Cytokine expression in human monocyte cell line infected with *Aspergillus flavus* is mediated by MAPK

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**ABSTRACT**

*Aspergillus flavus* is liable to mediate immune response in a wide variety of cells by inducing varied host pathogen interactions, being the most etiological agent in India. The culture filtrate and spores of fungal species are implicated in the onset of allergic responses finally leading to the initiation of proinflammatory reactions. Human monocytic U937 cells were exposed to varying concentrations of culture filtrate and spores as well, to establish their cytotoxic role and proinflammatory effect. The expression of a wide array of proinflammatory cytokines, particularly interleukin-8 (IL-8), was found to be induced in a dose-dependent manner upon stimulation of cells. The upregulation of cytokine IL-8 was further shown to be associated with the regulation of signalling pathways mediated via p38 and one of its prime downstream target molecules, activator protein-1 (AP-1) transcription factor. The p38 mitogen-activated protein kinase (MAPK) signalling pathway regulates the transcription factor AP-1 activity, the integration point of many signals that can differentially affect the expression and transcriptional activity of a cell. We observed activation of c-Jun, a critical component of the AP-1 complex, mediated by p38 MAPK upon stimulation with the culture filtrate. Thus, this study showed a crucial role of culture filtrate and spores of *A. flavus* in mediating the immune responses by the secretion of proinflammatory cytokines.

**Abbreviations** IL-8, Interleukin-8; TNF α, Tumor necrosis factor alpha; GM-CSF, Granulocyte macrophage colony stimulating factor

**Introduction**

*Aspergillus flavus*, a pervasive and inescapable pathogenic fungus, lies next to *A. fumigatus* in causing invasive as well as non-invasive pulmonary aspergillosis in immunocompetent persons (Denning 1998; Sharon et al. 2011). Generally, fungi are capable enough to endure in dynamic habitats by sensing their environs and retorting to cues in the particular habitat they live in through means of interaction with plants, animals or humans in multifaceted approaches, thereby establishing a range of host pathogen interactions. This involves particular reprogramming events which in turn facilitates them to become accustomed to the ecological conditions, struggle for nutrient acquirement and cope with the stress generated by host defence mechanisms (Romani 2004; Hube 2009). Quite a few biochemical mechanisms have been uncovered to complement the host cell attachment and infection process. Several fungi-associated factors possess an irresistible role in triggering out proinflammatory reactions by means of producing cytokines (Montone 2013). Up to date, clinical studies in a fraction of patient’s ordealing from fungal rhinosinusitis showed a prominent level in the production of proinflammatory cytokines (Kale et al. 2015). Even though it has been shown that conidia from *A. fumigatus* can induce interleukin-8 (IL-8) production (Balloy et al., 2008), the detailed mechanism of culture filtrate or spore-mediated host–*A. flavus* interactions barely exist. In this study, we investigated the culture filtrate and spore-mediated cytotoxicity and thereby focussed upon the host pathogen interactions brought about by the production of an array of cytokines. It is demonstrated that the proteases present in fungal extracts from both *A. fumigatus* and other non-aspergillus fungi interact with A549 cells, surpassing to morphological changes, cell desquamation and the induction of proinflammatory cytokines (Kauffman et al. 2000). In adenocarcinomic human alveolar basal epithelial cells, A549, the...
induction in the production of proinflammatory cytokines IL-6 and IL-8 followed by the activation of NF-κB is being reported upon treatment with the culture filtrate of A. fumigatus (Borger et al. 1999). Fungal secretome consists of many functional factors including cytokines, proteases and other molecules including secondary metabolites (Kauffman et al. 2000; Ghufran et al. 2016a), all of which are capable enough to induce multiple signalling pathways in order to initiate infection. There are reports affirming the destabilisation of cytoskeleton by proteases, the initiation of immunomodulatory and inflammatory signals by cytokines and the induction of carcinogenesis by toxins (Montone 2013; Kale et al. 2015; Ghufran et al. 2016b).

