Aspergillus fumigatus-induced IL-8 synthesis by respiratory epithelial cells is controlled by the PI3 kinase, p38 MAPK and ERK1/2 pathways and not by the TLR-MYD88 pathway.

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Previous studies have established that phagocytes are key cells of the pulmonary innate immune defense against A. fumigatus, an opportunistic fungus responsible of invasive pulmonary aspergillosis. Macrophages detect A. fumigatus via Toll-like receptors (TLR) 2 and 4, and respond by the MyD88-NF-κB-dependent synthesis of inflammatory mediators. In the present study, we demonstrate that respiratory epithelial cells also sense A. fumigatus and participate to the host defense. Thus, the interaction of respiratory epithelial cells with germinating but not resting conidia of A. fumigatus results in interleukin (IL)-8 synthesis that is controlled by PI3 kinase, p38 MAPK and ERK1/2. Using MyD88-dominant negative transfected cells, we also show that IL-8 production is not dependent on the TLR-MyD88 pathway, although the MyD88 pathway is activated by A. fumigatus and leads to NF-κB activation. Thus, our results provide evidence for the existence of two independent signalling pathways activated in respiratory epithelial cells by A. fumigatus, one which is MyD88 dependent and another that is My88 independent and involved in IL-8 synthesis.

INTRODUCTION

Aspergillus fumigatus is an opportunistic fungus responsible for invasive pulmonary aspergillosis (IPA), a life-threatening disease that usually only occurs in immunocompromised patients as inhalation of spores by those hosts results in fungal growth inside the lung and hematogenous dissemination (1,2).

The mechanisms of host resistance to IPA are not completely understood. It is generally accepted that macrophages and neutrophils represent the first two lines of innate host defense against *A. fumigatus* (3,4). Specifically, alveolar macrophages phagocyte and kill conidia while neutrophils lyse hyphae of germinated spores (5). However, it is now recognized that epithelial cells play also an important role in the innate defense (6,7,8). Rather than just representing a physical barrier to prevent pathogen gaining unwanted access to essential organs, the epithelial layer provides a surface where the host can interact with pathogens. The airway epithelium senses micro-organisms and responds accordingly by increasing its defence. This response consists of the synthesis and release of antimicrobial peptides, cytokines and chemokines. As do macrophages and neutrophils, epithelial cells can also sense microbial products through pattern recognition receptors that bind conserved molecular patterns expressed by micro-organisms.

Toll-like receptors (TLRs) have been identified as a major class of pattern recognition receptors (9-11). Recognition of pathogen-associated molecular patterns by TLRs triggers a cascade of cellular signals that culminates in the activation of NF-κB which leads to the inflammatory gene expression and ultimately to the clearance of the infectious agents (11-14). There is accumulating evidence, based on *in vitro* and *in vivo* studies, that supports a role for TLRs in *A. fumigatus* sensing. Findings from various studies demonstrated that *A. fumigatus* recognition by macrophages, neutrophils and dendritic cells, whether of mouse (15) or human (16) origin, depends on the presence of TLR2 and TLR4. The importance of TLR2 and TLR4 in IPA has been confirmed by the increased susceptibility of TLR2 and TLR4 knockout mice to *A. fumigatus* infections (17-19). However, very little knowledge exists concerning how the pulmonary epithelium responds to *A. fumigatus* infection and which receptors and signalling pathways might be involved. Most studies have investigated physical interactions between *A. fumigatus* and the

epithelium and found that *A. fumigatus* conidia can be internalized by epithelial cells (20,21). Once internalized, some conidia traffic to late endosomes/lysosomes where they can germinate. Those germlings are able to escape the phagosome and form extracellular hyphae without lysis of the host cells (20). *A. fumigatus* conidia have been also found to suppress apoptosis of epithelial cells induced by either tumor necrosis factor alpha (TNF-α) or staurosporine (22). These observations led to hypothesize that *A. fumigatus* uses epithelial cells as reservoirs for immune evasion and eventually a starting point for dissemination throughout the host (21,23).

As far as synthesis of cytokines and chemokines are concerned, it has been shown that *A. fumigatus* hyphae and proteases can activate cultured epithelial cells to produce interleukin (IL)-8 (24-26), a CXC chemokine that is a potent chemo-attractant for neutrophils. In support, CXC chemokines such as KC and MIP-2 have been shown to be components of neutrophil-mediated host defense in a mouse model of IPA (27,28). However, to date, there are no reports on the regulation of IL-8 gene expression by airway epithelial cells in response to *A. fumigatus* stimulation. This is of particular interest in the context of the involvement of TLRs in IPA, since respiratory epithelial cells express all the different types of TLRs and activation of these TLRs has been shown to induce the production of cytokines, chemokines and antimicrobial peptides (29-32). In this study, we investigated the signalling pathways that promote the generation of IL-8 by a human bronchial epithelial cell line stimulated with live *A. fumigatus*.

MATERIALS AND METHODS.

Reagents. F-12K nutrient mixture (Kaighn's modification), penicillin and streptomycin, glutamine and Trypsin-EDTA were from Invitrogen (Paisley, UK). The p38 MAP kinase inhibitor (SB203580) was obtained from Calbiochem (La Jolla, CA) and the PI3K (LY294002) and ERK1/2 (PD98059) inhibitors were from Cell Signaling Technology (Danvers, MA). MALP-2 was purchased from Alexis Biochemicals (San Diego, CA). The p38 MAP kinase, the Akt, the p44/42 MAP kinase, the phospho-p38 MAP kinase (Thr180/Tyr182), the phospho-Akt (Ser473) and the phospho-p44/42

MAPK (Thr202/Tyr204) antibodies were from Cell Signaling Technology. Duo-Set ELISA kit and the recombinant human TNF-α were obtained from R&D Systems (Minneapolis, MN). Leupeptin, aprotinin, soybean trypsin inhibitor, phenylmethylsulfonyl fluoride, benzamidine were from Sigma (Saint Louis, MI).

