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Aspergillus fumigatus Induces Innate Immune Responses in Alveolar Macrophages through the MAPK Pathway Independently of TLR2 and TLR4

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**Aspergillus fumigatus** causes invasive aspergillosis in immunosuppressed patients. In the immunocompetent host, inhaled conidia are cleared by alveolar macrophages. The signaling pathways of the alveolar macrophage involved in the clearance of *A. fumigatus* are poorly understood. Therefore, we investigated the role of TLRs in the immune response against *A. fumigatus* and their contribution to the signaling events triggered in murine alveolar macrophages upon infection with *A. fumigatus* conidia. Specifically, we examined the MAPKs and NF-κB activation and cytokine signaling. Our investigations revealed that immunocompetent TLR2, TLR4, and MyD88 knockout mice were not more susceptible to invasive aspergillosis as compared with wild-type mice and that the in vitro phosphorylation of the MAPKs ERK and p38 was not affected in TLR2, TLR4, or MyD88 knockout mice following stimulation with conidia. In vivo experiments suggest that ERK was an essential MAPK in the defense against *A. fumigatus*, whereas the activation of NF-κB appeared to play only a secondary role. In conclusion, our findings demonstrate that TLR2/4 recognition and MyD88 signaling are dispensable for the clearance of *A. fumigatus* under immunocompetent situations. Furthermore, our data stress the important role of ERK activation in innate immunity to *A. fumigatus*. The Journal of Immunology, 2006, 177: 3994–4001.

Aspergillus fumigatus is an opportunistic fungal pathogen responsible for invasive aspergillosis, a severe, life-threatening infection. Invasive aspergillosis is well known to occur in various immunocompromised populations such as neutropenic patients with hematologic malignancies, children with chronic granulomatous diseases, and transplant recipients (1). Diagnosis is difficult, treatment is inefficient, and, as a consequence, mortality is high.

One of the most striking conclusions of a literature survey on *A. fumigatus* is how little is known about the pathobiological features of this organism (2). This is especially true for the early stages of disease development. Following inhalation of airborne *A. fumigatus* conidia, the immunocompetent host is protected by pulmonary innate immunity, which includes phagocytosis by alveolar macrophages (AMs).3 Macrophages are key defenders of the lung and play an essential role in mediating the inflammatory response.

Killing of conidia by the AMs begins 6 h after phagocytosis. Swelling of conidia inside the AM is a prerequisite for killing. The contribution of phagolysosomal acidification as well as NADPH oxidase to the conidical activity has been demonstrated. This activity is inhibited by corticosteroids via impairment of the production of reactive oxidant intermediates (3, 4). If end-killing mechanisms are documented, the signaling pathways controlling these mechanisms are not known.

Recognition of invading microorganisms by the innate immune system is a first and essential step in their successful elimination. Essential receptors for the recognition of invading microorganisms are the TLRs and members of the NOD (nucleotide binding oligomerization domain) family of proteins, including Nod1 and Nod2. These receptors trigger the initiation of inflammatory signals. Deficiencies of specific TLRs and various adaptor proteins of the TLR signaling transduction pathway, including MyD88, have been shown to result in reduced pyogenic bacteria and Mycoplasma species clearance (5, 6).

Following microbial recognition, the macrophage effector functions are known to be dependent in part on the phosphorylation and activation of MAPKs. The ERKs and p38 are activated in macrophages (7) and AMs by a variety of stimuli (8, 9). After phosphorylation, the activated MAPKs translocate to the nucleus where they phosphorylate several targets, including transcription factors.

The involvement of TLRs in the recognition of *A. fumigatus* by macrophages has been investigated by different groups. Data on this subject are in conflict, because evidence both for and against the importance of TLRs in host defense has been reported (10–14). Other investigations attempted to clarify the role of TLRs against *A. fumigatus* by using adaptor protein MyD88 knockout mice. These findings are not without controversy, because macrophages from MyD88 knockout mice exposed to *A. fumigatus* hyphae, but not conidia, still have inflammatory responses (15).

In this study we investigated the role of TLRs in *A. fumigatus* pathogenesis. We found that TLRs are dispensable for the survival
of mice during infection or for phagocytosis, killing efficiency, cytokine production, and the initial signaling events triggered upon infection with *A. fumigatus* conidia. Overall, our data suggest that, for the parameters that we examined, innate immune signaling by *A. fumigatus* is independent of TLR2 and TLR4 recognition and MyD88-dependent signaling.