Mitogen-activated protein kinases (MAPKs), implicated in the expression of proinflammatory cytokines, get activated via dual phosphorylation of certain amino acid residues through multiple signal transduction pathways (Lee et al. 1994; Raingeaud et al. 1995). The cytokines are reported to be potent mediators in regulating immune and inflammatory responses during fungal infection. Recent advancements in the field of MAPK signalling following a fungal infection reveal the inevitable role of MAPKs in mediating the fungal virulence. The expression of a particular cytokine, IL-8, is barely detectable in the absence of an external stimulation; on the contrary, upon stimulation, its expression gets activated at both the transcriptional and post-transcriptional level. The transcription in such cases is reported to be enhanced by either the derepression of the gene promoter or the activation of NF-κB and MAPK pathways (Hoffmann et al. 2002). In the present study, we used the A. flavus strain isolated from rhinosinusitis patient, which triggers the inflammatory reactions through cytokine production. We focused upon divulging the expression pattern of cytokines, their mode of regulation and thereby the operable signalling mechanisms to exemplify the apparent role of an array of proinflammatory cytokines, especially, IL-8, in fungal pathogenesis.

Materials and methods

Chemicals and reagents

Acrylamide, N, N'-methylene bis acrylamide, Tris base (Trizma), TEMED (Tetramethylethylenediamine), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), SB203580, radioimmunoprecipitation assay (RIPA) buffer, protease inhibitor cocktail (PIC), were purchased from Sigma-Aldrich chemicals (St Louis, MO, USA). 12-O-tetradecanoylphorbol-13-acetate (TPA) was procured from Cell Signaling Technology, Inc., (Danvers, MA, USA). Roswell Park Memorial Institute (RPMI) medium, fetal bovine serum (FBS), antibiotic–antimycotic solution, phosphate-buffered saline (PBS), trypsin–EDTA solution, malt extract, Czapek yeast extract broth (CYB) and agar were obtained from Himedia Laboratories (Mumbai, India). CytoTox 96® Non-Radioactive Cytotoxicity Assay kit was purchased from Promega (Madison, WI, USA). Immobilon western chemiluminescent horseradish peroxidase (HRP) substrate and polyvinylidene fluoride (PVDF) membrane were procured from Merck-Millipore (Darmstadt, Germany). All other chemicals and reagents were of the analytical grade.

Antibodies

The primary antibodies against p38, phospho-p38, c-Jun, c-Fos, FosB, and the rabbit and mouse secondary antibodies were purchased from Sigma-Aldrich (St Louis, MO, USA). Primary antibodies against ERK1/2, phospho-ERK1/2, JNK1/2 and phospho-JNK1/2 were purchased from Invitrogen (Waltham, MA, USA). Mouse primary anti-β-actin antibody was procured from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Fungal culture

A. flavus (MTCC strain 8834) was used for the present study. This strain was originally isolated from a sinusitis patient, was obtained from the microbial type culture collection and gene bank, Institute of Microbial Technology (MTCC), Chandigarh, India. The culture conditions were maintained according to the supplier’s instructions in malt extract agar and Czapek yeast extract agar growth medium, for 5 days at 25°C, until the maturation of conidia.

Preparation of A. flavus culture filtrate

The spore suspension was prepared aseptically according to the method of Balloy et al. The spores were collected in a 0.1% Tween 20 solution, washed twice in PBS and 0.1% Tween 20 solution. The optical
density of the suspension was recorded at 600 nm, and 0.6 optical density corresponds to $2 \times 10^7$ spores/ml. The suspension was then diluted accordingly in order to get the desired concentration.

For preparation of culture filtrate/secretome, 200 ml of the CYB medium was inoculated with A. flavus spore suspension at a concentration of $1 \times 10^7$ spores/ml. Fungal cultures were grown at 25ºC in stationary condition for 5 days. The culture supernatant was collected after centrifugation at 12,000 rpm for 20 min to remove spores and debris using a Sorval ST 16 R centrifuge at 4ºC. The supernatant was then lyophilised and stored at $-20^\circ$C for further analysis. The protein concentration was determined by the method of Bradford (1976). Bovine serum albumin was used as reference standard.

**Cell culture**

Human monocyte cell line (U937) was procured from the National Centre for Cell Science, Pune, India. The cells were maintained in serial passage in RPMI medium supplemented with 10 mM HEPES, 2 mM L-glutamine, 10% heat-inactivated FBS and 1% antibiotic–antimycotic solution in 25 cm$^2$ culture flasks at 37ºC in a humidified incubator at 5% CO$_2$.

**Treatment conditions**

For treatment, U937 cells were incubated with different concentrations of both culture filtrate as well as spores for different time period and plated into 96-well plates, 24-well plates or 60 mm dishes as per the experiment. Since PBS was used for the solubilisation of culture filtrate, the same volume of PBS was added to the control for the assay purpose.

