Medical Mycology September 2006, **44**, S213–S217

Taylor & Francis
Taylor & Francis Group

Interaction of *Aspergillus fumigatus* with the alveolar macrophage

M. DUBOURDEAU*, R. ATHMAN†, V. BALLOY‡, B. PHILIPPE§, L. SENGMANIVONG#, M. CHIGNARD‡, D. J. PHILPOTT†, J. P. LATGÉ* & O. IBRAHIM-GRANET*

**Unité des Aspergillus, †Immunité Innée et Signalisation, ‡Défense Innée et Inflammation INSERM E336, §Hôpital Foch, Suresne, and #Plate-forme d'Imagerie Dynamique, Institut Pasteur, Paris, France*

Aspergillus fumigatus is a human pathogen, able to cause invasive aspergillosis in immunosuppressed patients. In the immunocompetent situation inhaled conidia are easily cleared by the immune system. Knowledge of the cellular pathways involved in the innate immunity against *A. fumigatus* is poorly represented. Therefore, we aimed to investigate the immune response against *A. fumigatus* in murine alveolar macrophages in terms of MAP kinases, NF-kappaB and cytokine signalling. Our investigations revealed that in murine alveolar macrophages, MAP kinases, ERK and p38 are activated under *in vitro* conditions, following addition of *A. fumigatus* conidia. *In vivo* experiments, however, showed that only ERK is directly involved, because activation of p38 was negligible. Immunosuppression with corticosteroids inhibited phosphorylation of ERK and was directly accompanied with a strongly decreased level of TNF-alpha and additional cytokines. In addition, killing of *A. fumigatus* conidia is reduced using the ERK inhibitor. Therefore, ERK appears to be an essential MAP kinase in the defence against *A. fumigatus*. Activation of the transcription factor NFkappaB appeared only at late times after infection suggesting an association with the intracellular swelling of conidia.

Keywords *Aspergillus*, alveolar macrophage, MAP kinases, cytokines, transcription factor

Introduction

Aspergillus fumigatus is an opportunistic fungal pathogen responsible for invasive aspergillosis (IA), a severe, life-threatening infection in various immunocompromised patients [1]. Diagnosis is difficult, treatment is poorly efficient and as a consequence, mortality is high. In addition very 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 normal immunocompetent host is protected by pulmonary innate immunity, which includes phagocytosis by alveolar macrophages. Macrophages are key defenders of the lung and play an essential role in mediating the inflammatory response.

We have studied the engulfment and intracellular trafficking of *A. fumigatus* conidia in alveolar macrophages. Phagocytosis of conidia required an important membrane ruffling activity, actin polymerization and phosphatidylinositol 3-kinase activity. We also have demonstrated that the maturation of *A. fumigatus* phagosomes results from fusion with compartments of the endocytic pathway [3].

The mechanisms of killing of conidia by the alveolar macrophages begin 6 h after phagocytosis. Swelling of conidia inside the alveolar macrophage is a prerequisite for killing. The contribution of phagolysosomal acidification as well as NADPH oxidase to the conidicidal activity has been demonstrated. This

Correspondence: O. Ibrahim-Granet, Institut Pasteur, Unité des *Aspergillus*, 25 rue du Dr Roux, 75724 Paris cedex 15, France. Tel: +33 1 40 61 32 45; Fax: +33 1 40 61 34 19. E-mail: ogranet@pasteur.fr

DOI: 10.1080/13693780600904900

activity is inhibited by corticosteroids via the impairment of production of reactive oxidant intermediates [3,4].

Recognition of invading microorganisms by the innate immune system is a first and essential step in their successful elimination. Following microbial recognition, the macrophage effector functions are known to be dependent in part on the phosphorylation of mitogen-activated protein kinases (MAPKs). The extracellular signal-regulated kinases (ERKs) and p38 have been shown to be activated either in macrophages [5] or in alveolar macrophages using a variety of stimuli [6,7]. After activation by phosphorylation, the MAPKs translocate to the nucleus where they phosphorylate several targets including transcription factors that then mediate the expression of a number of pro-inflammatory genes.