**Materials and Methods**

**Reagents and antibodies**

All reagents were obtained from Sigma-Aldrich unless otherwise stated. Phospho-specific Abs to the MAPKs ERK1/2 and p38 and HRP-conjugated anti-mouse and anti-rabbit Ig were purchased from Cell Signaling Technology. The mouse mAb to MAPK phosphate (MKP) 2, specific for the MKP carboxyl-terminal catalytic domain, was obtained from BD Transduction Laboratories. Rabbit polyclonal NF-κB p65 and IκB-α Abs were obtained from Santa Cruz Biotechnology. The MEK inhibitors PD98059 and U0126 and the p38 inhibitor SB203580 were purchased from Cell Signaling Technology.

**Fungal strains**

The *A. fumigatus* CBS 144.89 strain was a clinical isolate. Resting conidia were harvested by washing a 7-day-old slant culture with PBS supplemented with 0.1% Tween 20 (PBST). The suspension was filtered through a 40-μm cell strainer (Falcon) to separate conidia from the contaminating mycelium and verified microscopically (100% resting conidia). In the killing experiments, the conidia were labeled with FITC as previously described. Briefly, freshly harvested conidia (2 × 10^7 per 10 ml of 0.05 M sodium carbonate buffer (pH 10.2)) were incubated with FITC at a final concentration of 0.1 mg/ml at 37°C for 1 h and washed by centrifugation three times in PBST (4).

**Mouse strains and infections**

Several wild-type and knockout mouse strains were used. TLR2, TLR4, and MyD88−/− mice were obtained from S. Akira (Osaka University, Osaka, Japan) and backcrossed eight times with C57/BL6 to ensure similar genetic backgrounds. C57/BL6 mice were used as a control. These mice, as well as 20- to 24-g, 6- to 8-week-old male out-bred Swiss OF1 mice were supplied by the Centre d’Elevage R. Janvier and used at ~8 wk of age. Mice were fed normal mouse chow and water ad libitum and were reared for in accordance with Pasteur Institute guidelines in compliance with European Union animal welfare regulations.

Mice were expressing with 25 μg of cortisone acetate (Sigma-Aldrich) injected i.p. at day 3 and immediately before intranasal inoculation of conidia (day 0) for infection experiments. Before infection, mice were anesthetized with an intracranial injection of 0.1 ml of a solution containing 10 μg/ml ketamine (Merial) and 2 μg/ml xylazine (Bayer) per mouse. Conidia in 25 μl of PBST were inoculated intranasally using an automatic pipetting device. Control nonimmunosuppressed mice received the same conidial suspension. Survival of mice was followed daily over a period of 17 days.

**Histology**

Lungs were collected at day 4 after intranasal infection with 1 × 10^7 conidia. Organs were fixed in 3.7% neutral-buffered formaldehyde, embedded in paraffin, and cut into 5-mm-thick sections. Sections were stained with methylene blue for fungal detection and examined microscopically (16).

**Cells**

MH-S murine AMs (MAMs) (17), derived by SV40 transformation of MAMs, were cultured in RPMI 1640 complete medium (supplemented with HEPEs and sodium pyruvate) containing 10% FCS and maintained in an incubator at 37°C under 5% CO₂. MH-S cells were plated 24 h before experimentation and starved for at least 5 h before stimulation.

Primary MAMs were harvested from mouse lungs with 15 ml of ice-cold Ca²⁺− and Mg²⁺−free PBS through a 18-gauge plastic catheter inserted into the trachea after cervical dissection as described previously (3). Primary MAMs were separated from the lavage fluid by centrifugation (400 X g for 8 min at 4°C), and the cell pellet was suspended in RPMI 1640 complete medium containing 10% FCS and plated on 12-mm-diameter glass coverslips at a density of 5 × 10^7 or 1 × 10^8 cells per coverslip in 96-well ELISA plates. Nonadherent cells were removed by washing after a 2-h incubation at 37°C in an atmosphere of 5% CO₂. The viability of the AM preparations was >99% as judged by trypan blue dye exclusion.

**Coculture of conidia with AMs**

For MAPK phosphorylation experiments, AMs were starved in RPMI 1640 medium without FCS for 5–16 h, challenged with 10 conidia per macrophage, and subsequently incubated at 4°C for 30 min. Unbound conidia were removed by washing with complete cold RPMI 1640 medium, and the cells were shifted to 37°C in an atmosphere of 5% CO₂.

In some experiments, the MEK inhibitors PD98059 and U0126 and the p38 inhibitor SB203580 were added 30 or 60 min before infection at appropriate concentrations as indicated in Fig. 4.