**Cell viability assay**

Cell viability assay was performed by measuring the reducing potential of the cells using a colorimetric MTT assay (Mosmann 1983). Briefly, U937 cells were incubated with varying dose of culture filtrate (200–1000 ng/ml) or spores ($0.5–10 \times 10^5$ spores/ml), in a 96-well plate at a density of $5 \times 10^5$ cells/ml for 6 and 12 h. Cells treated with PBS were used as control. After incubation periods, MTT reagents (0.5 mg/ml) were added to each well following 3 h incubation in dark at 37ºC. After washing the cells three times in PBS, finally formazan crystals were dissolved in dimethyl sulfoxide, and the optical density was recorded at 570 nm (background wavelength 630 nm) using Enspire* Multimode plate reader from Perkin Elmer Inc. (Waltham, MA, USA).

**Cell cytotoxicity assay**

Cell cytotoxicity was performed by measuring the cell membrane integrity using lactate dehydrogenase (LDH) leakage assay, a soluble cytosolic enzyme released into the culture medium as a marker of dead cells. Briefly, U937 cells were incubated with varying dose of culture filtrate (200–1000 ng/ml) in a 24-well plate at a density of $5 \times 10^5$ cells/ml for a period of 6 and 12 h. Cells treated with 100 µl of Triton X-100 (1% v/v final concentration) or PBS were used as positive and negative control, respectively. Cells without exposure to culture filtrate were used as control. After incubation, the supernatant was transferred in a microfuge tubes and then centrifuged at 12,000 rpm at 4ºC for 5 min. LDH assay was performed using CytoTox 96® Non-Radioactive Cytotoxicity Assay kit from Promega (Cat No. G1780). Next, 50 µl of cell-free supernatant was incubated with 50 µl of LDH substrate, and the plate was kept for 30 min incubation at room temperature in the dark, followed by addition of 50 µl of stop solution to each well. The optical density was recorded at 490 nm using Enspire* Multimode plate reader from Perkin Elmer.

**Total RNA isolation and quantitative real-time polymerase chain reaction**

The U937 cells were incubated with different doses of A. flavus culture filtrate or spores in serum-free culture media for 12 h. Following incubation the cells were homogenised and the total RNA was isolated using TRIzol reagent (Ambion Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer’s instructions. For the synthesis of cDNA, reverse-transcription reactions were performed using cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s instructions. The LightCycler 480 system (Roche Life Science, Basel, Switzerland) was used to measure mRNA expression level. The primer sequences (Table 1) used for the gene expression analysis include the interleukin genes IL-1α, IL-1β, IL-6 and IL-8; granulocyte-macrophage colony stimulating (GM-CSF), tumour necrosis factor (TNFa)
and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The $\Delta\text{Ct}$ value for IL-8 gene was determined relative to the endogenous control GAPDH. The fold change data for the treated group were calculated from the $\Delta \Delta\text{Ct}$ values standardised against GAPDH abundance in the control group. All primer efficiencies were higher than 95%, and specificity was confirmed by sequence blast and melting curve analysis. All quantitative reactions were conducted in triplicates.

**Western blot analysis**

The U937 cells were treated with two different doses 250 and 500 ng/ml of *A. flavus* culture filtrate as explained earlier. After washing with PBS, cells were lysed with RIPA buffer containing 1 mM PMSF, 1 mM DTT and PIC on ice. The cellular lysates were cleared by centrifugation at 10,000 rpm for 10 min at 4ºC. Equal amounts of proteins were resolved in a 10% SDS-PAGE according to the method of Laemmli (Laemmli 1970) and transferred onto PVDF membranes for immunoblot analysis using diluted (1:5000) primary antibodies against a target protein. HRP-coupled secondary antirabbit antibodies (1:10,000) were used for recognition of primary antibody bound to antigen. The chemiluminescence was detected with the HRP substrate and measured directly by a Bio-Rad Versadoc Imaging System. Signal intensities were analysed using Image Lab 5.1 software (Bio-Rad). Beta-actin was used as a loading control.