We investigated the signalling events that occur when *A. fumigatus* conidia encounter a murine alveolar macrophage. We report on the involvement of MAPKs and the activation of the transcription factor NF-kappaB in the regulation of events leading to the activation of the immune response and killing of conidia [8].

Results

Study of the activation of ERK and p38 in response to A. fumigatus *stimulation*

To investigate whether MAP kinases play a role in the responses of immunocompetent and cortisone acetate immunosuppressed mice infected with conidia of *A. fumigatus*, we studied activation of ERK and p38 in alveolar macrophages isolated 4 h after intranasal infection with 1×10^7 conidia. Alveolar macrophages were recovered from bronchoalveolar and the activation of MAP kinases was investigated in the cell lysates by Western blot using phosphospecific antibodies. The level of phosphorylation was compared separately between immunocompetent non infected and infected mice as well as between immunosuppressed non-infected and infected mice (Fig. 1).

After 4 h of infection with conidia in immunocompetent mice, the infection resulted in a major activation of ERK with a phosphorylation fold of 2.9 and 1.9 for p44 and p42 subunits, when compared to non-infected mice $P < 0.05$ (Fig. 1A).

In immunosuppressed mice, levels of phosphorylated ERK are lower than in immunocompetent mice $P < 0.05$ (Fig. 1A). In addition, it was unexpected that the phosphorylation level of ERK even decreased after infection with conidia.

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. 1B, a similar level of phosphorylation of p38 was observed in both non-infected and infected immunocompetent and immunosuppressed mice.

Similar to the observations *in vivo*, the stimulation *in vitro* of murine primary alveolar macrophages or immortalized murine alveolar macrophages MH-S cells with conidia of *A. fumigatus* for different periods of time resulted in a significant fold of phosphorylation of ERK after 1 h stimulation and unlike *in vivo* experiments, p38 phosphorylation has been observed after *in vitro* stimulation (Fig. 2).

Translocation of NF-kappaB by A. fumigatus in alveolar macrophages

Translocation of NF-kappaB was analysed by immunofluorescence using a Rel-A specific antibody. MH-S cells were stimulated with conidia for 1, 4 and 6 h. As a positive control of NF-kappaB translocation, cells were treated with TNF-alpha for 30 min. As shown in

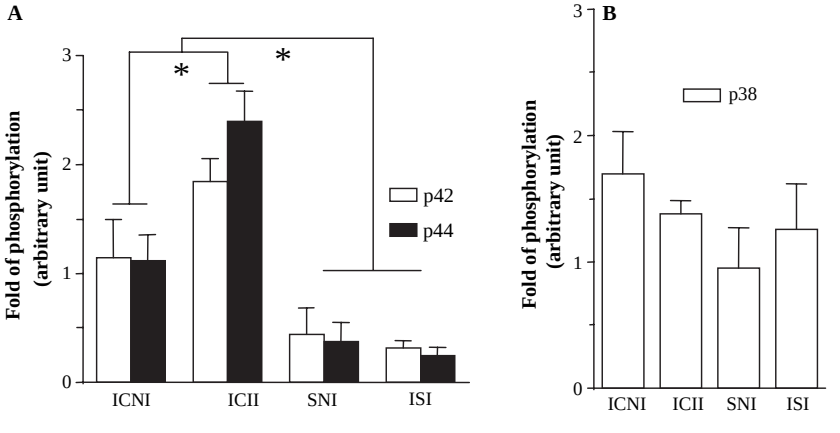

Fig. 1 *Aspergillus fumigatus* conidia induce phosphorylation of ERK and not p38 after 4 h *in vivo* infection. Alveolar macrophages were harvested by bronchoalveolar lavage from immunocompetent (IC) and immunosuppressed mice (IS) 4 h after intranasal infection with 1×10^7 conidia (I) or from PBS inhaled control mice (NI). Mice were immunosuppressed with 25 mg of cortisone acetate (Sigma) injected intraperitoneally at day 3 and immediately before intranasal inoculation of conidia (day 0**).** Phosphorylated and non phosphorylated forms of ERK and p38 were analysed by western blotting using phosphospecific antibodies. Levels of phosphorylation were quantified after normalization to the level of total ERK and p38 using densitometry scanning and the imageQuant program. Each bar represents average of fold phosphorylation of ERK (2A) or p38 (2B) from three independent mice ($P < 0.005$).