**Phagocytosis assay**

Phagocytosis assays were performed as previously described (4). AMs were challenged with two conidia per macrophage and were subsequently incubated at 4°C for 30 min. Unbound conidia were removed by washing with cold RPMI complete medium, and phagocytosis was initiated at 37°C in an atmosphere of 5% CO₂. Phagocytosis was stopped after 2 h by washing the AMs with ice-cold PBS and subjecting them to fixation with 3% formaldehyde in PBS (10 min at room temperature) followed by three washes with 50 mM NH₄Cl in PBS. The number of *A. fumigatus* conidia engulfed by macrophages was determined using a polyclonal anti-conidia Ab and a secondary Ab conjugated to Texas Red. This procedure labels only uningested conidia. The percentage of ingestion was estimated as the ratio of the number of ingested conidia to the total number of conidia bound to 100 macrophages.

**Killing experiments**

MAMs or MH-S cells were stimulated for 16 h with FITC-labeled conidia at a ratio of 1:10 and lysed with 0.2 ml of water. The lysate was suspended by pipetting, incubated overnight at 4°C, supplemented with 0.2 ml of a medium containing 4% glucose and 2% mycoplante, and incubated for 6–8 h at 37°C. The killing percentage (number of nongerminated conidia per 100 FITC-labeled-conidia) was assessed by fluorescent microscopy.

**Preparation of protein extracts and immunoblot analysis**

After incubation with conidia for various times, cells were washed with ice-cold PBS and lysed with ice-cold lysis buffer (150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1% Nonidet P-40, 0.1% SDS, and 0.5% sodium deoxycholate) supplemented with 1 mM PMSF, 1 mM NaVO₄, and 50 mM NaF. Lysates were cleared by centrifugation at 13,000 rpm for 15 min at 4°C. Equal amounts of total protein extracts were suspended in 2× Laemmli buffer under reducing conditions, boiled for 5 min, and separated by SDS-PAGE. The MAPKs ERK, p38, and MKP-2 were detected by immuno blotting according to standard procedures.

Blots were blocked for 1 h with 5% low-fat lyophilized milk in TBS, 50 mM Tris-HCl (pH 7.5), and 0.15 M NaCl and supplemented with 0.1% Tween 20 (TBST).

Membranes were washed three times with TBST and probed overnight with specific anti-phospho-ERK1/2 rabbit Ab (1/1000), anti-phospho p38 mAb (1/500), and anti-MKP-2 polyclonal Ab or with anti-FRB-α or anti-p38 rabbit Ab (1/1000) for total protein. Membranes were subsequently treated with HRP-conjugated Abs (1/1000). The blots were developed with an ECL kit. Levels of phosphorylation were quantified after normalization to the level of total ERK and p38 using densitometry scanning and the ImageQuant program.

**Cytokine expression**

For in vitro cytokine expression, MAMs or MH-S cells were added to 96-well plates at a density of 1 × 10⁶ cells and then stimulated with conidia at a conidium-to-macrophase ratio of 10:1 for 6 h. Supernatants were recovered and frozen at −70°C before analysis. For other experiments, MAPK inhibitors were added before stimulation as indicated earlier. As a positive control, cells were treated with LPS at a concentration of 5 μg/ml.

The expression of TNF-α in the cell supernatants was analyzed by ELISA using the mouse TNF-α detection kit (BD Biosciences).

For in vivo experiments, cytokine expression was measured in bronchoalveolar lavage (BAL) fluids of mice infected for 24 h with 1 × 10⁷ conidia.

IL-6, MCP-1, and TNF-α were analyzed by cytometry using the In vitro Nitrogen Life Technologies mouse inflammation cytometric bead array six bead kit according to manufacturer’s instructions. RANTES, KC, and G-CSF were measured by ELISA.
NF-κB activation

NF-κB nuclear translocation. MH-S cells (5 × 10^5) were attached to glass coverslips (25-mm diameter) and starved overnight before stimulation with A. fumigatus conidia. Resting conidia were incubated with MH-S cells at a conidium-to-macrophage ratio of 10:1. Phagocytosis was synchronized by a 30-min incubation of cells on ice. Cells were rinsed and incubated with serum-free medium at 37°C. Stimulation of MH-S cells was stopped at 1, 4, and 6 h.