**Enzyme linked immunosorbent assay (ELISA)**

Interleukin-8 concentration in cell culture supernatant was determined by sandwich ELISA using human IL-8 ELISA kit from Elabscience, Beijing (E-EL-H0048), following manufacturers protocol. The cells were treated with *A. flavus* culture filtrate (250 and 500 ng/ml) or spores (1 \times 10^5, and 2 \times 10^5 spores/ml) in 24-well plates for a period of 8 and 16 h. Cells treated with 100 nM TPA were used as positive control. The supernatants were collected at each time interval and concentration of IL-8 was measured. Untreated cells were taken as negative control.

**Inhibition study**

For inhibition study, U937 cells were pre-incubated with specific p38 MAPK inhibitor (SB203580) at concentrations of 20 μM for 1 h and then stimulated with 500 ng/ml of culture filtrate following 24 h of incubation. Cells without stimulation were used as control. The concentration of IL-8 was measured as explained earlier.

**Statistical analysis**

Each experiment was performed at three times with triplicates, and all the data were represented as mean ± SE of triplicate (standard error), and representative result was presented. The statistical significance of differences among the groups or one-way analysis of variance (ANOVA) was assessed by Student’s t-test with a threshold of $p < 0.05$ and considered as statistically significant. ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; n.s. indicates no statistical significance for all the figures.

**Results**

**Effect of culture filtrate and spores on cell viability**

The viability of U937 cells was subdued when incubated with different concentrations of the culture filtrate (200–1000 ng/ml) or spores (0.5–10 \times 10^5 spores/ml) of *A. flavus*. The cytotoxic effect was measured by MTT assay, which showed a significant inhibition of cell viability in treated cells in a dose-dependent manner (Figure 1(a) and 1(b)) when compared with the untreated cells. The $IC_{50}$ value in case of culture filtrate and spores was found to be 530 ng/ml and 3.37 \times 10^5 spores/ml.
Figure 1. Cell viability and cytotoxicity profile of human monocytic U937 cell line. For viability assay cells were treated with varying concentrations of (a) culture filtrate (b) spores of *A. flavus* for 24 h. Cell viability, as determined by MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cells treated with PBS were used as control and considered as 100% viable. (c) Lactate dehydrogenase assay, as performed using CytoTox 96® Non-Radioactive Cytotoxicity Assay kit. The data are presented as mean ± SD of three individual experiments that gave similar results. *p < 0.05, **p < 0.01, ***p < 0.001, and “n.s.” no statistical significance versus control.
**LDH assay**

LDH, a soluble cytosolic enzyme, present in most eukaryotic cells, can be used as marker of cell death due to damage in plasma membrane thereby distressing the cell permeability. In order to verify whether *A. flavus* has any cytotoxic effect on the cells, we measured the release of the cytosolic enzyme LDH into the supernatant after 6 and 12 h of stimulation with varying concentrations of the culture filtrate in U937 cells. There was a marked cytotoxic effect in treated cells compared to the untreated ones (Figure 1(c)).

**Induction of IL-8 production upon exposure to the culture filtrates/spores of *A. flavus***

A rapid and instinctive defence response is carried out upon exposure of cells with different stimuli (bacteria, virus or fungus) and results in the release of cytokines which in turn attract inflammatory effector cells. The succession in the cytokine production after the activation of epithelium is of meticulous interest in allergic pathologies (Mills et al. 1999). Disclosure of monocytic cells (THP-1) to the culture filtrate is reported to play a role in eliciting the production of an array of cytokines (Pei & Gunsch 2013).

Moreover, to completely analyse the mode of infection pattern, we treated the human U937 cells with varying concentrations of the filtrate and spores as well and monitored the mRNA expression of an array of cytokines out of which GM-CSF, IL-6, IL-1α, TNF-α, IL-1β and IL-8 showed a pertinent transformation in their expression level at 12 h after the treatment (Figure 2(a)–(f)). Since the variance in the expression of cytokine IL-8 was discernible, we went on with our experiments focussing on the cytokine IL-8. The secreted IL-8 protein concentration was estimated thereafter by ELISA. After incubating cells with 250 and 500 ng/ml of culture filtrate and 1–2 × 10⁵ spores/ml, the samples were collected at different time intervals (8 h and 16 h) and thereby assessed for IL-8 levels. A time-dependent increase in IL-8 secretion was observed, and it reached a significant level after 16 h of treatment compared with unstimulated control (Figure 3(a) and 3(b)). By now, it is reported that fungal hyphae and proteases can activate cells to produce the cytokine IL-8 (Balloy et al., 2008). Keeping this in mind, we moved on to appraise whether proteases do have a role in the induction of IL-8 production here in this context. For that, we performed further experiments by treating the cells synchronously with PIC along with the culture filtrate and spores. The results show that there was not much significant deviation in the secretion of IL-8 in presence of PIC. This apparently suggests the compelling role of culture filtrate and spores in the secretion of proinflammatory cytokine IL-8 (Figure 3(c)). We also analysed the IL-8 expression by ELISA in the presence of p38 inhibitor; our results show that 20 µM p38 inhibitor blocks the IL-8 secretion (Figure 3(d)).