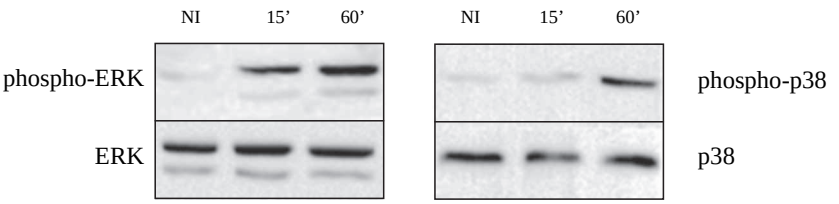

Fig. 2 Western blot analysis of phosphorylation of ERK and p38 MAP kinases in *Aspergillus fumigatus* conidia-stimulated murine primary alveolar macrophages. Primary alveolar macrophages were stimulated or not (NI) with conidia at a ratio of 10 conidia per macrophage for the indicated time. ERK and p38 phosphorylation was analysed by immunofluorescence using antibodies against phospho-ERK and phospho-p38. To confirm the sample loadings, the blots were stripped and probed with an anti- total ERK or anti-total p38 antibodies.

Fig. 3, TNF-alpha treatment resulted in complete nuclear translocation of NF-kappaB. Non stimulated control cells as well as cells stimulated with conidia for 1 h showed no Rel-A translocation but became apparent after 4 h of stimulation with conidia and reached 100% of translocation after 6 h. This result strongly implied that efficient Rel-A translocation only occurred after intracellular swelling of conidia. 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 (Trans-AM). Nuclear extracts were prepared from cells stimulated with conidia for 4 and 6 h. Results showed that resting conidia induce an activation fold of 1.7 after 6 h of stimulation compared to non-stimulated cells (data not shown).

Cytokine release from alveolar macrophages after stimulation with A. fumigatus

Primary murine alveolar macrophages were stimulated *ex vivo* with conidia for 6 h in presence or absence of inhibitors of MAP kinases and NF-kappaB. Supernatants were removed and used for determination of the TNF-alpha concentration.

Conidia induced a secretion of 137 ± 26 pg/ml of TNF-alpha 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 ERK-1/2, PD 98059 and UO126 used at the concentrations of 2.5, 5 and 10 μM, led to a dramatic decrease of TNF-alpha secretion between 46% and 86%, depending on the inhibitor and on the dose used. SB203580, the inhibitor of p38-MAPK used at the concentrations of 2.5 and 5 10 μM results in a minor inhibition of the expression of TNF-alpha**.** However this inhibitor did not induce any dose dependent inhibition. The effect of these two inhibitors on the capacity of killing of conidia by MH-S cells was also investigated. PD98059 added at the concentration of 5 μM, resulted in a percentage of killing of $53 \pm 4\%$ compared to $82.5 \pm 8\%$ for the