As a positive control for NF-κB translocation, cells were stimulated with TNF-α for 1 h. After different times of stimulation, cells were fixed with 3% formaldehyde (paraformaldehyde), permeabilized with 0.2% Triton X-100, and incubated with specific Abs. NF-κB was stained with a mouse mAb directed against the Rel-A p65 subunit, (1/200; Santa Cruz Biotechnology). Cells were then incubated with FITC-labeled mouse secondary Ab. Nuclei were visualized using 4',6-diamidino-3-phenylindole (10 μg/ml). Cells were mounted with PBS/glycerol (50%) and viewed using fluorescence microscopy (Leica). Images acquired at the same exposure time were processed by Photoshop software (Adobe).

1kB-α degradation. Cells were incubated with resting conidia for 4 and 6 h, rinsed with PBS, and collected by removing cells with Laemmli buffer (1×). Nonstimulated cells were used as controls. 1kB-α was detected by Western blotting. Proteins were separated on a 13% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (0.2 μm; Schleicher and Schuell). After incubation with the anti-1kB-α rabbit polyclonal Ab (1/1000), the membrane was washed in 0.5% PBS-Tween 20 and probed with a HRP secondary Ab (1/2000). The blots were developed with an ECL kit.

ELISA based NF-κB activation assay. To evaluate NF-κB activation, nuclear extracts were prepared using the nuclear extract preparation kit (TransAM) 4 and 6 h after stimulation with resting conidia. Nonstimulated cells and TNF-α–treated cells were used as negative and positive controls, respectively. Whole nuclear extracts were incubated with a double-stranded oligonucleotide containing the NF-κB consensus site (5'-GG GACCTTTCC-3') previously immobilized in the wells of a 96-well plate. After washing, bound NF-κB was detected with a p65 Rel-A-specific Ab. The reaction was developed colorimetrically with a HRP-conjugated secondary Ab and quantified spectrophotometrically at 450 nm.

Statistical analysis

Survival data were analyzed by means of log-rank comparisons of Kaplan-Meier survival curves followed by the Wilcoxon test using JMP 5.0 software (SAS). The Fisher test was used to determine the statistical significance of differences in phosphorylation of MAPK, cytokine expression, and inhibition of killing. As indicated in the legends to Figs. 1 and 3–5, significance was defined as p < 0.05. In vivo groups consisted of three animals. The data reported were mean values ± SEM of triplicates from a representative experiment.

Results

Role of TLR2, TLR4, and MyD88 in the innate immune response against A. fumigatus

Mice survival. Immunocompetent TLR2, TLR4, and MyD88 knockout mice as well as the wild-type C57BL/6 mice were resistant to infection with 1 × 10^7 conidia of A. fumigatus, even though this inoculum was able to kill C57BL/6 mice within five days after cortisone acetate immunosuppression. A Wilcoxon test on Kaplan-Meier revealed that the survival rate of MyD88 knockout mice was not statistically different between knockout and parental mice (p > χ^2 = 0.29) (Fig. 1A). Thin sections of lungs from

![Figure 1](http://www.jimmunol.org/)
MyD88 knockout infected mice (Fig. 1Ba) as well as lungs from wild-type infected mice (Fig. 1Bb) displayed normal lung histology in comparison to lung sections from immunosuppressed infected mice. Analysis of the lungs from later mice showed a typical invasive aspergillosis pattern with a massive fungal growth obstructing the bronchioli (Fig. 1Bc).

In vivo inflammatory response to *A. fumigatus*. The production of TNF-α and IL-6 was assessed in the BAL fluids of mice challenged with conidia of *A. fumigatus* for 24 h. These BAL fluids contained substantially more TNF-α (925 ± 171 vs 0 ± 0 pg/ml) and IL-6 (64 ± 18 vs 0 ± 0 pg/ml) than samples from noninfected control mice (p < 0.05) (Fig. 1C).

A similar pattern of expression of these two cytokines as well as MCP-1, RANTES, KC, and G-CSF was seen in BAL fluids of TLR2 knockout, TLR4 knockout, and wild-type mice after 24 h of infection. These cytokines did not show any significant difference between TLR-deficient and wild-type mice (data not shown).

In contrast, comparison of TNF-α and IL-6 recovered from BAL fluids of immunocompetent and immunosuppressed mice that were obtained after stimulation with *A. fumigatus* revealed that the concentration of these two cytokines was substantially lower in the immunosuppressed mice (Fig. 1C).

**Involvement of MyD88 in phagocytosis and killing of *A. fumigatus conidia***. We used immunofluorescence microscopy to measure the capacity of AMs isolated from wild-type C57BL/6 and MyD88−/− mice to ingest and kill the conidia of *A. fumigatus*. After 2 h of phagocytosis, conidia were efficiently internalized by C57BL/6 and MyD88−/− AMs with an ingestion capacity of 81 and 88%, respectively (see Fig. 2A as an example).