**Culture filtrate-induced activation of p38 MAPK and AP-1 transcription factor**

The transcription of IL-8 gene is allied with three dominant MAPK pathways, specifically, the extracellular regulated protein kinase (ERK), Jun N-terminal kinases (JNK) and p38 MAPK (Hoffmann et al. 2002). In order to conclude the role of these MAPKs in the context of our study, we first examined the phosphorylation of JNK1/2, ERK1/2 and p38 MAPK upon treatment of U937 cells with two different concentrations of the culture filtrate (250 and 500 ng/ml). Strikingly, we observed a remarkable increase in the level of p38, as shown in Figure 4(a). The phosphorylation of ERK1/2 and JNK1/2 did not show much difference as compared to p38. The p38-specific inhibitor SB203580 (data not shown) further confirms the role of p38 activation. The activation of p38 thereby resulted in the stimulation of downstream transcription factors, specifically, the activator protein-1 (AP-1) transcription factor, one of the leading downstream target molecules of p38. Consequently, for that reason, we analysed the expression level of AP-1 factors and observed a noteworthy increase in the expression of transcription factors c-Jun, c-Fos and Fos-B (Figure 4(b)).

**Discussion**

From the last few decenniums, an active field of research was intent to figure out the effect of fungal pathogens on cells paving a way to numerous host pathogen interactions which finally results in the secretion of cytokines and thus activates major signalling pathways. A wide variety of fungal species
have been reported to pose severe health adversities clearly indicating the subsistence of special elements causing the proinflammatory effects. Fungal spores are reported to secrete allergic proteins, a source of immunogenic molecules, which further results in the progress, commencement and worsening of allergic responses (Hogaboam et al. 2005; Babiceanu et al. 2013). Keeping our focus on A. flavus, the spores of this species have been reported to be able enough to get incorporated, withstand and germinate inside the epithelial cells. Not only this, the spores can also be consumed by the resident and inducted effector cells like macrophages (Han et al. 2011; Babiceanu et al. 2013). In this manner, it is evident that spores do have an important role in the activation of immunogenic responses in cells. In the present study, we have found the effect of culture filtrate and spores of A. flavus in U937 cells. An apparent alteration in the cell viability is observed upon treatment of cells with the culture filtrate or spores of A. flavus.

Figure 2. Cytokine expression profile upon treatment with culture filtrate and spores of A. flavus. Cells were treated with indicated concentrations of culture filtrate and spores for different time period. Total RNA was isolated and cDNA was synthesised as mentioned; mRNA expression level was determined by Q-PCR method. Time-dependent expression of (a) GM-CSF, (b) IL-6, (c) IL-1α, (d) TNF-α, (e) IL-1β, (f) IL-8 mRNA in U937 cells. Cells without treatment were used as control. The data are presented as mean ± SD of three individual experiments that gave similar results. *p < 0.05, **p < 0.01, ***p < 0.001, and n.s. no statistical significance versus control.
The results obtained in the present study show that both the culture filtrate and the spores of *A. flavus* are cytotoxic to U937 cells and play a major role in activating the inflammatory responses by secreting proinflammatory cytokines, particularly, the cytokine IL-8. Our study have provided for the first time an array of genes differentially expressed in human monocyte cell line U937 at the time of exposure to fungal culture filtrate and spores. The upregulation of different cytokines was clearly confirmed by monitoring the gene expression of an array of proinflammatory cytokines implicated in host pathogen interactions. Generally, our results substantially indicate the stimulation of many genes encoding cytokines brought into being correlated with the innate immune response. Besides the gene expression studies, an analytic biochemistry...
assay, ELISA, also affirmed the upsurge in IL-8 secretion in treated cells compared to the unstimulated control. Considering the fact that cytokines play a major role in the recruitment of effector cells involved in host-pathogen interactions, our data set appears to indicate that the cytokine IL-8 could be a vital moderator of inflammatory responses upon stimulation with culture filtrate/spores of *A. flavus* in monocyte U937 cell line.