Preparation of A. fumigatus *conidia.* A clinical isolate of *A. fumigatus* (Green strain CBS 144.89) was maintained on 2% malt extract agar slants at 22°C. Conidia were recovered from cultures grown for 7 days by washing the slant culture with a phosphate-buffered saline (PBS)-0.1% Tween 20 solution and gently shaked. Conidia were then washed by centrifugation (5 min at 10,000 x *g*) and suspended in a PBS-0.1% Tween 20 solution. Conidia concentrations were evaluated by measurement of the optical density of the suspension at 600 nm, with a 0.6 optical density corresponding to 2×10^7 conidia/ml. The suspension was then diluted as needed in order to reach the desired concentration.

Cell culture and stimulation conditions. The human bronchial epithelial cell line BEAS-2B obtained from the American Type Cell Collection (Manassas, VA) was maintained in serial passage in F-12K culture medium supplemented with 10% FCS, 1% penicillin and streptomycin, 1% glutamine and 10 mM HEPES in 75 cm2 culture flasks and seeded at 5×10^4 on 24-well plates 3 days before. In all experiments, except for the kinetic study, BEAS-2B cells were stimulated during 15 hours with conidia (3×10^5) in a 300 μl medium without penicillin and streptomycin.

Immunoblotting. Epithelial cells were washed once with cold PBS and then lysed on ice with a lysis buffer (10 mM Tris, 150 mM NaCl, 3 mM EDTA) supplemented with protein inhibitors (100 μM leupeptin, 10 μM aprotinin, 20 μg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine) and 1% (v/v) Triton X-100. After centrifugation for 30 min at 18,000 x *g* at 4 °C, the total protein concentration was determined using the Pierce protein assay. Samples were further solubilized prior to electrophoresis by adding SDS (2%, v/v), and disulfide bonds were reduced with 5% (v/v) β mercaptoethanol. An equal amount of protein (25 μg) was fractionated by SDS-PAGE on a 10% acrylamide gel, and proteins were further electrotransferred to a PVDF membrane

(Immobilon, Millipore Corporation, Bedford, M.A.) and probed by immunoblotting using specific antibodies as specified in the figure legends. Bound antibodies were detected using the ECL+ immunoblotting detection system (Amersham-GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Molecular masses were estimated from calibration standards included in each gel.

Transient and stable transfections of BEAS-2B cells and reporter gene studies. BEAS-2B cells were seeded at 10^4 cells/well on 24-well plates 72 h before transient transfection using FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, USA), according to the manufacturer's instructions. Each sample contained either 200 ng of NF-κB-luciferase (kindly provided by Dr. A. Israel, Pasteur Institute, Paris, France) or 200 ng of a luciferase reporter gene driven either by a long IL-8 promoter construct (-1481 bp relative to the transcription start site) or by a short IL-8 promoter construct (-133 bp relative to the transcription start site) containing or not site-specific mutations for the binding of NF-κB, activating protein-1 (AP-1) or nuclear factor–IL-6 (NF-IL-6) (gifts from Dr. N. Mukaida, Kanazawa University, Japan). After 24 h, cells were stimulated with *A. fumigatus* (3×10^5 conidia), TNF-α (0.05 ng/ml) or MALP-2 (30 or 100 ng/ml). Following 15 h with the indicated treatments, cells were lysed in a lysis buffer (25 mM Tris (pH 7.4), 8 mM MgCl2, 1 mM DTT, 1% (v/v) Triton X-100, and 15% (v/v) glycerol), and luciferase activity was measured in the cell lysates using an EGNG Berthold luminometer. Results are expressed as relative luciferase units.

BEAS-2B cells were stably transfected with 500 ng of vector expressing dominant-negative form of MyD88 (MyD88-DN), a kind gift from Dr. M. Muzio (Mario Negri Institute, Milan, Italy) or 500 ng of the empty plasmid (Invitrogen, Carlsbad, USA) used as control, by using the FuGENE 6 transfection reagent. The transfected cells were selected for MyD88-DN integration by incubation with the selection medium containing 1.5 mg/ml of Geneticin. Clones resistant to the selection medium were isolated, cultured and screened for MyD88-DN expression by stimulation with specific agonists of the MyD88 pathway.

Quantification of IL-8 gene expression by real-time PCR. Total RNA of epithelial cells was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Purified RNA was reverse-transcribed using M.MLV reverse transcriptase (Promega, Mannheim, Germany) and a random primer p(dN)6 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. The mRNA levels of IL-8 and β-actin were determined by real-time quantitative PCR. QuantiTect SYBR Green PCR kit (Qiagen) was used following the manufacturer's instructions. The PCR mixture was composed of 12.5 μl QuantiTect SYBR Green Mix (2x), 5 μl of cDNA (125 ng of total RNA) and corresponding primers (IL-8 F : AGAGACAGCAGAGCACACAA, IL-8 R : TTAGCACTCCTTGGCAAAAC, β-actin F : GGAAATCGTGCGTGACAT, β-actin R : TGGCGTACAGGTCTTTGC) (final concentration: 300 nM), and then distilled water was added to a final volume of 25 μl. PCR was done using 7500 Real-time PCR Systems (Applied Biosystems, Foster City, CA) according to the manufacturer's suggestions following thermal cycling program (enzyme activation 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 45 s, and extension at 72 °C for 45 s). Relative gene expression was calculated by comparing the number of thermal cycles that were necessary to generate threshold amounts of product (CT). CT was calculated for the IL-8 gene and for the housekeeping gene β-actin. For each cDNA sample, the CT for β-actin was subtracted from the CT for IL-8 to give the parameter ΔCT, thus normalizing the initial amount of RNA used. The amount of IL-8 mRNA was calculated as $2^{-\Delta\Delta CT}$, where ΔΔCT is the difference between the ΔCT of the two cDNA samples to be compared.

