Characterization and pro-inflammatory responses of spore and hyphae samples from various mold species

Running title: Mold characterization and inflammatory responses

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Abstract

Mold particles from *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Aspergillus versicolor* and *Stachybotrys chartarum* have been linked to respiratory-related diseases. Here we characterized X-ray-inactivated spores and hyphae fragments from these species by number of particles, morphology, and mycotoxin, β-glucan and protease content/activity. The pro-inflammatory properties of mold particles were examined in human bronchial epithelial cells (BEAS-2B) and THP-1 monocytes and phorbol 12-myristate 13-acetate (PMA)-differentiated

THP-1. Spores from *P. chrysogenum* and *S. chartarum* contained some hyphae fragments, whereas the other preparations contained either spores or hyphae. Each mold species produced mainly one gelatin degrading protease that was either of the metallo- or serine type, while one remains unclassified. Mycotoxin levels were generally low. Detectable levels of β-glucans were found mainly in hyphae particle preparations. PMA-differentiated THP-1 macrophages were by far the most sensitive model with effects in the order of 10 ng/cm^2. Hyphae preparations of *A. fumigatus* and *P. chrysogenum* were more potent than respective spore preparations, whereas the opposite seems to be true for *A. versicolor* and *S. chartarum*. Hyphae fragments of *A. fumigatus*, *P. chrysogenum* and *A. versicolor* enhanced the release of metalloprotease (pro-MMP-9) most markedly. In conclusion, species, growth stage and characteristics are all important factors for pro-inflammatory potential.

Key words: Mold/Mould particles, cytokines, proteases, mycotoxins, β-glucans, morphology.

Practical implications:

Pro-inflammatory effects of mold particles were detected at very low doses/concentrations supporting the notion that mold exposure could be linked to health effects. There were large differences in the pro-inflammatory potential between spores and hyphae fragments, and between various species. There were, however, no direct links between the observed pro-inflammatory response and sample characteristics including number, size and shape of particles, mycotoxins, β-glucan and protease content/activity. The results from the present study suggest that more direct analyzes of pro-inflammatory responses of samples from indoor environments should be performed as this may give valuable information to epidemiological studies exploring adverse health effects of indoor dampness/mold.

Background

There is sufficient evidence from epidemiological studies of associations between indoor dampness/mold and adverse health effects including respiratory symptoms, respiratory infections and exacerbation of asthma [1, 2]. Several kinds of indoor air pollution agents may contribute. Mold has been suggested to be particularly important, since it may not only cause infection and toxic effects, but also trigger allergic and non-allergic inflammatory reactions that may be linked to various respiratory-related diseases [3-5]. Quantitative guidelines (thresholds) for acceptable levels of indoor contamination of microorganisms/mold have not been suggested [1]. However, for the work environment where exposure levels can be much higher, a proposal has been made [6].

Species that commonly occur in moist indoor environments include *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Aspergillus versicolor* and *Stachybotrys chartarum*. The reproductive spores may disperse into the air. When they deposit they may germinate into multicellular filamentous structures called hyphae, which can grow further into tangled mass of networks known as mycelia. Spores from many species, but not all are easily aerosolized. Spores from some species e.g. *S. chartarum* are produced in slimy aggregates which are dispersed by water and may become airborne after secondary dispersion [6]. Furthermore, experimental studies have demonstrated that not only spores but also hyphae fragments can be liberated from fungal cultures [7-9]. Recently, an immune-microscopic method for their detection has been described [10].

In addition to direct microscopic quantification [9], components like ergosterol [11], polysaccharides such as β(1→3)-glucans [12] and enzymes such as proteases have been used as measurement units for total fungal exposure. Under certain growth conditions the fungi may

synthesize and excrete mycotoxins which are active secondary metabolites that are reported to induce toxic and inflammatory effects in experimental studies [13, 14]. The cell wall of both spores and hyphae consists of a matrix containing e.g. β-glucans, glycoproteins, and lipids, reinforced by chitin fibrils, but the 3-dimensional structure varies between species and between spores and hyphae [6]. Spores are considered to be largely inert towards recognition by the immune system, partly due to the rodlet layer composed of the hydrophobic RodA protein on the surface of resting spores. However, swelling and germination of spores leads to a loss of this protective hydrophobin layer, resulting in an increased exposure of immunological surface components [15, 16]. Beta-1,3-glucans are constituents of the fungal cell wall, and contain molecular structures called pathogen-associated patterns (PAMPs). PAMPs are recognized by pattern recognition receptors (PRRs) like Toll-like receptors (TLRs), and may thereby induce and/or modify inflammatory responses to antigens/allergens [17].

Furthermore, molds may secrete various proteases that have been linked to different health effects such as chronic inflammation and tissue damage [18, 19]. Most of the mold proteases belong to either the cysteine (C), serine (S) or metallo (M) class of proteases [20]. Matrix metalloproteinases (MMPs) are produced by many cell types, in particular by activated macrophages [21, 22]. They have broad substrate specificity and can process almost all extracellular matrix proteins as well as non-matrix proteins including cytokines, chemokines, growth factors and cell receptors.

Epithelial cells and alveolar macrophages constitute the first line of defense against inhaled molds [23, 24]. In addition, monocytes may migrate to the infected site in the alveoli during inflammation [25]. The molds are recognized by cellular membrane receptors, including toll-like receptors (TLR), protease-activated receptors (PAR) or C-type lectin receptors (CLR) [26].

In general, TLR2 and TLR4 are considered to be key recognition components for host innate defense system against fungi [27]. TLR5 has been reported to be involved in the immunological response to *A. fumigatus* in THP-1 monocytes [28], and TLR9 in response to *S. chartarum* [29]. Protease activated receptors (PAR)-2 is a G-protein coupled receptor that can be activated by proteases produced by molds [30]. Furthermore, various recognition receptors are found on different cell types and they differentially recognize molds in various growth stages [31-35].

As a response to the activation of different receptors, cells secrete signaling molecules, such as cytokines/chemokines which play a central role in orchestrating the immune response. Pro-inflammatory cytokines (interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor; TNF-α) act on surrounding cells, regulate adhesion molecules, other cytokines and recruit immune cells to the site of infection [36].

A number of studies have characterized biological effects of spores or germinating spores of *A. fumigatus* [31, 33, 37-39]. However, to our knowledge no other studies have characterized aerosolized spores and hyphae fragments from different fungal species, and tried to link this subsequently to pro-inflammatory responses in human cells.

In the present study spores collected by air- (aerosolized-) and liquid flow (washed spores) and hyphae fragments from *A. fumigatus, P. chrysogenum, A. versicolor,* and *S. chartarum* were characterized with regard to number, size and shape of particles, occurrence of mycotoxins, β-1,3-glucans and proteases. Finally, their pro-inflammatory potentials were examined using human bronchial epithelial cells (BEAS-2B) and THP-1 monocytes and PMA-differentiated THP-1 macrophages as representative lung cell models.

Materials and Methods

Fungal samples:

Three types of fungal samples from four pure fungal isolates were prepared, characterized and used in the present study. Isolates comprised *Aspergillus fumigatus* Fresenius 1863 (strain A1258 FGSC) purchased from the Fungal Genetics Stock Center (University of Missouri, Kansas City, KS), *Aspergillus versicolor* Tirobaschi 1908 (strain VI 03554), *Penicillium chrysogenum* Thom 1910 (strain VI 04528) and *Stachybotrys chartarum* (Ehrenb) S. Hughes (VI 03618) obtained from the Section of Mycology at the Norwegian Veterinary Institute (Oslo, Norway). The characteristics of these fungal isolates and the procedure for inoculum preparation, except for *Stachybotrys chartarum,* have been previously described elsewhere [8]. Three types of fungal particles (washed and aerosolized spores, and hyphae fragments) were prepared and characterized. Detailed linked to chemicals used and preparation procedures of these particles are presented in Supplementary 1. Cultures age and the media used are summarized in Supplementary 1, Table S1. Several preparations were tested, and similar results obtained (Øya et al. unpublished). Micrographs showing different types of fungal particles in washed spore samples from the four species are presented in Supplementary 1, Figure S1.

Characterization of fungal samples by gravimetry and microscopy

Hundred microliters of 10-100 fold diluted stock suspensions of the fungal samples were filtrated onto pre-weighed 25 mm polycarbonate filters (0.4 µm pores) which were dried in a desiccator overnight. The dried filters were weighed once more and the mass of fungal sample determined as the difference between the pre-weight and the final weight. Fungal samples were characterized by mass content (µg dry weight/mL) and by number of particles (n particles/mL). Spores and fragments present in each sample type were visualized by field emission scanning

electron microscopy (FESEM SU 6600, Hitachi Ibaraki-Ken, Japan) following filtration of 0.5 mL of particle suspension (concentration =100 µg/mL) through a 37 mm diameter polycarbonate filter with 0.2 µm pore size (Millipore, Tullagreen Cork, Ireland) as previously described [8]. Spores and hyphae were recognized by their morphological features and classified in 5 groups based on the number of spores per aggregate and the microscopic length, respectively. At least 200 particles were counted at 3000x magnification and the particle composition was determined by calculating the percentages of each type of particles. Further details are given in Afanou and coworkers [8]. The purity of the spore preparation was based on fragment/spore number after microscopic examination; however, estimates based on volume (closer to mass) were also done.

