than untreated THP-1 cells did. The VRC-induced increase in TLR2 mRNA was greater than that induced by *Aspergillus* hyphae. The combination of VRC and hyphae induced the highest TLR2 mRNA (Fig. 3A). In contrast, the TLR4 mRNA levels of THP-1 cells exposed to VRC and/or hyphae did not differ significantly from those of untreated THP-1 cells (Fig. 3B). TNF-α exhibited a profile similar to that of TLR2 for VRC alone or for the combination ($P < 0.01$; Fig. 3C), but not for hyphae.

TLR2 and TLR4 protein expression in THP-1 cells incubated with VRC and *A. fumigatus* hyphae. In untreated THP-1 cells, the levels of expression of TLR2 and TLR4 proteins were almost below the detection limits of the assay. However, when THP-1 cells were incubated with hyphae, VRC, or both, TLR2 protein showed patterns of expression corresponding to the mRNA levels. Larger amounts of TLR2 were observed in THP-1 cells exposed to VRC than to hyphae, while the largest amounts were observed with the combination treatment (Fig. 4). TLR4 protein showed very weak expression with a similar expression profile under all experimental conditions (Fig. 4).

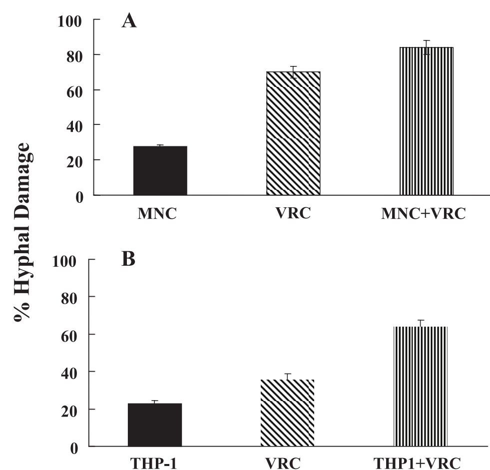

FIG. 2. Percent damage induced by freshly isolated human MNC and THP-1 cell line on *A. fumigatus* hyphae. Hyphae (5×10^4/ml) were incubated with differentiated MNC (A), THP-1 cells (B) at an E/T ratio of 10:1, VRC at 0.5 μg/ml (A and B), or MNC plus VRC or THP-1 plus VRC (A and B) for 6 h. The bars represent means ± SEs (error bars) derived from three independent experiments.

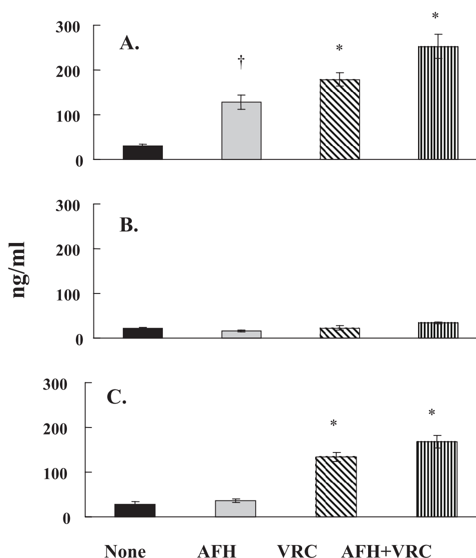

FIG. 3. TLR2 (A), TLR4 (B), and TNF-α (C) mRNA expression after induction of THP-1 cells with VRC or *A. fumigatus* hyphae (AFH). THP-1 cells (1×10^6/ml) were incubated with hyphae at an E/T ratio of 10:1 or with VRC at 0.5 μg/ml for 6 h. The production of TLR2, TLR4, and TNF-α mRNA was assessed in the intracellular extracts of unchallenged THP-1 cells (None) (black bars) and after incubation with AFH (gray bars), VRC (diagonal striped bars), and the combination of AFH plus VRC (vertical striped bars). Data represent means ± SEs (error bars) of three independent experiments. Differences in the values for treated MNC from those of untreated MNC were tested by ANOVA with Dunnett's posttest for multiple comparisons. Values that were significantly different from the values for untreated MNC are indicated as follows: *, $P < 0.01$; †, $P < 0.05$.

Induction of NF-κB expression in nuclear extracts of THP-1 cells in response to VRC and hyphae. More NF-κB p65 was present in nuclear extracts of THP-1 cells treated with VRC than with hyphae (Fig. 5). The combination of VRC and hyphae induced the highest level of NF-κB protein expression ($P < 0.01$).