**Impact of TLR2, TLR4, and MyD88.** To examine the involvement of the TLR family as well as that of the adaptor protein MyD88 in the phosphorylation of the MAPKs of AMs in response to *A. fumigatus*, murine primary AMs from TLR2, TLR4, and MyD88 knockout mice and their parental strains were stimulated ex vivo with conidia for different periods of time. The activation kinetic of ERK and p38 MAPKs in the MAMs was assessed by Western blot analysis using phospho-specific Abs.

Phosphorylation of macrophage MAPKs derived from the knockout mice strains were compared with those of murine primary AMs from control C57BL/6 wild-type mice after stimulation with conidia. In Fig. 3A, we show that the activities of both ERK and p38 were very low in uninfected macrophages. Upon stimulation with conidia, either in wild-type AMs or AMs from TLR2, TLR4, and MyD88 knockout mice, ERK and p38 were strongly activated in response to the conidial stimulation with a significant mean fold of phosphorylation of 7 ± 1.5 and 3 ± 0.25 (p < 0.05) for the ERK p44 and p42 subunits, respectively, and for p38 it was 7 ± 3 (p < 0.01) after 1 h as compared with noninfected cells. These results strongly suggest that these receptors as well as the adaptor protein MyD88 are not required for the phosphorylation of MAPKs in response to *A. fumigatus*.

**Phosphorylation of MAPKs in response to *A. fumigatus***

**Kinetics of ERK and p38 phosphorylation.** Because the MAPKs were phosphorylated similarly in primary AMs and the cell line, the follow-up of the activation of MAPKs in AMs in response to *A. fumigatus* at later time points postinfection was done using MH-S cells, because such an assay required a high number of cells (Fig. 3B). Incubation for up to 8 h increased the levels of phosphorylation even further with a significant fold of phosphorylation of 8 ± 1.5 and 6.5 ± 0.25 for the ERK p44 and p42 subunits, respectively, and for p38 it was 14 ± 3 (p < 0.005) after 8 h as compared with noninfected cells. An incubation time longer than 8 h could not be used because the high ratio (10 conidia per macrophage) resulted in overgrowth of nonphagocytosed conidia.

In mammalian cells, inactivation of MAPKs is primarily conducted by a group of dual-specificity MKPs (18). To examine whether MKP-2 plays a role in the regulation of MAPKs in AMs, we investigated the activation of MKP-2 after stimulation of MH-S macrophages with the conidia of *A. fumigatus* for different times. As shown in Fig. 3C, MKP-2 was not activated even after the stimulation of cells with conidia for 8 h. This result is in accordance with the long-term phosphorylation of MAPKs after stimulation with conidia.

**Activation of ERK and p38 in vivo; impact of immunosuppression.** To investigate whether MAPKs play a role in the responses of immunocompetent and immunosuppressed mice infected with conidia of *A. fumigatus*, we studied the activation of ERK and p38 in AMs isolated after intranasal infection with $1 \times 10^7$ conidia. MAMs were recovered as indicated in Materials and Methods, and the phosphorylation of MAPKs was investigated in the cell lysates by Western blotting. The level of phosphorylation was compared separately between immunocompetent noninfected and infected mice as well as between immunosuppressed noninfected and infected mice. Similar to what was observed ex vivo, after 4 h of
when 1 translation of ERK in vivo, the activation of p38 could not be observed low after infection with conidia (Fig. 3). In addition, the phosphorylation level of ERK remained extremely low after infection with conidia of A. fumigatus in immunocompetent and immunosuppressed mice (Fig. 3A, B). The data shown are representative of three independent experiments. NI is the noninfected control.

The phosphorylation of ERK was lower than that in immunocompetent mice (Fig. 3C). In contrast, in immunosuppressed mice the level of phosphorylated ERK was also investigated. Because MH-S cells behave like murine primary cells in terms of cytokine production as well as infection with conidia in immunocompetent mice the infection resulted in a major activation of ERK with a phosphorylation fold of 2.4 and 1.9 for the p44 and p42 subunits, respectively (p < 0.05). In contrast, in immunosuppressed mice the level of phosphorylated ERK was lower than that in immunocompetent mice (p < 0.05). In addition, the phosphorylation level of ERK remained extremely low after infection with conidia (Fig. 3D).