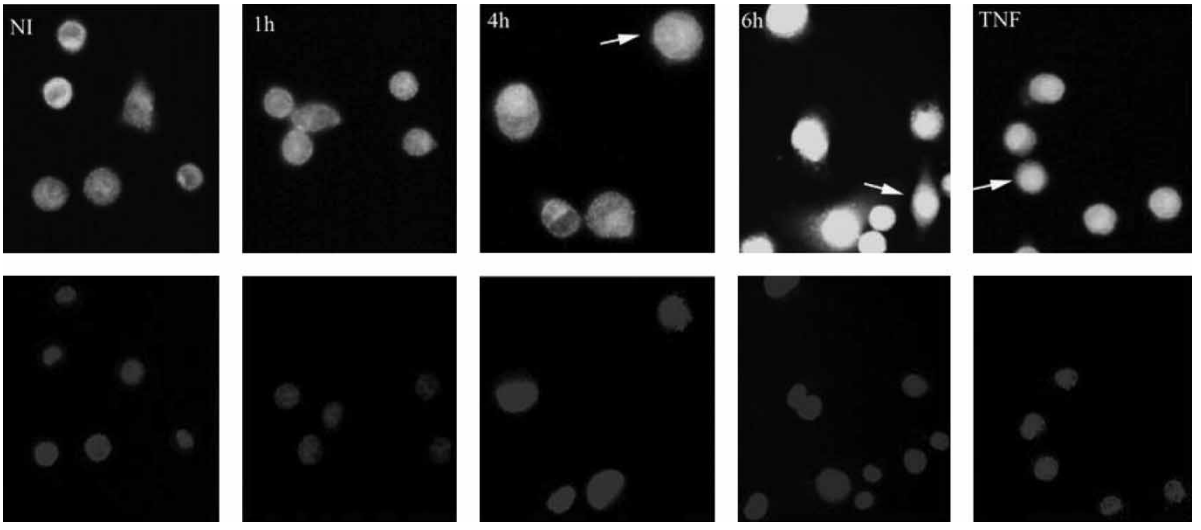

Fig. 3 Translocation of NF-kappaB in MH-S cells after stimulation with the conidia of *Aspergillus fumigatus*. MH-S cells were challenged or not (NI for none infected) with the conidia at the ratio of 10 conidia per macrophage for different times (1, 4, and 6 h) or with TNF-alpha for 1 h as positive control. NF-kappaB translocation was visualized using a Rel-A specific polyclonal antibody. The upper lane shows the Rel-A staining and the lower lane the nuclear staining. Stained cells were observed using conventional fluorescence microscopy. Arrows indicate nuclei with prominent concentrations of translocated Rel-A.

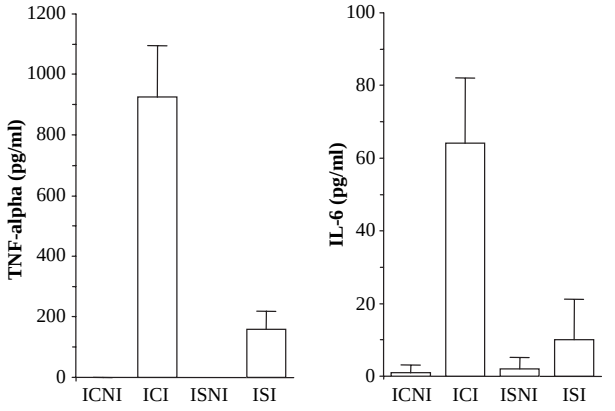

Fig. 4 Release of TNF-alpha and IL-6 from broncho alveolar lavage of mice infected intranasally with *Aspergillus. fumigatus* conidia. *In vivo* release of TNF-alpha and IL-6 was measured in the broncho-alveolar lavage of immunocompetent (IC) or immunosuppressed (IS) 24 h after infection. Infected (I) were compared to non infected mice (NI). Data are expressed as the mean $\pm$SEM of three independent experiments done with 3 mice for each point.

control cells without inhibitor. Although SB203580 negatively affects TNF-alpha production, the percentage of killing obtained in presence of this inhibitor was none significantly reduced.

We also conducted *in vivo* experiments to measure the production of TNF-alpha, IL-6 and MCP-1 in the broncho alveolar lavages of mice challenged with conidia of *A. fumigatus* for 24 h. These BAL fluids contained substantially more TNF-alpha (925 ± 171 vs. 0 ± 0 pg/ml), IL-6 (64 ± 18 vs. 0 ± 0 pg/ml), MCP-1 (51 ± 1 vs. 0 ± 0 pg/ml), than samples from non-infected control mice. Comparison of TNF-alpha and IL-6 recovered from BAL fluids of immunocompetent and immunosuppressed mice which were obtained after stimulation with *A. fumigatus* revealed that the concentrations of these cytokines were substantially lower in the immunosuppressed situation (Fig. 4).