Inactivation of fungal samples

In order to specifically examine immunologically responses from spores and hyphae, we inactivated the samples. More specifically, fungal samples were irradiated on ice with x-rays (17,45 Gy/min, 225 kV, 13 mA, no filter, 5 cm distance to the source) from X-RAD 225 (Precision X-ray Inc., North Branford) at the Norwegian Institute of Public Health (NIPH) receiving a total dose of 5 kGy.

Sample preparation for mycotoxin analyses

Samples were extracted and analyzed for their respective mycotoxins with Liquid chromatography – high resolution mass spectrometry (LC-HRMS) according to procedures described in Supplementary 2.

Extraction and analysis of β-glucans in fungal suspensions

Spores or hyphae fragments (approximately 10^7 spores) were centrifuged and resuspended in 0.05% Tween 20 and β-1,3-glucans were extracted by adding equal volume of 0.6 M NaOH and shaking for 1 h at room temperature. The extracts were neutralized with equal volume of 0.6 M Tris HCl (pH 7.4), and frozen at -20°C until analysis. Beta-1,3-glucans were quantified in duplicates using kinetic rate assay of the Glucatell β-1,3-glucan detection kit (ACC) according to the manufacturer's descriptions. Different dilutions of the extracts were tested, and possible interfering effects of the sample matrix were controlled by spike recovery tests of each sample using glucan standard.

Characterization of proteases by gelatin and collagen zymography

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described previously [40] using 4% and 10% (w/v) polyacrylamide in stacking and separating gel, respectively and 0.1% (w/v) of gelatin or collagen in the separating gel. The gel was run at 18 mA/gel at 4°C, washed twice in 2.5% (v/v) Triton X-100 and stained with 0.2% Coomassie brilliant blue R-250 (30% methanol). After destaining (30% methanol and 10% acetic acid), proteinase activity was evident as cleared (unstained) regions against the stained background. After electrophoresis, gels were divided into two or more parts, each part washed and incubated in buffer containing either no additives (control), 10 mM EDTA (metalloprotease inhibitor), 1.0 mM Pefabloc (serine protease inhibitor) or 2.8 µM E64 (cysteine protease inhibitor). The approximate molecular size of the proteases was determined by comparing their migration distance with the migration distances of proteases with known molecular size. These molecular size markers were; proMMP-9 dimer (225 kDa), proMMP-9 monomer (92 kDa), processed form of MMP-9 (83 kDa); proMMP-2 (72 kDa), processed form of MMP-2 (62 kDa) and trypsin (24 kDa).

Cell culture and exposure conditions

The BEAS-2B cell line, which was established after SV-40 hybrid (Ad12SV40) transformation of human bronchial epithelial cells, was purchased from European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were maintained in serum-free LHC-9 medium on collagen-coated flasks in a humidified atmosphere at 37°C with 5% CO_2, with refreshment of medium every second day and passaged twice per week. Two days prior to exposure, cells were plated into collagen-coated 10 mm 12-well culture dishes ($8x10^4$ cells/well) and grown to near confluence. In all experiments, medium was changed the day after seeding and before exposure. Cells were treated with 0-100 µg/mL fungal preparations (suspended in LHC-9 media) for 24 h. Medium blanks were included as control.

The THP-1 human monocytic leukemia cell line was purchased from American Tissue Type Culture Collection (Rockville, MD, USA), and maintained in a humidified atmosphere at 37°C and 5% CO_2, in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES, 1 mM pyruvic acid and 0.1% gentamycin, with refreshment of medium every second day and passaged once per week. On the same day as exposure, cells were plated into 35 mm 6-well culture dishes ($8x10^5$ cells/well) containing RPMI 1640 medium. In experiments with monocytes cells were treated with 0-100 µg/mL fungal preparations for 24 h, 3 h after seeding. THP-1 cells ($2x10^5$ cells/well) were also differentiated into macrophages in 10 mm 12-well culture dishes containing 1 mL of RPMI 1640 medium using 200 nM PMA for 3 days. Differentiation of PMA treated cells was enhanced after the initial 3 days stimulus by removing the PMA-containing media then incubating the cells in fresh RPMI 1640 with FBS for additional 3 h. In experiments with macrophages, medium was changed directly before exposure and cells were treated with 0-50 µg/mL fungal particles for 24 h.

THP-1 monocytes used in MMP analysis were cultured as follows. At the same day as exposure, cells were plated into 10 mm 12-well culture dishes ($1.4x10^6$ cells/well) containing RPMI 1640 media without FBS. In experiments with monocytes, cells were treated 3 h after seeding with 0 or 10 µg/mL fungal preparations for 72 h. THP-1 cells ($2x10^5$ cells/well) were differentiated into macrophages as previously described. In experiments with macrophages, medium was changed to PMA-containing media without FBS immediately before exposure and cells were treated with 0 or 1 µg/mL fungal preparations for 72 h.

Cytotoxicity

After exposure to the test compounds for 24 h or 72 h, the cell morphology of the cultures was visually examined with a light microscope. Toxicity was assessed and categorized as the relative amount of floating cells (dead cells) in the cultures versus attached cells (living cells). Cell shape and vacuoles were recognized and documented. Cells were trypsinised and stained with Hoechst 33342 and propidium iodide (PI) for determination of plasma membrane damage, changes in nuclear morphology associated with apoptosis and necrosis, using fluorescence microscopy (Nikon Eclipse E 400 with a 330- to 380 nm UV-2A excitation filter). A minimum of 300 cells per slide were counted (original magnification ×400).

Cytokine release

After exposure, the medium was harvested and centrifuged at 290×g for 10 min to remove mold and floating cells. The final supernatants were stored at -80°C. TNF-α IL-1β, IL-6 and IL-8 protein levels were determined by sandwich ELISA (Enzyme-Linked ImmunoSorbent Assay) according to the manufacturers‘ guidelines. Absorbance was quantified using a plate reader (TECAN Sunrise, Phoenix Research Products, Hayward, CA, USA) complete with software (Magellan V 1.10).

Statistical analysis

The statistical analysis was performed in GraphPad Prism 7 (GraphPad Software, CA, USA). One-way ANOVA was used to analyze the data sets, and Dunnett's post hoc tests were used to compare groups for one-way ANOVA. In all figures, spores and hyphae were plotted separately in two figures. However, the statistics were done as separate one-way ANOVA for each spore or hyphae (i.e. including all applied concentrations) due to differences in number of experiments performed per species. Therefore, no comparison between the responses to the different species was performed. As indicated in the figure legends, all data were log transformed before performing ANOVA to achieve a normal distribution and to fulfil the assumption of equal standard deviations of all sets of replicates, as recommended by the program developers.

Results

Composition and particle size distribution of exposure materials

The exposure materials were characterized with regard to morphology, size and composition by scanning electron microscopy to evaluate the distribution of particle types in all samples. As presented in Table 1, aerosolized spore samples of *P. chrysogenum* had 30% contamination of hyphae fragments, whereas the spore preparation from *S. chartarum* contained a mixture of spores and hyphae fragments with only 26% spores. However, microscopic examination revealed large spores when compared to small hyphae fragments (Supplementary 1, Figure S1). Therefore, volumes of the different components were estimated by microscopic examination. This estimate which is closer to mass of a sample, gave a contamination of only 8.4 and 21% hyphae fragments for the spore preparations of *P. chrysogenum* and *S. chartarum,* respectively. All other samples were mainly spore (90-97%) or hyphae fragment (99-100%) preparations. In

the spore preparations 87-91% of the spore particles were single spores, while in the hyphae samples fragments of 1-10 µm (64-84%) dominated. Washed spores appeared to be smaller and/or have less dry weight than aerosolized spores, thus for the same mass, samples with washed spores contained 2 - 3 times more particles than aerosolized spore preparations.

Characterization of mycotoxin content

We tested for a large number of relevant mycotoxins by various LC-HRMS analysis. The amounts were in general small and insignificant (Supplementary 3, Figure S2, 3 and 4). In short, we did not find any detectable concentrations of gliotoxin, fumitremorgins A-C, citrin or PR-toxin, penicillin G or any non-targeted tricothecenes. However, *A. versicolor* and *P. chrysogenum* contained minor amounts sterigmatocystin and roquefortine C, respectively (Table 2). The mycotoxins detected, seemed to be at somewhat higher levels in washed spores of both species when compared to hyphae fragments.

Measurement of β-1,3-glucans in fungal samples

The amounts of β-1,3-glucans in hyphae fragments were similar in *P. chrysogenum, A. versicolor* and *S. chartarum,* whereas *A. fumigatus* had somewhat lower amounts (Supplementary 4, Table S2). Washed spores of *A. versicolor* and *S. chartarum* also contained some β-glucans, but much less than hyphae did. Aerosolized spores of *A. fumigatus* and *P. chrysogenum* contained both minor amounts of β-1,3-glucans.

Characterization of proteases

Gelatin zymography showed that hyphae fragments of *A. fumigatus*, *P. chrysogenum*, and washed spores of *S. chartarum* contained a gelatin degrading protease with molecular sizes of approximately 44 kDa, 52 kDa and 60 kDa, respectively (Figure 1A). Hyphae fragments of *S.*

chartarum contained one or several proteases with larger molecular size, one just entered the separating gel. The latter had an estimated molecular size of around 300 kDa based on previous experience with proMMP-9/CSPG complexes in THP-1 cells [40, 41]. However, no gelatin degrading proteases were detected in washed spores of *A. fumigatus*, *P. chrysogenum*, *A. versicolor* and hyphae of *A. versicolor.* Furthermore, none of the species/cellular states contained collagen degrading enzymes (data not shown).