DISCUSSION

The results of this study indicate the following. (i) VRC and MNC have an additive antifungal effect against *A. fumigatus* hyphae. (ii) VRC induces a more pronounced profile of TLR2 and TNF-α mRNA expression in THP-1 cells than hyphae do, while the combination of VRC and hyphae increases both mRNAs to even higher levels. (iii) TLR4 mRNA expression is low under all experimental conditions and similar to untreated cells. (iv) TLR2 and TLR4 protein levels correlate with the corresponding mRNA levels. (v) More NF-κB is present in nuclear extracts of THP-1 cells treated with VRC than with hyphae, while the combination of VRC and hyphae induces the

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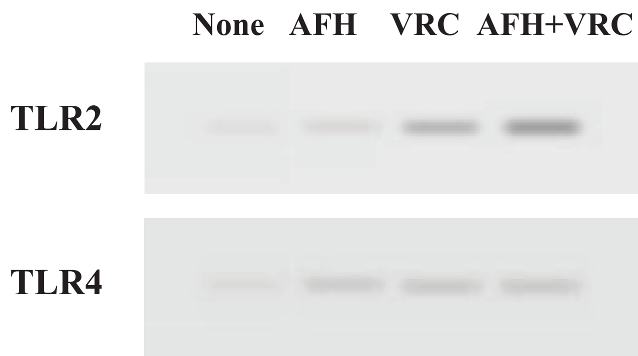

FIG. 4. TLR2 and TLR4 protein expression after induction of THP-1 cells with VRC or *A. fumigatus* hyphae (AFH). THP-1 cells (1×10^6/ml) were incubated with hyphae at an E/T ratio of 10:1 or with VRC at 0.5 μg/ml for 6 h. Equal amounts of protein samples were electrophoresed on a 10% SDS-polyacrylamide gel followed by blotting with mouse anti-human TLR2 and TLR4 monoclonal antibody. The signal was detected with HRP-conjugated horse anti-mouse immunoglobulin G antibody by standard techniques. Lanes: None, unchallenged THP-1 cells; AFH, incubation of THP-1 cells with hyphae; VRC, incubation of THP-1 cells with VRC; AFH+VRC, incubation of THP-1 cells with VRC and hyphae.

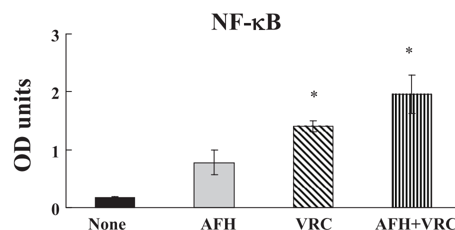

FIG. 5. NF-κB p65 gene expression in nuclear extracts of THP-1 cells is upregulated after induction of THP-1 cells with VRC or *A. fumigatus* hyphae (AFH). THP-1 cells (10^7/experimental condition) were incubated with hyphae at an E/T ratio of 10:1 or with VRC at 0.5 μg/ml for 6 h. Unchallenged THP-1 cells (None) (black bar) and THP-1 cells were incubated with AFH or VRC alone and the combination of AFH plus VRC are sown. Data expressed in OD units represent means ± SEs (error bars) from three independent experiments. Differences of treated MNC from untreated MNC were tested by ANOVA by Dunnett's posttest for multiple comparisons. Values that were significantly different ($P < 0.01$) from the value for untreated MNC are indicated by an asterisk.

highest NF-κB expression. These results provide evidence that, following challenge with hyphae, VRC enhances phagocyte proinflammatory programs by signaling the upregulation of TLR2, and this effect is mediated by NF-κB activation and nuclear translocation.

After the initial proinflammatory response to *A. fumigatus* associated with TLRs, phagocytes use both oxidative and nonoxidative mechanisms in concert to damage the hyphae of *A. fumigatus* (9, 29). While a number of cytokines upregulate these antifungal phagocytic activities (12, 14, 16), antifungal agents may assist in further damaging hyphae. Sau et al. found that amphotericin B induces signal transduction and inflammatory cytokine release from cells expressing TLR2, and the adapter protein MyD88 responds to amphotericin B with NF-κB-dependent reporter activity and cytokine release (28a). In addition, Bellocchio et al. found that neutrophil activation by *Aspergillus* occurs through TLR signaling pathways that affect the oxidative and nonoxidative killing mechanisms differently by diverting signaling from TLR-2 to TLR-4 (4).