Discussion

Aspergillus fumigatus is a human pathogen, which is able to cause invasive aspergillosis in immunosuppressed patients. In the immunocompetent situation, 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 which 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 tumor necrosis factor alpha (TNF-alpha) and interleukins 1α and 6 (IL1-α and IL-6). One of the major cytokines involved in immune defence against *A. fumigatus* is TNF-alpha. A high level of TNF-alpha synthesis is directly accompanied by an increased resistance against *A. fumigatus* infection [9]. We investigated the involvement of different MAP kinases on the response towards *A. fumigatus* conidia, because MAP kinases are directly involved in TNF-alpha production [10,11]. However, their exact functions in the response to fungal infection are poorly studied [12]. Even less is known about the involvement of MAP kinases in signalling and killing of conidia of *A. fumigatus* by alveolar macrophages.

Through the use of phospho-specific antibodies we found an activation of ERK and p38 after *in vitro* stimulation with conidia. However, *in vivo* activation of p38 was only observed when 2×10^8 conidia were applied whereas a strong response of ERK was observed by the use of at least ten times less conidia. This implied that p38 might only be of minor importance in recognition and defence against *A. fumigatus*.

MAP kinases and NF-kappaB are known to be involved in the control of cytokine gene expression including TNF-alpha, IL-1α and IL-6. In the immune response towards *A. fumigatus*, TNF-alpha has been shown to play a critical role in innate immunity in both immunocompromised and immunocompetent hosts. Pre-treatment with TNF-alpha agonist peptides have been demonstrated to significantly enhance resistance to *A. fumigatus* in neutropenic animals [9]. Therefore, the reduction in the level of cytokines released by macrophages after treatment with corticosteroids leads to insufficient clearance of conidia [13]. In our study, conidia induced a significant increase in TNF alpha production by alveolar macrophages after 6 h stimulation *in vitro*. When mice were challenged *in vivo* for 24 h, in addition to TNF alpha production, IL-6 as well as MCP-1 increased substantially.

Investigations with MAP kinases inhibitors were performed to determine the relation between production of TNF-alpha and phosphorylation of ERK and p38. The use of SB203580, the classically known inhibitor of p38, only had a non dose dependent effect on TNF-alpha production. Additionally, we were not able to observe any significant variation in the killing of conidia under *in vitro* conditions by use of SB203580, which underlines the minor role of p38. In contrast to p38, we revealed that after application of conidia, blocking of the ERK pathway inhibited TNF-alpha synthesis in a dose-dependent manner. In addition, treatment with PD98059 resulted in a reduction of 30% in killing of conidia compared to untreated control cells. This manifests an important

role of the ERK pathway in the defence against conidia.

Besides the role of MAP kinases in the production of cytokines, we were interested in the activation of NF-kappaB, since this transcription factor controls the expression of many cytokines [11]. In this study, we have shown that resting conidia do not induce the translocation of NF-kappaB at early time points. However, we observed a translocation of NF-kappaB after 6 h incubation of conidia with macrophages. From these data it can be assumed that NF-kappaB activation may be associated with the intracellular swelling of conidia, which is the first step of conidial germination [4]. Swelling of conidia significantly increases their size within the phagolysosome, which might be a trigger for NF-kappaB translocation. In addition, swelling of conidia is an absolute requirement for production of reactive oxygen intermediates that are responsible for conidial killing [4]. On the other hand, it was demonstrated by Meier *et al*. [14] that not only living conidia, but also killed conidia and dead hyphae are able to induce NF-kappaB translocation. Therefore, in our system, the late activation of NF-kappaB may also be due to soluble factors produced by the macrophages themselves such as cytokines, chemokines or lipid derivatives, which are involved in the inflammatory response.

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 [15]. Glucocorticoids have been shown to inhibit members of the MAP kinases family and p38 by sustaining the expression of MKP-1 [16]. When broncho alveolar macrophages are treated with dexamethasone and stimulated with living conidia of *A. fumigatus*, production of IL1-alpha and TNF-alpha is much less pronounced [17]. In our *in vivo* model, immunosuppression resulted in a significant decrease in the phosphorylation of pERK and, as a consequence, a strong decrease in TNF-alpha and IL-6 production.