The tested fungal materials contained gelatin degrading proteases that belong to different protease classes. The 44 kDa protease from hyphae fragments of *A. fumigatus* was partly inhibited by Pefabloc (serine protease inhibitor) but not by EDTA (metalloprotease inhibitor) or E64 (cysteine protease inhibitor) (Figure 1B and C). Thus, this protease is likely a serine protease. The 52 kDa protease from hyphae fragments of *P. chrysogenum* was inhibited by EDTA but not by Pefabloc or E64. Therefore, this protease is deemed a metalloprotease. The 60 kDa protease from washed spores of *S. chartarum* was not stable during the storing conditions and due to the low activity seen in the inhibitory experiments, it was not possible to classify this protease. The 300 kDa protease from hyphae fragments of *S. chartarum* was not inhibited by any of the three inhibitors used and may therefore belong to one of the new families of proteases found in microorganisms or an unassigned family.

Cell viability / Cytotoxicity

As judged by light microscopy, neither of the test materials seemed to affect viability after neither 24 h nor 72 h at these concentrations (not quantified). In addition, neither apoptosis nor necrosis was observed in any of the cell models (data not shown).

Cytokine release

IL-1β and TNF-α release from BEAS-2B cells was not increased even after 24 h exposure to 100 µg/mL dry weight mold samples (data not shown). However, at this high concentration marked increases in IL-6 and IL-8 were seen (Supplementary 4, Figure S5). The IL-6 response in pg/mL was greater than that of IL-8. The relative potential of the hyphae fragments was *A. fumigatus*>*P. chrysogenum*>*A. versicolor*>*S. chartarum*. Whereas, the hyphae fragments of *A. fumigatus* and *P. chrysogenum* in general were more potent than the respective spore fraction, responses of *A. versicolor* and *S. chartarum* spores were rather similar to that of their respective hyphae sample.

A. fumigatus and *P. chrysogenum* hyphae fragments induced a marked release of both IL-1β and TNF-α, starting at concentrations of 0.1 and 50 µg/mL dry weight in THP-1 monocytes, respectively (Figure 2A). In contrast, none of the other hyphae preparations had any significant effects. Washed spores of *A. fumigatus* and also *A. versicolor* induced low, but significant increase in both IL-1β and TNF-α release at concentrations ≤50 µg/mL dry weight.

Due to their greater sensitivity, THP-1 macrophages were exposed to mold samples at lower concentrations than the monocytes. All mold preparations significantly induced IL-1β release except aerosolized spores of *P. chrysogenum* (Figure 2B). The relative potential of the hyphae fragments was *A. fumigatus*>*P. chrysogenum*>*A. versicolor*>*S. chartarum*. A significant induction of both IL-1β and TNF-α by *A. fumigatus* hyphae fragments was observed at concentrations as low as 10 ng/mL. *A. fumigatus* and *A. versicolor* washed spores were the most potent of all spores with regard to both IL-1β and TNF-α. They induced a significant response at 1 µg dry weight/mL. In THP-1 macrophages, the aerosolized spores of *A. fumigatus* also significantly increased IL-1β. Most interestingly, *A. versicolor* and *S. chartarum* spores were at least as potent as their respective hyphae preparations.

Protease release from THP-1 monocytes and macrophages

THP-1 monocytes and PMA-differentiated macrophages were treated with the mold samples in order to examine their effect on cellular release of proteases. Based on previously published studies [40, 42, 43] an exposure period of 72 h was chosen in these experiments. As can be seen from the data presented in Figure 3A, gelatin zymography of the THP-1 monocyte in serum free conditioned medium showed only a weak band at 92 kDa, whereas the medium from cells exposed to washed spores of *A. fumigatus* and *P. chrysogenum*, hyphae fragments of *A. fumigatus*, *P. chrysogenum*, *A. versicolor* and *S. chartarum* as well as aerosolized spores of *A. fumigatus* and *P. chrysogenum* had increased levels of this gelatin degrading enzyme. The strongest stimulation was observed with hyphae fragments of *A. fumigatus* and *P. chrysogenum* in addition to washed spores of *A. fumigatus*. The 92 kDa band disappeared when the gels were washed and developed in the presence of EDTA (data not shown), and hence this metalloprotease is most likely proMMP-9 as it was previously shown to be the only gelatin degrading enzyme secreted from THP-1 monocytes [42].

Compared to media from THP-1 monocytes, the media from PMA-differentiated THP-1 macrophages showed an increased level of a 92 and a 225 kDa protease (Figure 3B upper panel). As shown previously, these two proteases are the monomer and homodimer forms of proMMP-9 [41]. A very faint band also appeared at 72 kDa, the synthesis of which is not affected by incubating the cells with the various mold species. This 72 kDa protease is likely proMMP-2 since these cells have been shown to be able to produce this protease [44]. Due to the large amount produced already in the controls of the 92 kDa protease, it was not possible to evaluate if the mold affected the secretion of this protease without dilution of the cell conditioned media. As seen in figure 3B lower panel, in spite of the large induction of the 92 kDa protease following

PMA-differentiation of THP-1, hyphae fragments from the *A. fumigatus*, *P. chrysogenum* and *A. versicolor* could further stimulate the production of this gelatin degrading enzyme. In addition, *A. fumigatus* washed spores also induced this protease but to a lesser extent. As expected, this band disappeared when the gels were washed and developed in the presence of EDTA (data not shown), confirming that the protease belonged to the metalloprotease family and it is most likely proMMP-9 that has been further induced by the mold.

No collagen degrading enzymes could be detected by collagen zymography in the conditioned media from THP-1 monocytes, macrophages or mold stimulated THP-1 monocytes and macrophages (data not shown).

Discussion

Spores, crude aqueous extracts and/or isolated single components including various β-glucans and proteases from *A. fumigatus* have been used in most of the studies published on immune responses following exposure to mold [28, 45-47]. Studies elucidating effects of viable spores or hyphae versus inactivated spores from *A. fumigatus* have reported that the growth stage (hyphae) and swelling spores have stronger pro-inflammatory potential than inactivated spores [28, 33, 38, 39]. In the present study, we have systematically characterized spore and hyphae fragment preparations from four species common in indoor environments and compared their pro-inflammatory potentials in different experimental models. We treated the samples with X-ray [28], since this procedure inactivates the specific mold stage without making changes to the structural features and/or surface molecules of fungal samples important for the immune responses (Øya el al. unpublished). An important finding is that the generalized hypothesis/dogma that "hyphae are more potent and give stronger pro-inflammatory responses than spores" does not necessarily hold true for all mold species, since *A. versicolor* and *S.*

chartarum spores were found to be at least as potent as their hyphae counterpart. Furthermore, no single characterized component in the fungal preparations could predict the pro-inflammatory response to the particles.

We aimed to use fungal preparations as close as possible to naturally occurring airborne particles, thus spores were preferentially collected by air flow (aerosolized). Aerosolized spores were only obtained in sufficient quantity from *A. fumigatus* and *P. chrysogenum.* These preparations were slightly contaminated with hyphae fragments, 10 and 30%, respectively as measured by number of fragments. Washed spores appeared to be smaller and/or have less dry weight than aerosolized spores, probably due to the presence of immature spores. The quality of the samples was considered very high except the spore samples from *S. chartarum* and partly *P. chrysogenum.* However, due to the relative large size of spores compared to the small size of the hyphal fragments, the purity of these samples was also estimated by volume which are closer related to mass. A relative high purity by mass was supported by the differences found in β-glucans, protease activities as well as their pro-inflammatory responses. It should also be noted that there are no direct correlations between inflammatory responses and particle number between the various fungal particle samples.

Several studies have reported that mycotoxins may suppress the inflammatory responses through cytotoxicity [48, 49]. Thus, in order to remove any possible toxic effect of mycotoxins that might mask the pro-inflammatory potential, all spore preparations were washed after harvesting. Low levels of mycotoxins in the preparations after washing were confirmed by chemical analysis, and none of the preparations induced cytotoxicity. Small amounts of sterigmatocystin and roquefortine C were detected in some of the samples. Since neither of

these have been reported to have pro-inflammatory effects, their presence did not explain the observed inflammatory responses.

Beta-glucans are mainly exposed during hyphae growth [50]. These carbohydrates are masked by rodlets, hydrophobins or α-glucans on dormant spores [15, 37, 51]. In accordance with this, no or only low levels of β-glucans were found in preparations from *A. fumigatus* and *P. chrysogenum,* or *A. versicolor* and *S. chartarum,* respectively, whereas the levels in hyphae preparations were at least 10x higher.

Beta-glucans seem to elicit a strong immune response [50], and some studies have linked exposure to β-glucans to inflammation-related health factors [52, 53]. In line with this, we find a lower pro-inflammatory potential of *A. fumigatus* and *P. chrysogenum* spores when compared to their respective hyphae preparations. However, in the current study there was no correlation between the total level of β-glucans in the various hyphae preparations and their pro-inflammatory potential. Although the importance of that β-glucans for the triggering of immune responses is well documented, it is not likely that their total content necessarily reflects the pro-inflammatory potential. In addition to β-glucans, also other components of the cell wall contribute, including ergosterol and chitin. Most importantly, the availability of such molecules at the surface, as well as the topical structures of the particle are important determinants of the pro-inflammatory effects [50, 54].