Although an infection for 4 h showed a significant phosphorylation of ERK in vivo, the activation of p38 could not be observed when 1 × 10^7 conidia were used. As shown in Fig. 3E, a similar level of phosphorylation of p38 was observed in both noninfected and infected immunocompetent and immunosuppressed mice.

Study of the inhibition of MAPKs; effect on cytokine release and killing of A. fumigatus. Primary MAMs were stimulated ex vivo with conidia for 6 h in the presence or absence of inhibitors of MAPKs. Supernatants were removed and used for determination of the TNF-α concentrations.

As shown in Fig. 4A, conidia induced a secretion of 137 ± 26 pg/ml TNF-α after 6 h of stimulation, which corresponds to an 8.6-fold increase in comparison with the basal level of secretion of this cytokine in unstimulated cells (16 ± 2 pg/ml). The two inhibitors of MEK1/2, PD 98059 and UO126, caused a significant decrease of TNF-α secretion between 46 and 86%, depending on the inhibitor and the dose used (p < 0.05). A minor but significant inhibition (p < 0.05) was obtained with SB203580, which is an inhibitor of p38, at all concentrations tested. However, in contrast to the inhibitors of ERK, SB203580 did not induce any dose-dependent inhibition of TNF-α synthesis. Similar results on cytokine production and inhibition have been obtained using the MH-S cells.

The effect of PD98059 and SB203580 in the capacity of killing conidia was also investigated. Because MH-S cells behave like murine primary cells in terms of cytokine production as well as secretion, we were interested in comparing the effect of the inhibitors of ERK and p38 on the ability to kill conidia.

As shown in Fig. 4B, conidia induced a secretion of 137 ± 26 pg/ml TNF-α after 6 h of stimulation, which corresponds to an 8.6-fold increase in comparison with the basal level of secretion of this cytokine in unstimulated cells (16 ± 2 pg/ml). The two inhibitors of MEK1/2, PD 98059 and UO126, caused a significant decrease of TNF-α secretion between 46 and 86%, depending on the inhibitor and the dose used (p < 0.05). A minor but significant inhibition (p < 0.05) was obtained with SB203580, which is an inhibitor of p38, at all concentrations tested. However, in contrast to the inhibitors of ERK, SB203580 did not induce any dose-dependent inhibition of TNF-α synthesis. Similar results on cytokine production and inhibition have been obtained using the MH-S cells.

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MAPK phosphorylation in response to the stimulation with *A. fumigatus*, the effect of MAPK inhibitors on the killing of conidia was studied using the MH-S cells. Fig. 4B shows that PD98059 resulted in a percentage of killing of 53 ± 4% compared with 83 ± 8% for the control cells without inhibitors (*p < 0.05*). SB203580 caused a slight, nonsignificant reduction in the percentage of killing.

Translocation of NF-κB by *A. fumigatus* in macrophages

Because NF-κB is associated with the regulation of immune responses in a variety of infection models, we analyzed the activation of this transcription factor following stimulation of macrophages with *A. fumigatus*. Translocation of NF-κB was analyzed by immunofluorescence using a Rel-A specific Ab. MH-S cells were stimulated with conidia for 1, 4, and 6 h. As a positive control of NF-κB translocation, cells were treated with TNF-α for 1 h. As shown in Fig. 5A, TNF-α treatment resulted in complete nuclear translocation of NF-κB after 1 h of incubation. Nonstimulated control cells as well as cells stimulated with conidia for 1 h showed no Rel-A translocation but showed ~25% translocation after 4 h of stimulation with conidia and reached 100% after 6 h. This result implied that efficient Rel-A translocation was triggered only after intracellular swelling of conidia or, indeed, by paracrine effects of factors released following infection. To go further, we tested the ability of the translocated Rel-A subunit to bind to its target DNA sequence by use of an ELISA-based assay (TransAM). Nuclear extracts were prepared from cells stimulated with conidia for 4 and 6 h. As shown in Fig. 5B, resting conidia induce an activation fold of 1.6 after 6 h of stimulation compared with nonstimulated cells. This fold activation is statistically identical with that after stimulation with TNF-α.

Another way to evaluate NF-κB activation is to examine IκB-α degradation. MH-S macrophages were stimulated either with TNF-α as a control or with resting conidia for 4 and 6 h. As shown in Fig. 5C, the addition of conidia resulted in an efficient degradation of IκB-α only after 4 and 6 h of stimulation, similarly as the TNF-α stimulation control.