We have demonstrated that the MAP kinase pERK, plays an essential role in the inflammatory host response against *A. fumigatus*. Although widely studied, the question concerning the implication of one or different types of pattern recognition receptors or other receptors specific for cell wall carbohydrates in the recognition of *A. fumigatus* by the macrophage is not completely addressed.

References

1 Denning DW. Epidemiology and pathogenesis of systemic fungal infections in the immunocompromised host. *J Antimicrob Chemother* 1991; **28**(Suppl. B): 1–16.

2 Latge JP. *Aspergillus fumigatus* and aspergillosis. *Clin Microbiol Rev* 1999; **12**: 310–350.

3 Ibrahim-Granet O, Philippe B, Boleti H, *et al*. Phagocytosis and intracellular fate of *Aspergillus fumigatus* conidia in alveolar macrophages. *Infect Immun* 2003; **71**: 891–903.

4 Philippe B, Ibrahim-Granet O, Prevost MC, *et al*. Killing of *Aspergillus fumigatus* by alveolar macrophages is mediated by reactive oxidant intermediates. *Infect Immun* 2003; **71**: 3034–3042.

5 Rao KM. MAP kinase activation in macrophages. *J Leukoc Biol* 2001; **69**: 3–10.

6 Carter AB, Monick MM, Hunninghake GW. Both Erk and p38 kinases are necessary for cytokine gene transcription. *Am J Respir Cell Mol Biol* 1999; **20**: 751–758.

7 Monick MM, Carter AB, Gudmundsson G, *et al*. A phosphatidylcholine-specific phospholipase C regulates activation of p42/44 mitogen-activated protein kinases in lipopolysaccharide-stimulated human alveolar macrophages. *J Immunol* 1999; **162**: 3005–3012.

8 Dubourdeau MAR, Balloy V, Huerre M, Chignard M, *et al*. *Aspergillus fumigatus* induces innate immune responses in alveolar macrophages through the MAP kinases pathway independent of TLR2 and TLR4. *J Immunol* 2006.

9 Mehrad B, Strieter RM, Standiford TJ. Role of TNF-alpha in pulmonary host defense in murine invasive aspergillosis. *J Immunol* 1999; **162**: 1633–1640.

10 Saklatvala J. The p38 MAP kinase pathway as a therapeutic target in inflammatory disease. *Curr Opin Pharmacol* 2004; **4**: 372–377.

11 Yankun Li RFS, De Vries-Seimon T, Pin Mei Yao, *et al*. Free cholesterol-loaded macrophages are an abundant source of tumour necrosis factor-alpha and interleukin-6. *J Biol Chem* 2005; **280**: 21763–21772.

12 Ibata-Ombetta S, Jouault T, Trinel PA, *et al*. Role of extracellular signal-regulated protein kinase cascade in macrophage killing of *Candida albicans*. *J Leukoc Biol* 2001; **70**: 149–154.

13 Balloy V, Huerre M, Latge JP, Chignard M. Differences in patterns of infection and inflammation for corticosteroid treatment and chemotherapy in experimental invasive pulmonary aspergillosis. *Infect Immun* 2005; **73**: 494–503.

14 Meier A, Kirschning CJ, Nikolaus T, *et al*. Toll-like receptor (TLR) 2 and TLR4 are essential for Aspergillus-induced activation of murine macrophages. *Cell Microbiol* 2003; **5**: 561–570.

15 Munck A, Mendel DB, Smith LI, Orti E. Glucocorticoid receptors and actions. *Am Rev Respir Dis* 1990; **141**: S2–10.

16 Lasa M, Abraham SM, Boucheron C, Saklatvala J, Clark AR. Dexamethasone causes sustained expression of mitogen-activated protein kinase (MAPK) phosphatase 1 and phosphatase-mediated inhibition of MAPK p38. *Mol Cell Biol* 2002; **22**: 7802–7811.

17 Kamberi M, Brummer E, Stevens DA. Regulation of bronchoalveolar macrophage proinflammatory cytokine production by dexamethasone and granulocyte-macrophage colony-stimulating factor after stimulation by Aspergillus conidia or lipopolysaccharide. *Cytokine* 2002; **19**: 14–20.