Exposures to proteases, including those from molds, have been shown to stimulate inflammatory signaling pathways in airway cell models [45, 55]. Often the triggering pathways involve crosstalk between PARs and various TLRs-mediated signaling pathways [56]. The characterization of the samples with regard to gelatin degrading protease activities revealed that

the hyphae from *A. fumigatus*, *P. chrysogenum* and *A. versicolor* contained more protease activity than their respective washed/non-aerosolized spore preparations, corresponding with higher inflammatory potential. However, as the two *S. chartarum* samples contained high protease activity despite low pro-inflammatory potential, protease activity as such is not a good parameter for these inflammatory responses. A further characterization of the protease activity revealed that hyphae fragments of *A. fumigatus* contained a gelatin degrading serine protease, whereas hyphae fragments of *P. chrysogenum* consisted of a gelatin degrading metalloprotease. Thus, this serine protease could be more potent than the metalloprotease with respect to pro-inflammatory potential.

IL-1β and TNF-α are the early mediators of inflammatory reaction, which initiate and amplify a wide variety of effects associated with innate immunity and host responses to fungi [57, 58]. Previous studies have suggested that phagocytes and epithelial cells are the primary target cells producing these inflammatory mediators in the lung [59], while monocytes are recruited to the lung during inflammation [25]. The enhanced production of pro-inflammatory mediators is seen at non-cytotoxic concentrations, illustrating that inflammation/pro-inflammatory responses are the primary response to these fungal particles and that IL-1β may be secreted without membrane damage. The THP-1 macrophages model was the most sensitive model with respect to triggering inflammatory responses, followed by the undifferentiated THP-1 monocytes. This is in line with the notion that macrophages represent an important role of in the first line of defense towards molds [16].

Although the BEAS-2B cells did not increase the release of IL-1β and TNF-α, it should be noted that at high concentrations hyphae fragments from all of the species increased the secretion of in particular IL-6 and for *A. fumigatus* and *P. chrysogenum* also IL-8. Furthermore,

while the pro-inflammatory response in BEAS-2B appeared to be low, it is important to remember that the epithelial layer and its' mucus layer represent an important barrier towards various microbes. The differences in sensitivity between various models are greatly reflected by the fact that the more differentiated THP-1 macrophage stage has receptors involved in phagocytosis and/or fungi recognition that will trigger pro-inflammatory responses when compared to monocytes and epithelial cells. In accordance with this, the CLRs dectin-1 and 2 are highly expressed in macrophages [33, 60].

It is interesting to note that washed spores from *A. fumigatus* seem to give more pro-inflammatory effects, than the aerosolized spores. A possible explanation could be that more immature, smaller and/or lighter spores were collected by the washing technique than via the aerosolization. Mature spores will have a more complete hydrophobin layer and thicker cell wall making them less immune-reactive [15, 51].

With regard to *A. fumigatus* and *P. chrysogenum*, the hyphae preparations seem to have a larger potential to trigger IL-1β than their corresponding spore preparations, which is in accordance with the current dogma that spores are less pro-inflammatory than hyphae [6, 15, 61]. However, *A. versicolor* and *S. chartarum* washed spores seemed to give as least as large responses as their respective hyphae preparation. Dectin-1 recognizes β-glucans, whereas Dectin-2 binds to α-mannans both being common in the fungal cell wall [33, 60]. As the above mentioned receptors preferentially should bind to hyphae/germinating spores and not to resting spores, other receptors must also be involved/responsible for the spore-induced pro-inflammatory effects. Possibly THP-1 macrophages have receptors specifically recognizing some topical structures/epitopes on the spores irrespectively of their protease activity, mycotoxin or β-glucan content [33].

MMPs are secreted as pro-enzymes and their activity is carefully regulated. The longer exposure of both THP-1 monocytes and PMA-differentiated THP-1 macrophages to hyphae fragments resulted in increased release of proMMP-9. The basal release from the macrophages was found to be particularly high as also reported by others [21, 22]. Although mold proteases may activate PAR-2, and PAR-2 is known to mediate upregulation of MMP-9 [62, 63], these two events are probably not linked, as the release was first seen after longer periods of exposure. More probably, the release of proMMP-9 is a secondary event due to release of IL-1β and/or TNF-α as these cytokines are known to trigger the release of MMPs [22].

Based on equivalent dry weight concentrations of hyphae fragments added to cell cultures, the overall potential to stimulate the production of pro-inflammatory mediators decreased in the order A. fumigatus>P. chrysogenum>A. versicolor>S. chartarum. By looking at their relative characteristic as determined by relative number, size and shape of particles, content of mycotoxins, β-glucan and crude proteases activity we find no single explanation to this observation. Thus, the pro-inflammatory reactions seems to be a complex combinations of several factors.

In the present study, pro-inflammatory effects of molds were detected at very low doses/concentrations (in the order of 10 ng/cm^2) when compared to other studies [31, 39, 64]. This is rather relevant levels, as airborne concentrations in indoor environments are found to be in the range 10^2 - 10^4 spores/m^3 [6]. As chronic inflammatory responses as such are linked to adverse health effects, the present findings support the notion that mold exposure could be linked to health effects. However, further studies should be conducted in order to explore the relevance of these models and the possible unwanted implications of these type of responses.

In conclusion, the present study has characterized aerosolized spores and hyphae fragments and washed/non-aerosolized spores from different fungal species common in moist indoor environments and compared their characteristics in relation to their inflammatory potential. The pro-inflammatory effects of molds were detected at low doses/concentrations supporting the notion that mold exposure could be linked to health effects. *A. fumigatus* seemed to be the most potent species. THP-1 macrophages were clearly the most sensitive model followed by THP-1 monocytes and BEAS-2B cells. There was no clear link between various sample characteristics and the observed pro-inflammatory response, illustrating the complexity of the many combined factors involved in such immune responses. Most interestingly, while the hyphae preparations of *A. fumigatus* and *P. chrysogenum* were more potent than the respective spore preparations, the opposite seems to be true for *A. versicolor* and *S. chartarum*.

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Table 1: Distribution of fungal particle types in the various mold samples

	Aspergillus fumigatus			*Penicillium chrysogenum*			*Aspergillus versicolor*		*Stachybotrys chartarum*	
	Aerosolized spores	Washed spores	Hyphae fragments	Aerosolized spores	Washed spores	Hyphae fragments	Washed spores	Hyphae fragments	Washed spores	Hyphae fragments
Number counted	200	201	200	201	204	201	201	204	214	201
Fragments, %										
0.2-1µm	4	0	5.5	1.5	0.5	9.5	0	4.4	10.8	9.5
1-2µm	3	0	28	18.9	2.9	16.9	1.5	5.4	29	27.4
2-5µm	3	1.5	36.5	9	3.9	43.3	1.5	28.4	28	27.4
5-10µm	0	1.5	19	1	0	17.4	1	30.4	5.1	12.9
>10µm	0	0	11	0	0	11.9	0	31.4	1.4	22.9
All	10	3	100	30	4	99	4	100	74	100
Spores, %										
Single	82	85.1	0	63.7	82.4	1	83.1	0	25.7	0
Aggregates of 2	6	10.5	0	4	6.4	0	10.5	0	0	0
Aggregates of 3	1.5	1	0	0.5	1.5	0	2	0	0	0
Aggregates of 4	0	0.5	0	0	2	0	0	0	0	0
Aggregates ≥5	0.5	0	0	1.5	0.5	0	0.5	0	0	0
All	90	97	0	70	96	1	96	0	26	0
Particles/mg	0.31×10^8	0.30×10^8		0.40×10^8	0.57×10^8		0.44×10^8		0.13×10^8	
Particles/mL	9.52×10^5	2.90×10^6	1.23×10^6	7.57×10^5	1.47×10^6	1.85×10^6	2.83×10^6	1.72×10^6	2.26×10^6	1.34×10^6

Table 2: Detected fungal metabolites in the particle fractions and estimated concentrations in methanol supplemented and sonicated suspensions (ng/mL)

	Aspergillus fumigatus			*Penicillium chrysogenum*			*Aspergillus versicolor*		*Stachybotrys chartarum*	
Fungal metabolite	Aerosolized spores	Washed spores	Hyphae fragments	Aerosolized spores	Washed spores	Hyphae fragments	Washed spores	Hyphae fragments	Washed spores	Hyphae fragments
Sterigmatocystin	−	−	−	−	−	−	153	+	−	−
Methoxy-sterigmatocystin	−	−	−	−	−	−	*	−	−	−
Roquefortine C	−	−	−	11.5	67.3	1.23	−	−	−	−

(-): not detected; (+): detected, but not quantified; (*): tentatively identified, but no reference standard available

Figure legends

Figure 1. Gelatinolytic proteases in spores and hyphae fragments from various molds. A) Representative gelatin zymography of the different mold samples. **B)** and **C)** Representative gelatin zymography of various mold samples where the gels were washed and incubated without (control), with 1 mM Pefabloc, 10 mM EDTA or 2.8 μM E64, respectively. Arrows at the top of the gels indicate the bottom of the application well and the arrowheads the border between the stacking gel and the separating gel. The gelatin zymography shown in **A)** is from the same gel. This is also the case for the control, Pefabloc, EDTA and E64 in **B)** and **C)**.