One of the intriguing observations was the validation of the fact that the cytotoxic effect and further inflammatory responses are provoked only by the culture filtrate and spores and not by the proteases (Balloy *et al.*, 2008). Results evidently show the irresistible role of culture filtrate in inducing inflammatory responses, since, the treatment of cells with the culture filtrate in presence of PIC did not inhibit the level of IL-8 secretion effectively. Using pharmacological inhibitors, we observed that p38 inhibitor significantly blocked the IL-8 secretion, suggesting the involvement of p38-mediated pathway in culture filtrate-induced cytokine release.

For the better understanding of mechanisms occurring behind the secretion of the proinflammatory cytokine IL-8 upon exposure to the culture filtrate, we tested the activation of protein kinases like p38, JNK and ERK1/2 along with the involvement of transcription factors. As yet, the intentness of fungal MAPKs has been portrayed broadly, though, the counterpart regarding the MAPK intervened feedback in fungal infected cells has earned meagre consideration, in contempt of their importance (Román *et al.*, 2007; Osherov 2015). MAPKs are activated by extensive series of external stimuli, for example, by the activation of p38 and JNK next to UV response. A stress-activated signal transduction pathway and ERK1/2 stimulation regulate the action of p38 and JNK partly. Activation of MAPK and induction of cytokine IL-8 in different cells have been extensively reported (Kauffman *et al.*, 2000; Hoffmann *et al.*, 2002; Li *et al.*, 2002). One of such report is based on the secretion of IL-8 in response to the germinating spores of *A. fumigatus*, via activation of phosphatidylinositol 3 kinase, p38 and ERK1/2 (Balloy *et al.*, 2008).

In the current context of our work, culture filtrate of *A. flavus* stimulated human monocyte U937 cells resulted in the activation of p38 pathway (Figure 5). The expression of transcription factors like c-Jun is regulated by a surfeit of environmental and physiological stimuli; yet, its regulation by MAPK cascade is also reported in a study (Osherov 2015). Upon activation, c-Jun associates

![Figure 5](image.png)
with other AP-1 factor/s which can bind to specific sequence of DNA and thereby regulates gene transcription. Numerous reports have depicted the synchronised action of diverse signal transduction pathways and transcription factors in the induction of IL-8 production (Balloy et al., 2008; Harant et al. 1996; Hoffmann et al. 2002; Li et al. 2002). Maximum induction of IL-8 secretion is brought into action by the conjoint act of both AP-1 factors and NFkB. Role of AP-1 factor in the transcription of IL-8 is implicated by performing mutation studies at the binding site of AP-1/NFkB in IL-8 promoter (Balloy et al., 2008).

To be concise, the present study illustrates the pro-inflammatory effect of culture filtrate and spores likely to play a role in A. flavus infection (Figure 5). The p38 MAPK activation in U937 cells in response to the culture filtrate has extended to induce the proinflammatory signalling, helping in antifungal innate immune response. Since IL-8 synthesis is triggered by the activation of combination of different kinases (Balloy et al., 2008; Harant et al. 1996; Li et al. 2002), it is difficult to establish exactly the way how signalling is activated here. The production of cytokine IL-8 in lungs and human corneal epithelial cells is initiated by the stimulation of receptors expressed in human lungs, epithelia of airway and alveoli (Heyl et al. 2014; Peng et al. 2015). So we proposed that the response initiated, following a fungal infection, paves a way to the production of cytokine IL-8. However, further aspects need to be revealed to figure out the involvement of the culture filtrate and spores in infection and pathogenesis of A. flavus, and thus understanding the molecular mechanism of signalling pathways activated during infection could help in the advancement of new curative schemes.

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Conflict of interest

All authors declare that there is no conflict of interest.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Babiceanu MC, Howard BA, Rumore AC, Kita H, Lawrence CB. 2013. Analysis of global gene expression changes in human bronchial epithelial cells exposed to spores of the allergenic fungus, Alternaria alternata. Front Microbiol. 4:196.

Balloy V, Sallenave JM, Wu Y, Touqui L. 2008. Aspergillus fumigatus-induced interleukin-8 synthesis by respiratory epithelial cells is controlled by the phosphatidylinositol 3-kinase, p38 MAPK, and ERK1/2 pathways and not by the toll-like receptor-MyD88 pathway. J Biol Chem. 283:30513–30521.