Cytokine measurements and cytotoxicity of cultured cells during A. fumigatus infection. IL-8 concentrations in cell culture supernatants were determined using Duo-Set ELISA kits. To test the viability of the infected and uninfected cells, 50 μl of supernatant were removed at various time points post-infection and the level of lactate dehydrogenase (LDH) activity was determined using the Cytotox 96 Non-radioactive assay kit from Promega (Madison, WI). The assay quantitatively measures LDH, a stable cytosolic enzyme that is released upon cell lysis. Uninfected wells were treated with either 100 μl of 10%

Triton X-100 (1% v/v final concentration) or PBS to serve as positive and negative controls for LDH release respectively. Samples were incubated with LDH substrate for 30 min and then the absorbance was read on a microplate reader at 490 nm.

Statistical Analysis. Each point corresponds to the mean ± sem of the indicated number of experiments. The statistical significance of differences between groups was tested using the unpaired Student's t test with a threshold of p < 0.05.

RESULTS

A. fumigatus *induces IL-8 synthesis by respiratory epithelial cells.* Confluent monolayers of BEAS-2B cells were infected with $3x10^5$ conidia/0.3 ml per wells. At different time intervals postinfection, culture supernatants were collected and assayed for IL-8 concentrations. A time-dependent increase in IL-8 secretion was noted and reached significance after 8 h compared to unstimulated cells (Fig. 1), a time point at which all spores are swollen, as observed by light microscopy. To confirm that only the germinated state of *A. fumigatus* is able to induce IL-8 production, CEA17, a mutant strain of *A. fumigatus* unable to germinate, was tested under the conditions used with the wild type strain. As anticipated, 15 h post infection, IL-8 synthesis was not triggered by this mutant (Fig. 2).
In order to assess whether the release of soluble components were involved in the induction of IL-8 production, $3x10^5$ conidia/0.3 ml were incubated for 15 h at 37°C in complete F12K medium. After incubation, fungal cultures were centrifuged, supernatants were filtered, and added to BEAS-2B cells for 15 hours. The supernatants did not induce IL-8 synthesis (data not shown).
Taken together, these results indicate that the germinated form of *A. fumigatus* and not resting spores or released components induces IL-8 production by epithelial cells.
To determine whether *A. fumigatus* has any cytotoxic effect on the cells, we measured the release of the cytosolic enzyme LDH into the supernatant after 15 h stimulation. There was no difference in LDH release between infected and uninfected cells (data not shown).

A. fumigatus *induces NF-κB activation in respiratory epithelial cells.* NF-κB is a critical transcriptional activator involved in the expression

of many inflammatory genes of the innate immune response including IL-8 (33). To determine whether the interaction between epithelial cells and *A. fumigatus* induced NF-κB activation, we measured the transcription of a luciferase reporter gene containing in its promoter copies of the consensus sequence of NF-κB elements (NF-κB-Luc). Transfected cells were stimulated for 15 h with $3x10^5$ conidia or with MALP-2 (30 ng/ml), a TLR2 agonist used as positive control of NF-κB activation. As presented in Fig. 3, *A. fumigatus* and MALP-2 stimulation resulted in a three and a six fold increase of luciferase activity, respectively, indicating that NF-κB is activated by *A. fumigatus.*

IL-8 gene expression induced by A. fumigatus *is NF-κB-dependent.* As *A. fumigatus* is able to induce IL-8 production and NF-κB activation in epithelial cells, we therefore assessed whether NF-κB activation is involved in IL-8 production. In a number of studies (33-36), it was found that a sequence spanning nucleotides −1 to −133 within the 5' flanking region of the IL-8 gene is essential and sufficient for transcriptional regulation of the gene. This promoter element (short promoter) contains binding sites for NF-κB, activating protein-1 (AP-1) and nuclear factor–IL-6 (NF-IL-6). To determine the implication of each site in the context of *A. fumigatus* stimulation, cells were transiently transfected with a luciferase reporter gene driven by the IL-8 promoter (-133) containing site-specific mutations, and stimulated for 15 h with $3x10^5$ conidia. As shown in Fig. 4, the NF-κB and AP-1 site mutation reduced the IL-8 promoter activity by 99% and 40%, respectively. In contrast, NF-IL-6 site had no significant effect.

A. fumigatus *induces activation of PI3 kinase, p38 MAPK and ERK1/2.* The transmission of extracellular signals to their intracellular targets is mediated by a network of interacting proteins that relay biochemical messages and thus control multiple cellular processes. Several related intracellular signaling pathways, collectively known as protein kinase signaling cascades, have been demonstrated to play a role in many systems.
To determine whether *A. fumigatus* was able to activate PI3 kinase, p38 MAPK and ERK1/2, kinases that have been implicated in IL-8 production (33), we examined the phosphorylation of the Ser/Thr kinase Akt, identified as an important target of PI3 kinase (37,38) and of p38

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MAPK and ERK1/2. Phosphorylation was measured at different time intervals (3, 8, 10 and 13 hours) post infection by immunoblotting of whole cell lysates with a phosphospecific antibody that recognize Akt only when phosphorylated on Ser 473, a phospho-p38 MAPK specific antibody or a phospho-p44/42 MAPK specific antibody that recognizes the phosphorylated form of ERK1/2. *A. fumigatus* induced phosphorylation of Akt, p38 MAPK and ERK1/2 at 10 h (Fig. 5) that was still observed at 13 h. This result shows that stimulation of respiratory cells cells with *A. fumigatus* led to PI3 kinase, p38 MAPK and ERK1/2 phosphorylation and that this activation occurred at a late time point, suggesting that only germinated spores are able to activate these pathways.