Figure 2. Secretion of cytokines from THP-1 cells. A) THP-1 monocytes and **B)** PMA-differentiated THP-1 cells were exposed to spores and hyphae fragments from different species for 24 h at concentrations indicated in the figures. IL-1β and TNF-α secretion are means ± standard error of the mean (SEM) of separate experiment $n>3$; * $p<0.05$ control vs exposed. Statistical analysis was based on log-transformed data using analysis of variance with Dunnett's post hoc tests.

Figure 3. Secretion of gelatinolytic proteases from THP-1 cells. A) THP-1 monocytes were exposed to 10 μg and **B)** PMA-differentiated THP-1 cells to 1 μg dry weight/mL of spores and hyphae fragments from various species for 72 h. **A)** Representative gelatin zymography of undiluted conditioned serum-free media from monocytes. **B)** Representative gelatin zymography of undiluted (upper panel) and five times diluted (lower panel) conditioned serum-free media from PMA-differentiated THP-1 cells. The various undiluted and diluted media was treated and loaded to the gel as described in Figure 1A). Standard (St) are identical with St2 in figure 1 and the molecular sizes in kDa are shown. Arrowheads show the border between the

stacking gel and the separating gel. The gelatin zymography shown in **B)** lower panel are from the same gel.

Fig. 1

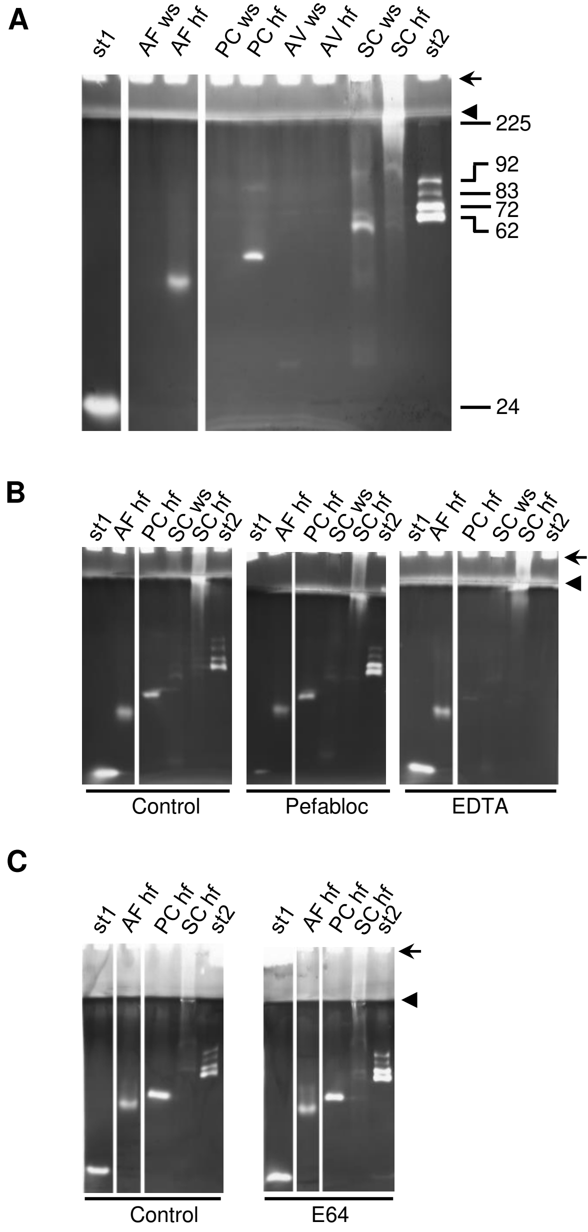


Fig. 2

A

IL-1b secretion (pg/ml)

Control
0,1 µg/ml
1 µg/ml
10 µg/ml
50 µg/ml
100 µg/ml

as ws as ws ws ws
AF PC AV SC

hf hf hf hf
AF PC AV SC

TNF-a secretion(pg/ml)

as ws as ws ws ws
AF PC AV SC

hf hf hf hf
AF PC AV SC

Aerosolized or washed spores (s)

Hyphae fragments (hf)

B

IL-1b secretion (pg/mL)

Control
1 ng/ml
10 ng/ml
100 ng/ml
1 µg/ml
10 µg/ml
50 µg/ml

as ws as ws ws ws
AF PC AV SC

hf hf hf hf
AF PC AV SC

TNF-a secretion (pg/ml)

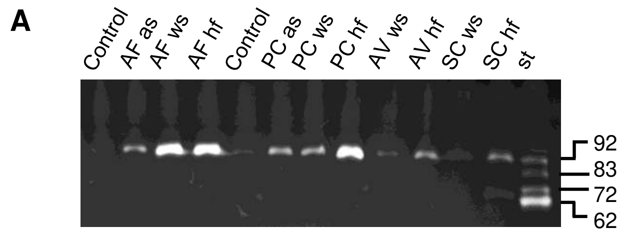
as ws as ws ws ws
AF PC AV SC

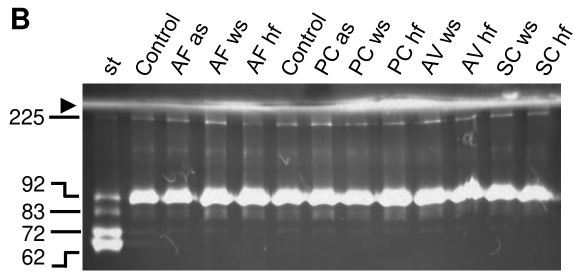
hf hf hf hf
AF PC AV SC

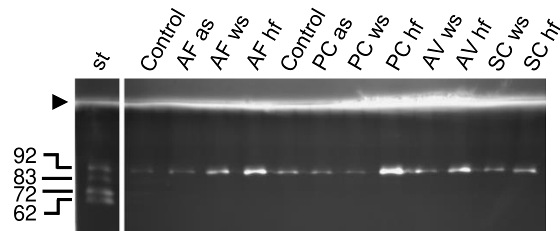
Aerosolized or washed spores (s)

Hyphae fragments (hf)

Fig. 3







Supplementary 1:

Materials and Methods

Chemicals

Bacterial collagenase 1A (C9891; 760U/mg), rat tail collagen type I (C 8897), porcine skin gelatin (G 2500), 4-(2-aminoethyl) benzenesulfonyl fluoride (Pefabloc), E64 (E-3132), verrucarol, aflatoxin B, B1, G, G1, gliotoxin, roquefortine C, PR-toxin, bovine trypsin, pro matrix metalloproteinases (MMP)-2, were all from Sigma-Aldrich (St. Louis, MO, USA), while citrinin and sterigmatocystin were from Romer Labs (Tulln, Austria). Roridin A was a gift from James J. Pestka (Michigan State University). Phorbol 12-myristate 13-acetate (PMA) was from Calbiochem, EMD Chemicals, Inc (San Diego, CA, USA. ProMMP-9 was purified from PMA stimulated THP-1 cells as described previously [1]. LHC-9 cell culture medium was from Invitrogen (Carlsbad, CA, USA) and PureCol™ collagen from Advanced BioMatrix (Carlsbad, CA, USA). RPMI 1640 medium was from Lonza (Verviers, Belgium). Cytokine ELISA assays for TNF-α (Human TNF-α Cytoset), IL-6 (Human IL-6 Cytoset) and CXCL8 (Human IL-8 Cytoset) were purchased from Life Technologies (Camarillo, CA, USA). Cytokine ELISA assay for IL-1β (Human IL-1β Duoset) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). All other chemicals used were purchased from commercial sources at the highest purity available.

Particle preparation methods

Spores collected by liquid (washed spores; ws)

Spores were gently scraped from respective fungal cultures grown on cellophane covered agar media by flooding with PBS containing 0.1% Tween 20 (PBST). The obtained spore suspension was resuspended by vortexing for 2x30 s followed by sonication for 5 min in an ultrasonic bath at a frequency of 35 kHz (Sonorex RK 510H, Bandalin Electric, Berlin, Germany). The spore

suspension was then filtered through a 10 µm nylon mesh filter mounted in Steriflip (Millipore, Tullagreen, Cork, Ireland) centrifuged at 5000×g for 10 min (Sigma 4k15, Osterode, Germany) and the pellet resuspended in appropriate volumes of LHC-9 cell culture medium. The prepared material was characterized by gravimetry and microscopy.

Spores collected by air (aerosolized spores; as)

Only the cultures from *A. fumigatus* and *P. chrysogenum* were subjected to aerosolization. Spores from the other isolates did not aerosolize sufficiently effective to give satisfactory amount of spores. The agar plates covered with fungal biomass were subjected to aerosolization at 20 L/min in Stami Particle generator (SPG) following the procedure described by Afanou and co-workers [2]. The released spores were collected onto 0.4 µm polycarbonate filters and eluted in a centrifuge tube by vortexing (1x5 min) and sonicating (1x5 min) in PBST. Suspended spores were filtered through 10 µm nylon mesh and the filtrate was centrifuged as described in the section above. The supernatant was carefully discarded and the pellet resuspended in LHC-9 cell culture medium to achieve a final stock suspension of 30 mg/mL. This suspension was further diluted and characterized by gravimetry and microscopy.