Borger P, Koëter GH, Timmerman JA, Vellenga E. 1999. Proteases from Aspergillus fumigatus induce interleukin (IL)-6 and IL-8 production in airway epithelial cell lines by transcriptional mechanisms. J Infect Dis. 180:1267–1274.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72:248–254.

Denning DW. 1998. Invasive aspergillosis. Clin Infect Dis. 26:781–803.

Ghufran MS, Ghosh K, Kanade SR. 2016b. Aflatoxin B1-induced upregulation of protein arginine methyltransferase 5 in human cell lines. Toxicon. 119:117–121.

Ghufran MS, Ghosh K, Kanade SR. 2016a. A fucose specific lectin from Aspergillus flavus induced interleukin-8 expression is mediated by mitogen activated protein kinase p38. Med Mycol. 10.1093/mmy/myw066.

Han X, Yu R, Zhen D, Tao S. 2011. β-1,3-Glucan-induced host phospholipase D activation is involved in Aspergillus fumigatus internalization into type II human pneumocyte A549 cells. PLoS One. 6:e21468.

Harant H, De Martin R, Andrew PJ, Foglar E. 1996. Synergistic activation of interleukin-8 gene transcription by all-trans-retinoic acid and tumor necrosis factor-alpha involves the transcription factor NF-kappa B. J Biol Chem. 271:26954–26961.

Heyl KA, Klassert TE, Heinrich A, Muller MM. 2014. Dectin-1 expressed in human lung and mediates the proinflammatory immune response to nontypeable Haemophilus influenzae. mBio. 5:e01492–14.

Hoffmann E, Dittrich-Breiholz O, Holtmann H, Kracht M. 2002. Multiple control of interleukin-8 gene expression. J Leukoc Biol. 72:847–855.
Hogaboam CM, Carpenter KJ, Schuh JM, Buckland F. 2005. Aspergillus and asthma—any link? Med Mycol. 43:S197–202.
Hube B. 2009. Fungal adaptation to the host environment. Curr Opin Microbiol. 12:347–349.
Kale P, Rudramurthy SM, Panda NK, Das A. 2015. The inflammatory response of eosinophil-related fungal rhinosinusitis varies with inciting fungi. Med Mycol. 53:387–395.
Kauffman HF, Tomee JF, Van De Riet MA, Timmerman AJ. 2000. Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production. J Allergy Clin Immunol Pract. 105:1185–1193.
Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:680–685.
Lee JC, Laydon JT, McDonnell PC, Gallagher TF. 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature. 372:739–746.
Li J, Kartha S, lasvovskaia S, Tan A. 2002. Regulation of human airway epithelial cell IL-8 expression by MAP kinases. Am J Physiol Lung Cell Mol Physiol. 283:L690–L699.
Mills PR, Davies RJ, Devalia JL. 1999. Airway epithelial cells, cytokines, and pollutants. Am J Respir Crit Care Med. 160: S38–43.
Montone KT. 2013. Role of fungi in the pathophysiology of chronic rhinosinusitis: an update. Curr Allergy Asthma Rep. 13:224–228.
Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 65:55–63.
Osherov N. 2015. Modulation of host-cell MAPkinase signaling during fungal infection. MAP Kinase. 4:5389.
Pei R, Gunsch CK. 2013. Inflammatory cytokine gene expression in THP-1 cells exposed to Stachybotrys chartarum and Aspergillus versicolor. Environ Toxicol. 28:51–60.
Peng XD, Zhao GQ, Lin J, Jiang N. 2015. Fungus induces the release of IL-8 in human corneal epithelial cells, via dectin-1 mediated protein kinase C pathways. Int J Ophthalmol. 8:441–447.
Raingeaud J, Gupta S, Rogers JS, Dickens M. 1995. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem. 270:7420–7426.
Román E, Arana DM, Nombela C, Alonso-Monge. R. 2007. MAP kinase pathways as regulators of fungal virulence. Trends Microbiol. 15:181–190.
Romani L. 2004. Immunity to fungal infections. Nat Rev Immunol. 4:1–23.
Sharon H, Amar D, Levdansky E, et al. 2011. PrtT-Regulated proteins secreted by Aspergillus fumigatus activate MAPK signaling in exposed A549 lung cells leading to necrotic cell death. PLoS One. 6:e17509.