A. fumigatus *induces IL-8 production via PI3 kinase, p38 MAPK and ERK1/2 pathways.* To investigate the implication of PI3 kinase, p38 MAPK and ERK1/2 in the production of IL-8, specific inhibitors were tested. The PI3 kinase (LY294002), p38 MAPK (SB203580) and ERK1/2 (PD98059) inhibitors incubated 1 h before and during the 15 h of stimulation with *A. fumigatus*, diminished IL-8 production in a concentration-dependent manner, LY294002 and SB203580 inhibited IL-8 synthesis by around 50% and by more than 95% at concentrations of 3 and 30 μM, respectively. The ERK1/2 inhibitor (PD98059) was less potent and inhibited the IL-8 synthesis by only 20% at 3 μM and 75% at 30 μM (Fig. 6). It is of note that each one of these inhibitors prevents the phosphorylation of its own target without affecting the phosphorylation of the target of the other inhibitors (data not shown). No difference in LDH release was found between treated and untreated infected cells (data not shown).
We conclude that PI3 kinase, p38 MAPK and ERK1/2 pathways are involved in IL-8 production by respiratory epithelial cells stimulated with *A. fumigatus.*

PI3 kinase, p38 MAPK and ERK1/2 are not implicated in the NF-κB translocation. In order to study the involvement of PI3 kinase, p38 MAPK and ERK1/2 in NF-κB activation, NF-κB-Luc transfected cells were treated with the different kinase inhibitors at 30 μM for 1 h before and during the 15 h of stimulation with *A. fumigatus* and then luciferase activity was measured. As shown in Fig. 7, LY294002,

SB203580 and PD98059 were not able to abolish NF-kB activation.
Thus, whereas the different kinase inhibitors abrogate IL-8 production, they do not affect NF-κB translocation into the nucleus. It is inferred from these data that PI3 kinase, p38 MAPK and ERK1/2 control either a pathway downstream of NF-κB translocation or the activation of another transcription factor necessary for IL-8 synthesis.

Implication of PI3 kinase, p38 MAPK and ERK1/2 in the activation of the IL-8 promoter. To understand the involvement of the protein kinases in the activation of the IL-8 promoter, cells were transfected with a luciferase reporter gene driven by the long IL-8 promoter (-1481) and then treated with concentrations of kinase inhibitors that inhibited by around 80% IL-8 synthesis *i.e* 10 μM for LY294002 and SB203580, and 30 μM for PD98059. As previously, kinase inhibitors were incubated 1 h before and during the 15 h (10 h only for the measurement of IL-8 mRNA) of stimulation with *A. fumigatus.* Luciferase activity and IL-8 mRNA and protein productions were then measured. Whereas all kinase inhibitors diminished IL-8 mRNA and protein productions by around 80% (Fig. 8B and C) only PD98059, the inhibitor of ERK1/2, blocked the luciferase activity of the IL-8 reporter gene (Fig. 8A).
These data indicate that, apart from NF-κB, another transcription factor is necessary for IL-8 gene expression and that the activation of this factor is controlled by ERK1/2.

MyD88 is involved in NF-κB activation induced by A. fumigatus. To determine whether TLRs were implicated in NF-κB activation of epithelial cells stimulated by *A. fumigatus*, we stably transfected BEAS-2B cells with a vector expressing a dominant negative form of MyD88 (MyD88-DN), a common adaptator molecule to the intracellular domain of all TLRs (except TLR3). For each particular experiment, cells were transiently transfected with the vector expressing NF-κB-Luc, stimulated for 15 h with $3x10^5$ conidia and luciferase activity was measured. As shown in Fig. 9, luciferase activity was deeply inhibited in cells expressing the MyD88-DN and stimulated by *A. fumigatus* compared to control cells expressing the empty control plasmid (pcDNA3). As expected, the luciferase activity was similar in the two cell lines when stimulated

with TNF-α used as a negative control, and was inhibited in MyD88-DN expressing cells when stimulated with MALP-2 used as positive control. From these experiments substantiating a role for MyD88, we speculate that possibly TLR pathways, although receptors for IL-1 and IL-18 operate also through MyD88, are important for the activation of NF-κB in epithelial cells stimulated with *A. fumigatus*.

MyD88 is not involved in IL-8 synthesis by A. fumigatus. Because our results implicated of NF-κB in IL-8 production by *A. fumigatus–*activated epithelial cells, we next evaluated whether MyD88 was involved in this production. IL-8 was measured in supernatants of BEAS-2B cells expressing MyD88-DN or pcDNA3 alone, and stimulated for 15 h with *A. fumigatus.* Surprisingly, no difference was observed for IL-8 production between the two cell lines stimulated with *A. fumigatus* (Fig. 10) or TNF-α used as negative control, while MALP-2-induced IL-8 production was completely abolished in the MyD88-DN cell line. These data indicate that whereas MyD88 is implicated in the signaling pathway leading to NF-κB activation and NF-κB is involved in IL-8 synthesis (see above), MyD88 is not implicated in the production of this chemokine by epithelial cells stimulated with *A. fumigatus.*

DISCUSSION

The dogma of the anti-*A. fumigatus* innate response is that the key cells are alveolar macrophages and neutrophils, two types of phagocytes, which eliminate conidia and hyphae, respectively (3-5). For a long time, the respiratory epithelial layer was described only as a physical inert barrier between the internal and external environments. However, recently numerous studies have demonstrated that respiratory epithelial cells have a great importance in the initiation of the host innate immune response by sensing the pathogens and secreting inflammatory mediators (6-8).

In the present study, we observed that germinating conidia are able to induce IL-8 synthesis, while resting conidia or mutant conidia that are unable to germinate, did not. This result is in accordance with a previous study showing a production of IL-8 significantly increased only when epithelial cells were stimulated with hyphae (26). The activation results from direct contact between epithelial cells and germinating conidia and not from released soluble molecule(s) as *A. fumigatus* culture supernatants are not able to induce IL-8 production. In contrast to our observations, others have shown that proteases obtained from filtrates of *A. fumigatus* cultures induced the release of IL-8 by respiratory epithelial cell lines (25). This discrepancy may be explained by the fact that concentrations of fungal proteases obtained from *A. fumigatus* culture filtrates were probably higher than those possibly produced in our experimental conditions that are more closely related to an *in vivo* infection process. This hypothesis is reinforced by the observation of epithelial cell detachment when stimulated with the proteases (24) while under our conditions, direct stimulation with *A. fumigatus* did not result in either detachment nor lysis of the cells.