Discussion

A. *fumigatus* is a human pathogen that is able to cause invasive aspergillosis in immunosuppressed patients. In the immunocompetent patient, inhaled conidia are easily cleared by the immune system. Macrophages play an essential role in this process. Their activation results in the acquisition of new molecular and cellular capacities that are determined largely by new gene expression. The up-regulation of specific genes depends mainly on the mode of activation and frequently results in production of the proinflammatory cytokines including TNF-α, IL1-β, and IL-6. Indeed, one of the major cytokines involved in immune defense against *A. fumigatus* is TNF-α. A high level of TNF-α synthesis is directly accompanied by an increased resistance against *A. fumigatus* infection (19).

Secretion of cytokines for immune defense often results from the stimulation of TLRs. Regarding *A. fumigatus*, the current literature on this topic is somewhat puzzling, because although it is extensively studied, conflicting results are presented (20). In mice, both conidia and hyphae stimulate cytokine production via TLR2 (11), whereas conidia signal solely through TLR4 to stimulate murine macrophages (12). In contrast to those findings, the results of Wang et al. (21) implicate TLR4 but not TLR2 for the stimulation of human monocytes by *A. fumigatus* hyphae. Furthermore, Mamula et al. (11) reported that TNF-α production in response to *A. fumigatus* is MyD88 dependent. Contrary to that finding, responses to *A. fumigatus* have been reported by Marr et al. (15) to occur in a MyD88-independent manner.

Our results support the idea that innate immune signaling by *A. fumigatus* in immunocompetent MAMs is largely TLR2 and TLR4 independent. First, we observed a late translocation of NF-κB in response to the conidia, suggesting that direct stimulation of the TLR- NF-κB axis does not occur following *A. fumigatus* stimulation of AMs. Indeed, stimulation of TLR by direct and specific binding of conidia would likely result in rapid translocation of NF-κB, which was not the case in our studies. Second, we analyzed phosphorylation of ERK and p38 after stimulation with conidia from *A. fumigatus in TLR2-, TLR4-, and MyD88-deficient AMs. Neither these receptors nor the adaptor protein MyD88 appeared to play a role in the phosphorylation of ERK and p38 in vitro. Third, analysis of the cytokine and chemokine profiles 24 h after infection of the TLR-deficient mice compared with wild-type mice gave the indication that these receptors are not involved in the innate immune response against *A. fumigatus*, because no significant differences were seen between the two groups. Furthermore, TLR2, TLR4, and MyD88 knockout immunocompetent

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**FIGURE 5.** Translocation and activation of NF-κB in MH-S cells after stimulation with the conidia of *A. fumigatus*. A, MH-S cells were challenged with the conidia at the ratio of 10 conidia per macrophage for different times or with TNF-α for 1 h as a positive control. NF-κB translocation was visualized using a Rel-A-specific polyclonal Ab (upper green panel). 4',6-Diamidino-3-phenylindole nuclear staining is shown in the lower panel. Stained cells were observed using conventional fluorescence microscopy. Arrows indicate nuclei with prominent concentrations of translocated Rel-A. NI is the noninfected control. B, Time course of NF-κB activation and IκB-α degradation in MH-S cells challenged with the conidia of *A. fumigatus*. MH-S cells were challenged as indicated in A. Nuclear extracts were assayed for NF-κB DNA binding activity by the TransAM method; *p < 0.05*. C, Western blot analysis of cytoplasmic levels of IκB-α in cells infected with *A. fumigatus conidia (A.f)* or treated with TNF-α.
mice challenged with $1 \times 10^7$ conidia did not develop aspergillosis similar to that seen in wild-type strain, which is in agreement with others stating that TLR2 $^{-/-}$ and TLR4 $^{-/-}$ immunocompetent mice do not display an increased susceptibility to aspergillosis (22). As well, the phagocytosis and intracellular killing of the conidia in MyD88 $^{-/-}$ mice was similar to that of wild-type mice (15).

We also investigated the involvement of different MAPKs in the response toward A. fumigatus conidia, because these kinases are important also for cytokine production (23, 24). However, their exact functions in the response to fungal infection are poorly studied (25). Even less is known about the involvement of MAPKs in signaling and the killing of conidia of A. fumigatus by AMs.