Hyphae fragments (hf)

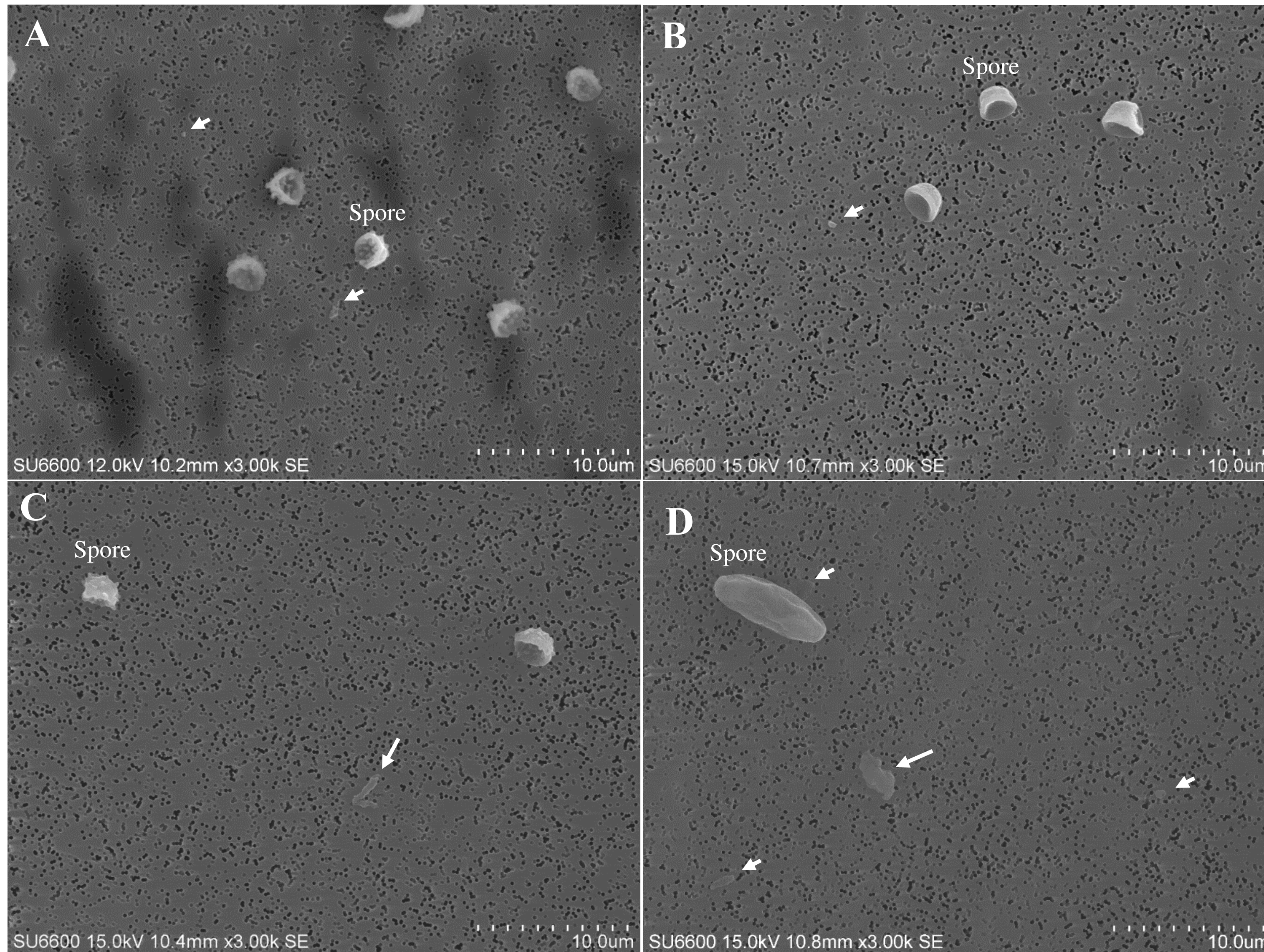
Fungi were grown for 2- to 7-days on cellophane covered Sabouraud Dextrose Agar (SAB) plates based on our observations revealing late sporulation of the tested isolates. The samples were harvested prior to sporulation by scraping the hyphae mass into a polypropylene centrifuge tube. The biomass was freeze-dried using a Drywinner 1.0 to 6.0 (Heto, Denmark) connected to a RZ2 vacuum pump (Vacuubrand Gmbh, Wertheim, Germany) for 22 to 48 h. The dried biomass was then ground in a RetchMM301 mixer mill (Retsch Gmbh, Haan, Germany), and the resulting hyphae powder was vacuumed through a cyclone (GK2.69 Cyclone; BGI,

Waltham, MA, USA) operated in the respirable mode at 4.2 L/min onto polycarbonate filters which were eluted as described in the section above. The obtained fragment pellet was resuspended in LHC-9 cell culture medium and incubated at 37ºC for 20 h.

Supplementary Table S1: Different types of fungal samples and growth conditions

A. fumigatus			*A. versicolor*			*P. chrysogenum*			*S. chartarum*		
Types	Media	Age (days)	Types	Media	Age (days)	Types	Media	Age (days)	Types	Media	Age (days)
Aerosolized spores	MEAC	14	-	-	-	Aerosolized spores	MEAC	14	-	-	-
Washed spores	MEAC	13	Washed spores	MEAC	21	Washed spores	MEAC	11	Washed spores	PDAC	10
Freeze dried hyphae fragments	SABC	4	Freeze dried hyphae fragments	SABC	2	Freeze dried hyphae fragments	SABC	7	Freeze dried hyphae fragments	SABC	3
Incubation during growth in the dark at 25 ± 1ºC and 90 ± 5% relative humidity. Fungal materials were inactivated with X-ray 5kGy and kept at -20ºC until cell exposure experiments.											

MEAC: cellophane covered malt extract agar; SABC: cellophane covered Sabouraud dextrose agar; PDAC: Cellophane covered potato dextrose agar.



Supplementary Figure S1. Micrographs showing different types of fungal particles (spores and fragments (arrows)) in washed spore samples from *A. fumigatus* (A), *P. chrysogenum* (B); *A. versicolor* (C) and *S. chartarum* (D).

References

1. Malla, N., E. Berg, L. Uhlin-Hansen, and J.O. Winberg, Interaction of pro-matrix metalloproteinase-9/proteoglycan heteromer with gelatin and collagen. J Biol Chem, 2008. 283(20): p. 13652-65.
2. Afanou, K.A., A. Straumfors, A. Skogstad, I. Skaar, L. Hjeljord, O. Skare, B.J. Green, A. Tronsmo, and W. Eduard, Profile and Morphology of Fungal Aerosols Characterized by Field Emission Scanning Electron Microscopy (FESEM). Aerosol Science and Technology, 2015. 49(6): p. 423-435.

Supplementary 2:

Mycotoxin methods

Sample preparation for mycotoxin analyses

Aliquots (500 µg dry weight) of the suspensions containing washed spores, aerosolized spores or hyphae fragments in LHC-9 cell culture medium were transferred to Eppendorf tubes. Methanol (1:1) was added, the suspensions vortex shaken (15 s) and sonicated for 15 min at room temperature, and then, centrifuged at 15,000×g for 5 min at room temperature. Supernatants were transferred to chromatography vials and sealed tight.

Base-hydrolysis of macrocyclic trichothecenes yielding verrucarol was tested using roridin A as a model compound and 0.1 M carbonate buffer (pH 9.2), 2% ammonia or 0.1 M sodium hydroxide as base. In the final protocol, 100 µL of 0.2 M sodium hydroxide was added to 100 µL-aliquots of the suspensions or 100 µL of a 10:1 concentrated supernatant from washing of the spores (see below). The mixture was vortex shaken and heated to 80°C for 90 min. The aliquots were centrifuged at 15,000×g for 5 min and supernatants transferred to chromatography vials for LC–HRMS analysis.

The supernatant from washing of the spores was concentrated by solid-phase extraction on a 100 mg Strata-X column (Phenomenex, Torrance, USA). The column was conditioned with 3 mL each of methanol and water, and then 10 mL of the supernatant was applied to the column. After washing with 3 mL of water and drying under vacuum, the column was eluted with 3 mL methanol. The solution was evaporated to dryness and the residue dissolved in 1 mL of water.

Liquid chromatography – high resolution mass spectrometry (LC–HRMS) procedure for mycotoxin analyses

Separation of the suspensions containing aerosolized spores, washed spores, or hyphae fragments was achieved using a 100×2.1 mm, i.d. 2.6 µm Kinetex EVO column (Phenomenex). Injection volumes were 1–5 µL. The mobile phase (400 µL/min) consisted of 5 mM ammonium acetate in 1% acetic acid (A), and 5 mM ammonium acetate in 95:5 acetonitrile–water containing 1% acetic acid (B). The column was eluted isocratically with 5% B for 0.5 min followed by a linear gradient from 5−100% B over 20 min, followed by a 2 min hold and a return to 5% B at 23 min, and equilibration with 5% B for 3 min using a Dionex UltiMate 3000 UPLC pump (Thermo Fisher Scientific, Waltham, MA). The detector was a Q-Exactive Fourier-transform high-resolution mass spectrometer (HRMS, Thermo Fisher Scientific) equipped with a heated electrospray ionization interface (HESI). The HRMS was run in positive and negative ion full-scan mode using fast polarity switching (i.e. alternating positive and negative ion scans), in the mass range *m/z* 250–800. The mass resolution was set to 70,000 at *m/z* 200. The spray voltage was 4 kV, the transfer capillary temperature was 250°C, and the sheath and auxiliary gas flow rates were 35 and 10 units, respectively. Exact values of *m/z* used for extracted ion LC–HRMS chromatograms as well as mass errors were obtained using Xcalibur 2.2 or 3.0 (Thermo Fisher Scientific). The mass spectral characteristics of available reference toxins were studied by infusion (5 µL/min) of a 1–10 µg/mL solution into a mobile phase consisting of 1:1 A/B.