These data show that respiratory epithelial cells play an active role in the antifungal innate immune response, by producing IL-8, a chemokine that contibutes to the effective recruitment and activation of neutrophils at the site of infection (39,40). However, it is a common thought that inflammation must be controlled much more tightly in the lungs than in other organs, in order to preserve a high degree of functionality allowing sufficient gas exchange. To this aim the epithelial cell response may be somewhat restricted to the presence of crucial pathogens to avoid frequent inflammation. Our results are in accordance with this view as respiratory epithelial cell responses differ from macrophages. Alveolar macrophages are able to recognize and eliminate resting conidia (3,5) whereas epithelial cells do not sense resting conidia but are able to recognize *A. fumigatus* during the first step of conidia swelling, the pathogenic state of *A. fumigatus* development. This delayed epithelial response may be useful to limit the inflammatory response. Indeed, conidia are first eliminated by resident alveolar macrophages while epithelial cells remain in an inactive state. However as soon as the capacity of alveolar macrophages to eliminate conidia is overwhelmed, free conidia swell and began to be recognized by epithelial cells. This interaction induces IL-8 production which contributes to the

recruitment of neutrophils that are able to eliminate hyphae.

As the molecular mechanism by which *A. fumigatus* induces IL-8 secretion by respiratory epithelial cells had not been studied, we examined the activation and the involvement of the transcriptional factor NF-κB and of protein kinases such as PI3 kinase, p38 MAPK and ERK1/2. NF-κB is an ubiquitous transcription factor playing a key role in the immune response to infections. In unstimulated cells, NF-κB dimers are sequestered in the cytoplasm by a family of inhibitors, called IκB. Activation of the IκB kinase induces the phosphorylation and degradation of IκB and allows translocation of NF-κB to the nucleus where it can bind to specific sequences of DNA and regulates gene transcription. The activation of these genes leads to the expression of a number mediators, including many cytokines, chemokines and cell adhesion molecules that are involved in the inflammatory response (41,42). In a number of studies, it was found that the IL-8 gene transcription requires activation of the combination of NF-κB and either AP-1 or NF-IL6, depending on the cell type (33,36). Using transient transfection of cells by the short IL-8 promoter driven reporter gene containing the binding site, mutated or not, for NF-κB, AP-1 and NF-IL6, we observed that NF-κB and AP-1 are implicated in the activation of the promoter.

It is well known that many stimuli able to induce IL-8 production activate a number of protein kinases (43,44). We therefore examined the contribution of PI3 kinase, p38 MAPK and ERK1/2. First, we observed that they were all activated upon *A. fumigatus* challenge. We then used specific inhibitors and observed that the blockade of PI3 kinase, p38 MAPK and ERK1/2 pathways inhibited IL-8 synthesis, indicating their involvement. However, inhibition of PI3 kinase and p38 MAPK did block neither the NF-κB transactivation nor the IL-8 promoter activity whereas inhibition of ERK1/2 that had also no effect on the NF-κB transactivation, did block the activation of the IL-8 reporter gene. These data infer that neither PI3 kinase, p38 MAPK nor ERK1/2 are involved in the nuclear translocation of NF-κB and the upstream signaling pathways since NF-κB is able to activate the promoter NF-

κB–Luc. The present results also indicate that PI3 kinase and p38 MAPK are not involved in the IL-8 promoter reporter gene activation although they are necessary for the IL-8 protein production. It is now well recognized that protein kinases can control the transcriptional process in many ways in particular through their ability to remodel chromatin structure by phosphorylation of histone (45-48). Under our experimental conditions, this activity cannot be highlighted as the transfected IL-8 promoter gene is not wrapped in nucleosomes or within native chromatin environment and consequently cannot recapitulate the specificity of the endogenous gene. Taking these data into account, our results suggest on the one hand that PI3 kinase and p38 MAPK may play a role in the downstream NF-κB translocation events like epigenetic modifications of chromatin or also possibly in mRNA stabilization (49-51) and on the other hand that ERK1/2 is involved in the translocation of one or several transcriptional factors other than NF-κB, and essential for the IL-8 gene expression.

Human TLRs are a family of 10 receptors, expressed in lung epithelial cells (30,52,53), that trigger innate immune reactions in response to various microbial products and are the first step of a signaling cascade that leads to the activation of various nuclear factors and, in particular of NF-kB. Recent studies have demonstrated a crucial involvement of TLRs in the recognition of *A. fumigatus*. *In vitro* studies suggest that TLR2 is clearly involved in the recognition of *A. fumigatus* by alveolar macrophages and neutrophils (19,54-56), while the contribution of TLR4 remains to be more firmly established (57,58). By studying fungal infection in mice deficient in either TLRs or TLR-associated adaptor molecules, it appeared that TLR2 and TLR4 play differential roles in the activation of the innate immune response (19,15,59). Taken together, these data led us to evaluate the role of the TLR pathways in the activation of the respiratory epithelial cells stimulated by *A. fumigatus*. Knowing that ligand-TLR interactions, except those involving TLR3, trigger the binding of at least one adaptor molecule, MyD88, to the intracellular domain of TLRs, we compared the response of cells stably transfected with a dominant negative form of MyD88 (MyD88-DN) or the empty plasmid and transiently co-transfected with NF-κB-Luc to *A.*

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fumigatus. Our results showed that NF-κB activation was abolished in the MyD88-DN cells suggesting an implication of TLRs in the activation of respiratory epithelial cells by *A. fumigatus.* Surprisingly, under the same experimental conditions, IL-8 production was not decreased in the MyD88-DN cells. These data differ from our other results showing a role for NF-κB in the activation of the short IL-8 promoter driven reporter gene (Fig. 4). A possible explanation for these discrepant results, is that a transcriptional factor (or several), apart from NF-κB, which is regulated by ERK1/2, possibly AP-1, is sufficient to trigger IL-8 synthesis. This interpretation agrees with a previous report on TNF-stimulated airway epithelial cells showing that ERK is required and sufficient to regulate IL-8 promoter transcription by activating the AP-1 site (43). It remains to establish whether the expression of other inflammatory genes apart from the IL-8 gene, is also activated through the MyD88-NF-κB independent pathway. With regard to the MyD88-NF-κB dependent pathway, the involvement of one or several TLR, possibly TLR2 and/or TLR4, has to be looked for.