Through the use of phospho-specific Abs, we found an activation of ERK and p38 after in vitro stimulation with conidia. The phosphorylation occurred at early time points postinfection and was sustained for at least 8 h, which correlated with the absence of MKP-2 induction. The sustained late activation of ERK and p38 may suggest the involvement of the swelling state of conidia in the innate response against A. fumigatus. We were the first to show that 6 h after phagocytosis, conidia significantly increase their size but are inhibited in germinating within the phagolysosome. We demonstrated that this swelling state is an absolute requirement for production of the reactive oxygen intermediates that are responsible for conidial killing (3). Follow-up studies have shown that conidial swelling is sufficient to induce inflammatory cytokine/chemokine production by dendritic cells, AMs, and human CD14$^+$ macrophages after stimulation either with conidia for 18 h or with swollen conidia (26, 27). Although ERK and p38 were activated after stimulation of conidia in vitro, activation of p38 in vivo was only observed when $2 \times 10^6$ conidia were applied, whereas a strong response of ERK was observed by the use of at least 10 times fewer conidia. This finding suggested that p38 might only be of minor importance in recognition and defense against A. fumigatus.

MAPKs and NF-κB are known to be involved in the control of cytokine gene expression, including that of TNF-α, IL-1β, and IL-6. In the immune response toward A. fumigatus, TNF-α has been shown to play a critical role in innate immunity in both immunocompromised and immunocompetent hosts. Pretreatment with TNF-α agonist peptides significantly enhance resistance to A. fumigatus in neutrophilic animals (19). Moreover, the reduction in the level of cytokines released by macrophages after treatment with corticosteroids leads to insufficient clearance of conidia (28). In our study, conidia induced a significant increase in TNF-α production by AMs after 6 h of stimulation in vitro. When mice were challenged in vivo for 24 h, TNF-α production and IL-6 increased substantially. Our results and those of others (26, 27, 29) on the modulation of the expression of TNF-α in macrophages after exposure to live but not heat-killed conidia for 6 h as well as to swollen conidia suggest that TNF-α and IL-6 are essential cytokines in the host defense against exposure to A. fumigatus conidia.

Investigations with MAPK inhibitors were performed to determine the relationship between the production of TNF-α and the phosphorylation of ERK and p38. The use of SB203580, the classically known inhibitor of p38, had a minor effect on TNF-α production independently of the dose used. Additionally, we were not able to observe any significant variation in the killing of conidia under in vitro conditions using SB203580, which underlines the minor role of this MAPK. In contrast to p38, we revealed that, after the application of conidia, blocking of the ERK pathway by the use of UO126 and PD98059 inhibited TNF-α synthesis in a dose-dependent manner. In addition, treatment with PD98059 resulted in a reduction of 30% in the killing of conidia as compared with untreated control cells. This demonstrates an important role of the ERK pathway in the defense against conidia.

We were further interested in the role of corticosteroids in the inflammatory response against A. fumigatus. Corticosteroids are potent anti-inflammatory drugs. They diffuse into target cells where they bind to their cytoplasmic receptor, which, in turn, translocates to the nucleus and blocks transcription of several cytokine genes (30). Glucocorticoids inhibit members of the MAPK family and p38 by sustaining the expression of MAPK phosphatase (31). When bronchoalveolar macrophages are treated with dexamethasone and stimulated with living conidia of A. fumigatus, the production of IL-1α and TNF-α is much less pronounced (32). In agreement with these data, we found that immunosuppression in vivo resulted in a significant decrease in the phosphorylation of ERK and, as a consequence, a strong decrease in TNF-α and IL-6 production.

In addition to the role of MAPKs in the production of cytokines, the activation of NF-κB was studied because this transcription factor controls the expression of many genes involved in the inflammatory response (24). As discussed earlier, we have shown that resting conidia do not induce the translocation of NF-κB at early time points. However, we observed a translocation of NF-κB after a 6-h incubation of conidia with macrophages. Our data are in agreement with results published during the review of our manuscript of showing that swollen conidia stimulated NF-κB to a greater degree than resting conidia (26).

In summary, we have demonstrated that the MAPK ERK plays an important role in the inflammatory host response against A. fumigatus. Although our data strongly suggest that TLR2 and TLR4 are not involved in this response, we cannot exclude the possibility that other TLRs and/or MyD88 may be of importance during different stages of infection. In the situation of neutrophil depletion, the lack of TLR2 apparently renders macrophages less responsive to A. fumigatus with a reduced level of TNF-α (14). The residual level of TNF-α that was observed in our system may be derived from an activation of these receptors and may be of importance under these conditions. Nevertheless, in the immunocompetent situation the contribution of TLRs appears to be less relevant. Further studies will be required to elucidate the receptor(s) that specifically senses A. fumigatus conidia. It is likely that multiple receptors are involved that recognize different components of the conidial cell wall to drive the innate immune response against A. fumigatus. In this regard, the combined contributions of TLRs as well as those of the scavenger family of receptors must be examined.

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References