The concentrations of sterigmatocystin and roquefortine C in the suspensions were estimated from external calibration of the LC–HRMS instrument using authentic standards dissolved in acetonitrile-water (9:1, v/v). We did not make efforts to determine the limits of detection of target compounds. However, in our experience the quadrupole–Orbitrap hybrid instrument we used for detection of fungal metabolites in general provides limits of detection in the range 0.1–5 ng/mL for small molecules.

Available relevant reference standards for metabolites related to *A. versicolor* included sterigmatocystin and aflatoxins, which all afforded $[M+H]^+$ ions using LC–HRMS in the positive ion mode. We also looked tentatively for methoxy-sterigmatocystin as well as versicolorins A and B by plotting extracted ion chromatograms for their $[M+H]^+$ ions assuming that these analogues of aflatoxins and sterigmatocystin have the same MS ionization characteristics.

In order to test if methanol supplementation was necessary for the extraction of sterigmatocystin from the spores, or if the compound was also present in the aqueous medium we added water (1:1) to an aliquot of the spore suspension and placed it in an incubator at 37°C for 24 h.

S. chartarum spore and hyphae suspensions were screened with regard to macrocyclic trichothecene mycotoxins (also known as type–D trichothecenes). Roridin A was used as a model compound and was found to afford $[M+acetate]^-$ ions upon electrospray ionization in the negative ion mode. The accurate *m/z* of the $[M-H]^-$ and $[M+acetate]^-$ ions were then calculated for a range of satratoxins (F, G, H, isosatratoxin F), roridins (A and E) and verrucarin J in order to plot extracted ion chromatograms from the full-scan LC–HRMS data.

Supplementary 3:

Mycotoxin results

Characterization of mycotoxin content

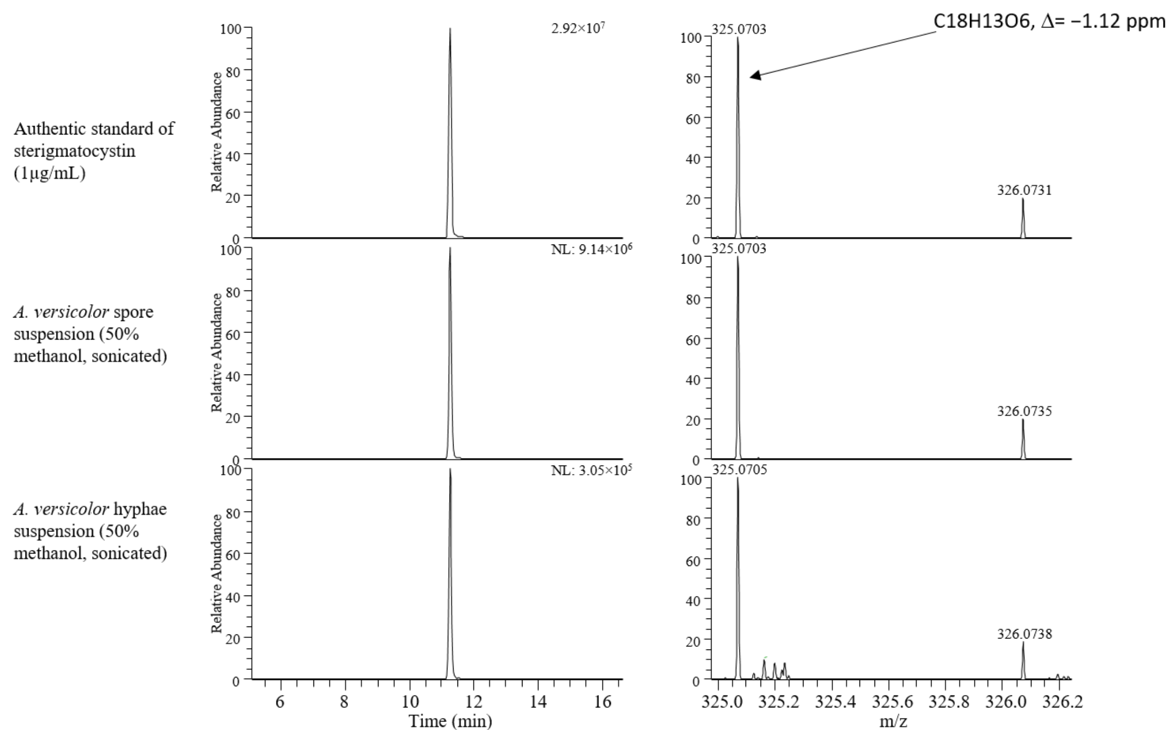
The various samples containing aerosolized spores, washed spores and hyphae fragments from *A. fumigatus* were screened for the presence of gliotoxin. Gliotoxin was available as reference standard and afforded $[M-H]^-$ ions using LC–HRMS in the negative ion mode. None of the samples contained gliotoxin in detectable concentrations. Furthermore, we plotted extracted ion chromatograms for sensible ions of fumitremorgins A–C, but could not detect any of these fungal metabolites.

Available metabolites related to *P. chrysogenum* included roquefortine C, PR-toxin and citrinin. Roquefortine afforded $[M+H]^+$ and $[M-H]^-$ ions upon positive and negative electrospray ionization, respectively. The former ions were, however, of approximately twice the intensity compared to the latter. Citrinin was found to afford primarily $[M+H_2O-H]^-$ ions and PR-toxin $[M+H]^+$ ions. The subsequent targeted analyses of the different suspensions of *P. chrysogenum* spores and hyphae fragments showed the presence of roquefortine C in all samples, but no citrinin or PR-toxin. The concentration of roquefortine C was highest in the medium suspension containing washed spores, and lowest in the medium suspension containing hyphae fragments (Table 2). We also looked for penicillin G by calculating the accurate *m/z* of sensible ions and plotting extracted ion chromatograms but could not detect this compound.

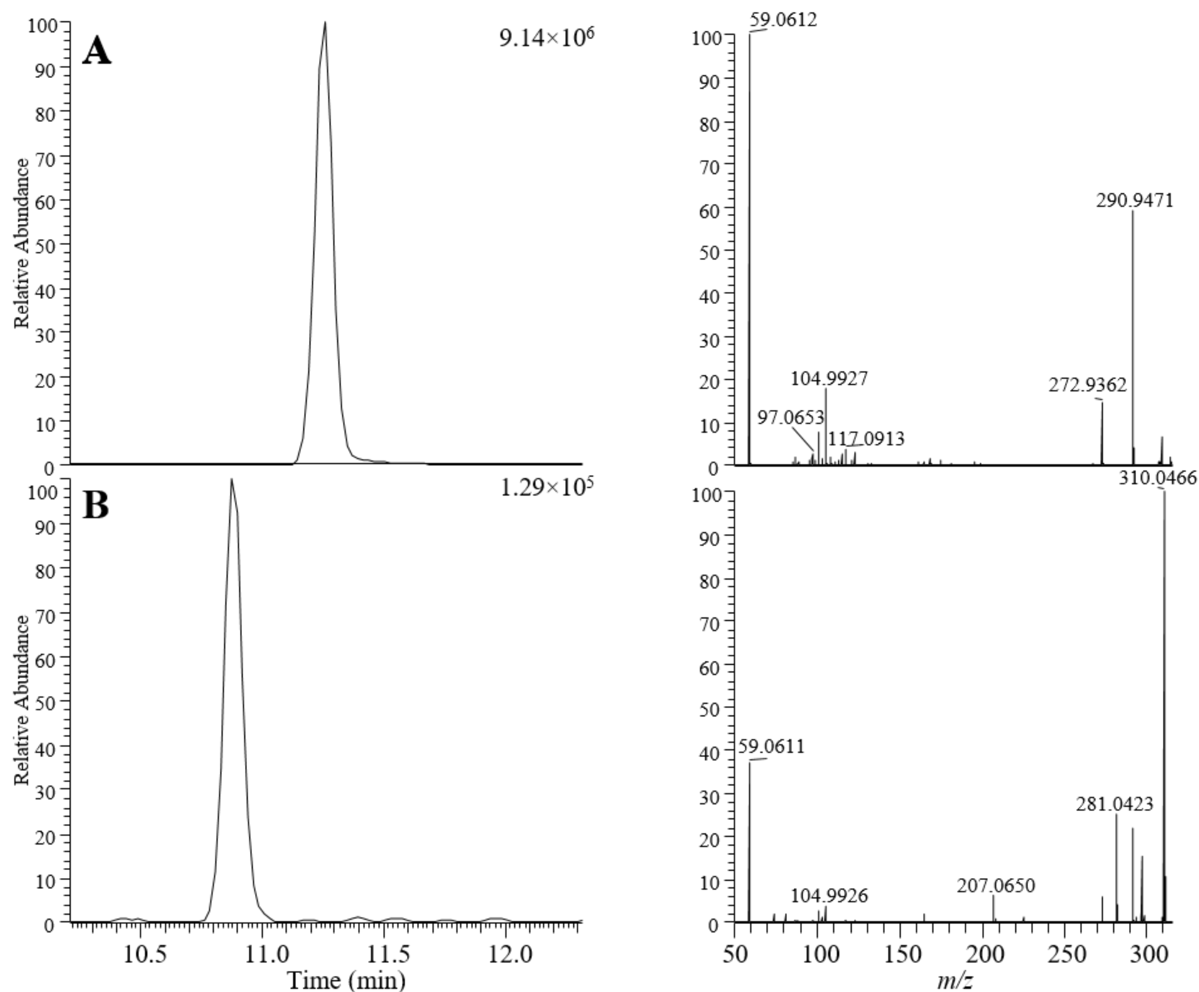
Both the methanol supplemented medium suspension with washed spores and the suspension with hyphae fragments contained detectable amounts of sterigmatocystin (Table 2, Supplementary Figure S2). In the washed spore suspension, a nearby eluting compound identified as methoxy-sterigmatocystin based on its calculated elemental composition and LC–