In summary, taking advantage of a cell line that can be easily transfected, our results suggest the existence of two independent signaling pathways in respiratory epithelial cells activated by *A. fumigatus.* One that does not result in IL-8 synthesis and is controlled by MyD88 and NF-κB, implicating one or several TLRs. The other which is probably the most important observation of our study as it does not fit with the classical paradigm (33-36), is MyD88 and NF-κB independent. This latter pathway leads to IL-8 synthesis through different kinase pathways including PI3 kinase, p38 MAPK and ERK1/2. It is difficult to speculate on the nature of the receptor that recognizes *A. fumigatus* and that triggers this second intracellular transduction. Indeed, Dectin-1 that ligates β1-3 glucans expressed by germinating conidia (60-61), would be the most obvious candidate but it seems not expressed by epithelial cells (62). DC-SIGN is a C-type lectin that mediates the binding and capture of *A. fumigatus.* Nonetheless, its expression is restricted to dendritic cells and subsets of macrophages (63). Whatever it could be, it also remains to demonstrate that this pathway is operative in primary cells in culture and under the *in vivo* situation when conidia invade the airspaces. In short, our results showing that the synthesis of IL-8 by epithelial cells in response to a fungal challenge is not controlled by the TLR-MyD88-NF-κB pathway, have to be extended in order to identify the host cell receptor(s) and its fungus ligand(s), the downstream signaling pathways and the genes regulated by the different kinase pathways, to allow the search of potential therapeutic targets able to manipulate the innate immune response of immunosuppressed patients suffering from invasive pulmonary aspergillosis.

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FOOTNOTES

figure legends

Fig. 1. Kinetic of IL-8 production by respiratory epithelial cells challenged with *A. fumigatus* conidia. Sub-confluent BEAS-2B cells cultured in 24-well plates were incubated in 300 µl medium with 3×10^5 conidia for different time intervals. At each time point, supernatants were collected and IL-8 concentrations were measured by ELISA. Each point is the mean ± sem of 3 experiments performed in triplicate. * $P < 0.05$, significantly different from baseline values.

Fig. 2. IL-8 synthesis by respiratory epithelial cells challenged with germinating or non-germinating conidia of *A. fumigatus*. Sub-confluent BEAS-2B cells cultured in 24-well plates were incubated in 300 µl medium with 10^5 or 3×10^5 of WT conidia (DAL) or mutated conidia unable to germinate (CEA 17). After 15 h, supernatants were collected and IL-8 concentrations were measured by ELISA. Each histogram is the mean ± sem of 3 experiments performed in triplicate. ** $P < 0.01$, significantly different from DAL values.

Fig. 3. *A. fumigatus*-induced NF-κB activation in respiratory epithelial cells. BEAS-2B cells cultured in 24-well plates were transiently transfected with a NF-κB driven luciferase gene (NF- κB-Luc). After 24 h, cells were incubated for 15 h with 3×10^5 conidia (Af), with 30 ng/ml MALP-2 or with the culture medium alone (NS). Then, cells were lysed and the luciferase activity was measured. Each histogram expressed in fold increase over the non stimulated cells, is the mean ± sem of 5 experiments performed in triplicate. ** $P < 0.01$, significantly different from NS values.

Fig. 4. Implication of NF-κB and AP-1 binding sites in the IL-8 promoter activity in *A. fumigatus*-activated respiratory epithelial cells. BEAS-2B cells cultured in 24-well plates were transiently transfected with a construct of a fragment of the IL-8 promoter (-133) containing binding site for NF-κB, AP-1 and NF-IL6 or the IL-8 promoter (control) containing specific mutations of binding site for NF-κB (Δ-NF-κB), AP-1 (Δ-AP-1) or NF-IL6 (Δ-NF-IL6), and fused to the luciferase gene. After 24 h, cells were incubated for 15 h with 3×10^5 conidia. Then, cells were lysed and the luciferase activity was measured. Each histogram expressed in % of the activity of the non mutated construct, is the mean ± sem of 3-4 experiments performed in duplicate. ** $P < 0.01$ and * $P < 0.05$, significantly different from values obtained with the non mutated construct.

Fig. 5. Activation of PI3 kinase, p38 MAPK, and ERK1/2 in *A. fumigatus*-activated respiratory epithelial cells. Sub-confluent BEAS-2B cells cultured in 24-well plates were incubated in 300 µl medium without (NS) or with (Af) 3×10^5 conidia. After 10 h, cells were lysed, and extracted proteins immunoblotted with specific antibodies for the detection of AKT (a PI3 kinase substrate) and its phosphorylated form (P-AKT), of p38 MAPK (p38) and its phosphorylated form (P-p38), of ERK1/2 (ERK) and its phosphorylated form (P-ERK). The upper panel shows the western blots of one experiment representative of four others. Levels of phosphorylation were quantified as described in the Materials and Methods section and presented in the lower panels. Each histogram is expressed in relative ratio of phosphorylated form *vs* the total amount of the studied kinase, of one experiment representative of four others.

Fig. 6. Inhibition of IL-8 synthesis in *A. fumigatus*-activated respiratory epithelial cells by specific inhibitors of PI3 kinase, p38 MAPK, and ERK1/2. Sub-confluent BEAS-2B cells cultured in 24-well plates were pre-incubated in 300 µl medium with different concentrations of LY294002, SB203850 or PD98059, three specific inhibitors of PI3 kinase, p38 MAPK and ERK1/2, respectively. One hour later, 3×10^5 conidia were added and following 15 h incubation, supernatants were collected for IL-8 measurement by ELISA. Each point is the mean ± sem of 3-5 experiments performed in triplicate, expressed in % of control value obtained in absence of inhibitor.

Fig. 7. Effect of specific inhibitors of PI3 kinase, p38 MAPK, and ERK1/2 on *A. fumigatus*-induced NF-κB activation in respiratory epithelial cells. BEAS-2B cells cultured in 24-well plates were transiently transfected with a NF-κB driven luciferase gene (NF-κB-Luc). After 24 h, cells were pre-incubated in 300 µl medium with 10 µM LY294002 (LY), 10 µM SB203850 (SB), or 30 µM PD98059 (PD), three specific inhibitors of PI3 kinase, p38 MAPK and ERK1/2, respectively. One hour later, 3×10^5 conidia were added and following 15 h incubation, cells were lysed and the luciferase activity was measured. Each histogram expressed in fold increase over the non stimulated cells, is the mean ± sem of 3-5 experiments performed in triplicate. ** *P*< 0.01, significantly different from baseline values.