In the present study, we have provided evidence for a differential regulation of TLR2 and TLR4 receptors in THP-1 cells in response to VRC or hyphae and have shown that compared to hyphae, VRC induces greater amounts of TNF-α expression, a proinflammatory cytokine associated with early host response to *A. fumigatus* (21, 28). We have further found that this event is associated with NF-κB activation.

It has previously been shown that when VRC enters human MNC, the intracellular phagocytic capacity of these cells against *Candida* spp. is enhanced (6). Our study extends the existing knowledge on the combinational antifungal activities of VRC with immune cells in response to *Aspergillus* and shows that, even at subinhibitory concentrations (0.1 μg/ml), VRC has an additive effect with MNC on damaging hyphae. This finding is in agreement with the report by Vora et al. where MNC and VRC at 0.5 μg/ml additively collaborated in inhibiting hyphal growth of *A. fumigatus* (31).

Pulmonary alveolar macrophages of TLR2 knockout mice are less responsive to *A. fumigatus* and cause reduced production of TNF-α during IA (2, 20). In comparison, Meier et al. (22) provide evidence that both TLR2 and TLR4 are required for an optimal response to *A. fumigatus* in vivo. Furthermore, Bellochio et al. (5) demonstrate that TLR2 signaling promotes antifungal activity of dexamethasone-treated polymorphonuclear leukocytes against hyphae through release of TNF-α, while TLR4 favors oxidative pathways with participation of interleukin 10 release. However, signaling of hyphae in MNC has been reported by Wang et al. (34) to occur in a TLR4-dependent manner.

Activation of most TLRs leads to the initiation of a signal transduction cascade with final activation and nuclear translocation of the transcription factor NF-κB, a key molecule for the inducible regulation of a number of genes, which modulate the innate immune response against invading microbes (24, 25). In this study, the subunit p65 (RelA) of the NF-κB family was detected in nuclear extracts of either untreated THP-1 cells or THP-1 cells incubated with hyphae or VRC alone or combined. Our results support the hypothesis that VRC increases the fungicidal activity of THP-1 cells challenged with hyphae, which is largely TLR2 dependent and is mediated by NF-κB activation with TNF-α, a cytokine known to be induced in macrophages after TLR2 production in experimental aspergillosis (2, 20, 22). Whether other azoles or other classes of antifungal agents have similar properties is presently unknown, but studies are under way or planned to delineate it.

In this study, the small amounts of TLR4 expression, both at the mRNA and protein levels, suggest that TLR4 may play a role in assisting fungal pattern recognition and ligand binding. The data provided by Wang et al. (34), while supporting TLR4 involvement in signaling by hyphae, do not exclude a role for TLR2 in hyphal signaling. These investigators used blocking monoclonal anti-TLR2 and anti-TLR4 antibodies to demonstrate partial inhibition of TNF-α released by MNC after challenge with hyphae. The weak inhibition of MNC activation observed with both monoclonal antibodies could not rule out a role for TLR2 in hyphal signaling or the possibility of cross-reactivity with other TLRs. Our data also cannot exclude the

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possible contribution of additional receptors. For example, the observations that dectin-1 receptor recognizes the hyphal form of *A. fumigatus* and is important for reactive oxygen species production (13) and that mannan-binding lectin receptor enhances uptake of *A. fumigatus* conidia by polymorphonuclear leukocytes (17) suggest that additional receptors participate in activating host defense against pathogens.

Accumulating evidence, based on in vitro and animal data, supports a role for several TLRs in sensing *A. fumigatus*. Clinical reports suggest that TLR gene polymorphisms are associated with invasive pulmonary aspergillosis but their involvement is affected by the immunological status of the patient. These studies support the argument that TLR2, acting as a heterodimer either with TLR1 or TLR6, is a major component of the host defense mechanisms against *Aspergillus* (8). A limitation of this study is that it evaluates cells from healthy donors, whereas aspergillosis occurs in immunocompromised patients and especially in profoundly neutropenic patients. However, the difficulty in obtaining large quantities of cells from such ill patients prohibits the performance of studies in these patients.

In conclusion, our data indicate that differential regulation of TLR2/TLR4 receptors mediated by activation of the NF-κB signal transduction pathway and facilitated by the presence of VRC leads to an augmented fungicidal activity of host cells upon challenge with hyphae. These results may lay the foundation for further studies aiming to improve management of invasive aspergillosis.

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