HRMS/MS data (Supplementary Figure S3). The relative peak area of the methoxy-analogue relative to sterigmatocystin was 1.5%. The peak area of sterigmatocystin in the aqueous medium suspension was 7% of that of sterigmatocystin in the methanol supplemented medium, and hence we conclude that the concentration of the fungal toxin in the cell incubations was significantly lower than the estimated 153 ng/mL in the methanol supplemented spore suspension (Table 2).

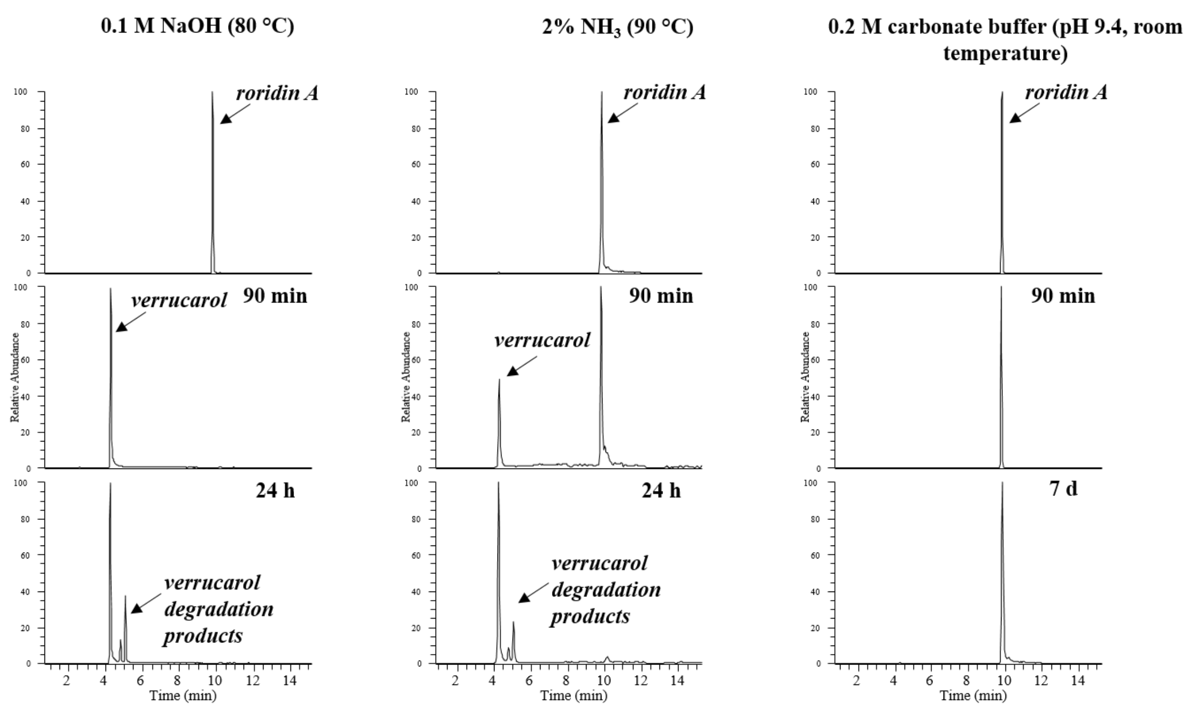
None of the targeted trichothecenes were detected in the samples. In order to further investigate the presence of possible other type–D trichothecenes, we set up a protocol with the aim to base-hydrolyze the ester linkages of the macrocyclic ring in any of the possibly present analogues. The expected product of the hydrolysis is verrucarol, which also was available as reference standard. The hydrolysis reaction was successfully tested using the roridin A (Supplementary Figure S4). However, base treatment of any of the *S. chartarum* samples, including the 10:1 concentrated supernatant from washing of the spores, did not result in the presence of verrucarol, and we thus conclude that none of the samples contained macrocyclic trichothecenes.



Supplementary Figure S2. LC-HRMS extracted ion chromatograms (left, $[M+H]^+$, ±5 ppm) for sterigmatocystin in a standard solution as well as methanol supplemented and sonicated *A. versicolor* spore and hyphae samples, and mass spectrum of the protonated molecular ions. The number in the top right-hand corner of each chromatogram is the intensity of the highest peak in that chromatogram (arbitrary units).



Supplementary Figure S3. LC-HRMS extracted ion chromatograms (left, $[M+H]^+$, ±5 ppm) for sterigmatocystin (**A**) and putative methoxy-sterigmatocystin (**B**) in a methanol supplemented and sonicated *A. versicolor* spore samples, as well as product ion spectra from fragmentation of the protonated molecular ions. The number in the top right-hand corner of each chromatogram is the intensity of the highest peak in that chromatogram (arbitrary units).

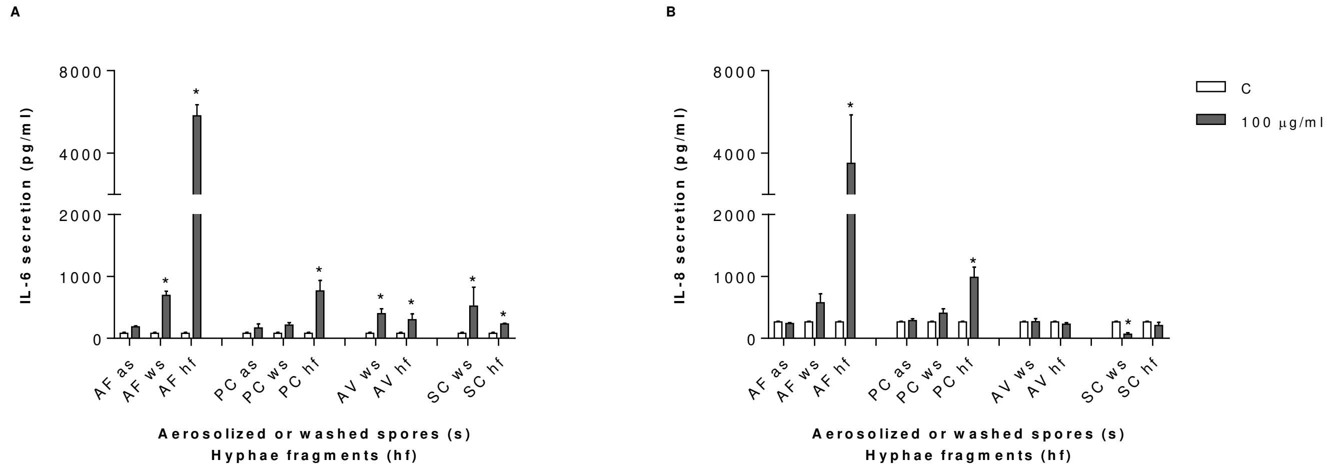


Supplementary Figure S4. LC-HRMS extracted ion chromatograms ([M+acetate]$^-$, ±5 ppm) from monitoring the base hydrolysis of roridin A in three different experiments employing different bases and temperatures.

Supplementary 4:

Supplementary Table S2: β-1,3-glucans content in mold samples

Mold samples	**β-1,3- glucans (ng/ml)**
A. fumigatus aerosolized spores	<2.53
A. fumigatus hyphae fragments	244
P. chrysogenum aerosolized spores	<2.53
P. chrysogenum hyphae fragments	400
A. versicolor washed spores	45.7
A. versicolor hyphae fragments	>404
S. chartarum washed spores	45.5
S. chartarum hyphae fragments	>404



Supplementary Figure S5. Cytokine/chemokine secretion in BEAS-2B cells induced by different molds. Cells were exposed to 0 or 100 µg dry weight /mL of spores or hyphae fragments from various species for 24 h. A) IL-6 secretion by ELISA after 24 h exposure of 100 µg dry weight /mL different species of mold in BEAS-2B cells. B) IL-8 secretion by ELISA after 24 h exposure of 100 µg/mL dry weight different species of mold in BEAS-2B cells. Protein release in cell culture supernatants was determined by ELISA. Bars represent protein levels detected in each separate experiment (mean ± SEM, n=3). * p<0.05 control vs exposed. Statistical analysis was based on log-transformed data using analysis of variance with Dunnett's post hoc tests.