Fig. 8. Effect of specific inhibitors of PI3 kinase, p38 MAPK, and ERK1/2 on the IL-8 promoter activity and IL-8 synthesis in *A. fumigatus*-activated respiratory epithelial cells. BEAS-2B cells cultured in 24-well plates were transiently transfected with a long construct of the IL-8 promoter (-1421) fused to the luciferase gene. After 24 h, cells were pre-incubated in 300 µl medium with 10 µM LY294002 (LY), 10 µM SB203850 (SB), or 30 µM PD98059 (PD), three specific inhibitors of PI3 kinase, p38 MAPK and ERK1/2, respectively. One hour later, cells were incubated for 15 h with 3×10^5 conidia (Af) and then surpernatants were collected for IL-8 protein measurement by ELISA, and cells were lysed for luciferase activity measurement. For IL-8 mRNA synthesis measurement, experiments were conducted exactly the same way except that incubations were stopped 10 h post-infection for mRNA extraction. Each histogram is expressed in % of the control-activated cells (Af) and depicts either the IL-8 promoter activation (Fig.8A), the IL-8 mRNA synthesis (Fig.8B) or the IL-8 protein secretion (Fig.8C). Each one is the mean ± sem of 4 experiments performed in triplicate for luciferase activity and IL-8 protein, and the mean ± sem of 3 single experiments for IL-8 mRNA. ** *P*< 0.01, significantly different from Af values.

Fig. 9. Involvement of MyD88 in *A. fumigatus*-induced NF-κB activation in respiratory epithelial cells. BEAS-2B cells stably transfected either with vector expressing a dominant-negative form of MyD88 (MyD88-DN) or with the empty plasmid (PcDNA3) used as a control, were cultured in 24-well plates and transiently transfected with a NF-κB driven luciferase gene (NF-κB-Luc). After 24 h, cells were incubated for 15 h with 3×10^5 conidia, 100 ng/ml MALP-2 or 0.05 ng/ml TNF-α. Then, cells were lysed and the luciferase activity was measured. Each histogram expressed in fold increase over the non stimulated cells, is the mean ± sem of 5 experiments performed in triplicate. ** *P*< 0.01 and * *P*< 0.05, significantly different from PcDNA3 values.

Fig. 10. Involvement of MyD88 in *A. fumigatus*-induced IL-8 synthesis in respiratory epithelial cells. BEAS-2B cells stably transfected either with a vector expressing a dominant-negative form of MyD88 (MyD88-DN) or with the empty plasmid (PcDNA3) used as a control were cultured in 24-well plates. Cells were incubated for 15 h with 3×10^5 conidia, 100 ng/ml MALP-2 or 0.05 ng/ml TNF-α. Then, supernatants were collected for IL-8 measurement. Each histogram expressed in ng/ml, is the mean ± sem of 5 experiments performed in triplicate. ** *P*< 0.01, significantly different from PcDNA3 values.
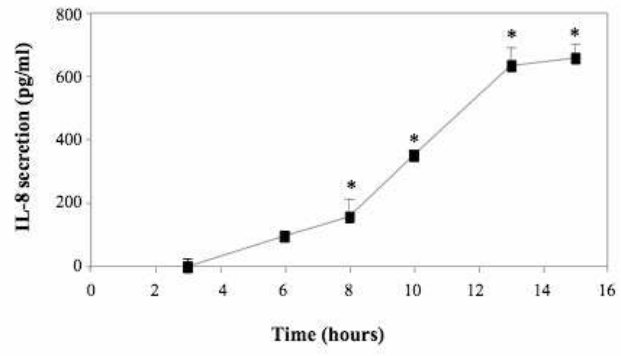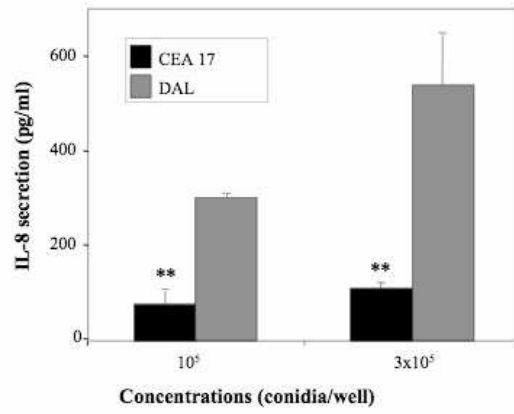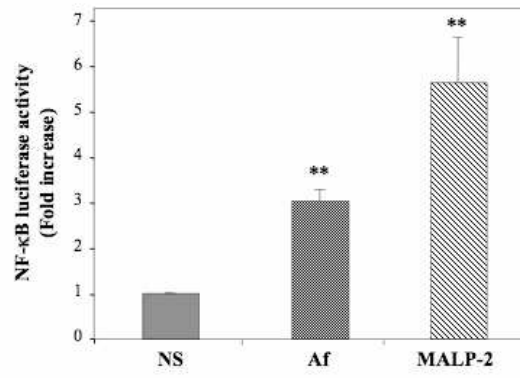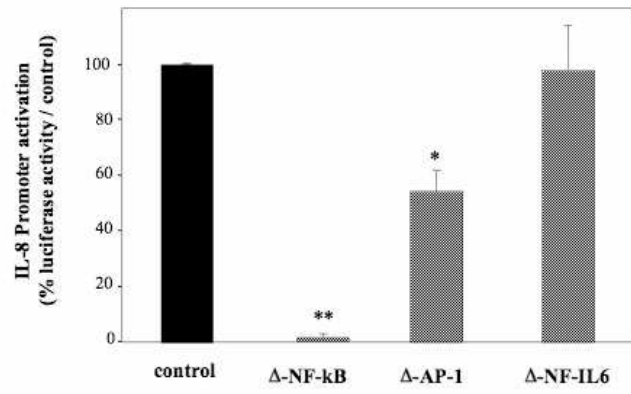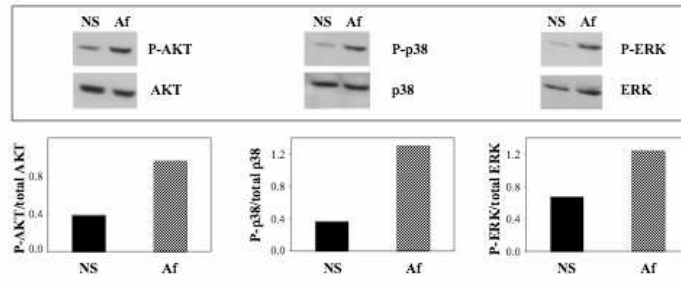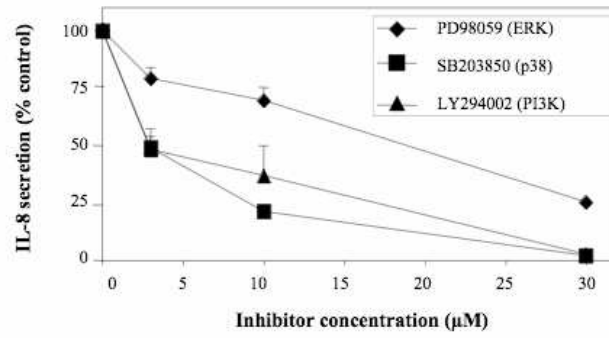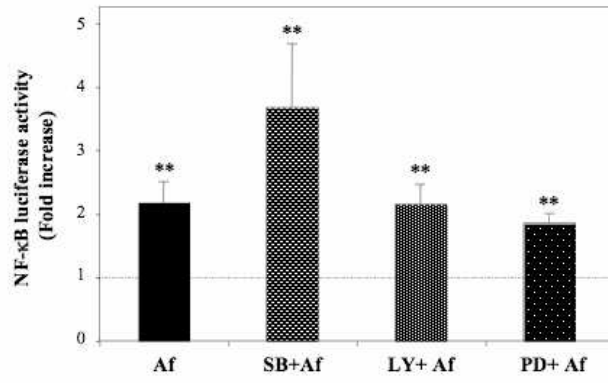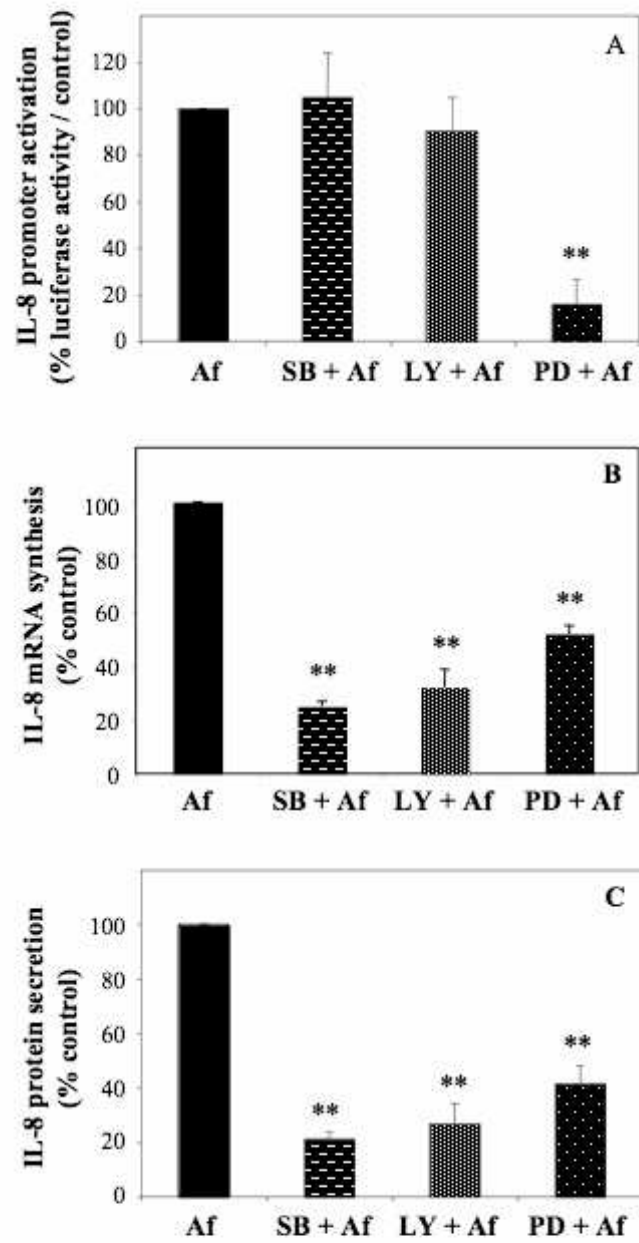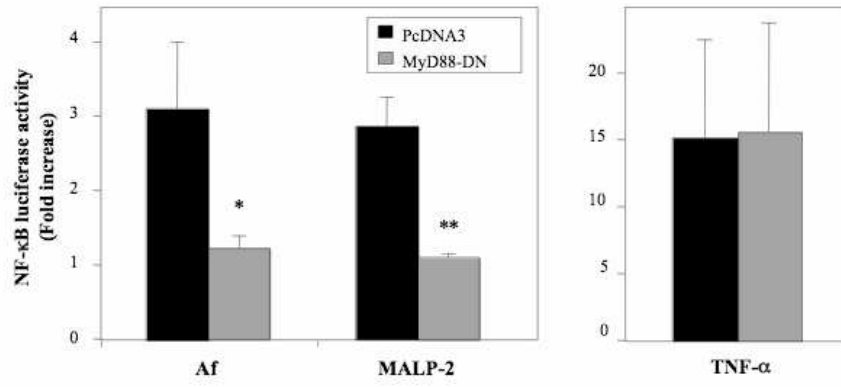
Figure 1

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