Characterization and pro-inflammatory potential of indoor mold particles

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Abstract

A number of epidemiological studies find an association between indoor air dampness and respiratory health effects. This is often suggested to be linked to enhanced mold growth. However, the role of mold are obviously difficult to disentangle from other dampness related exposure including microbes as well as non-biological particles and chemical pollutants. The association may partly be due to visible mycelial growth and a characteristic musty smell of mold. Thus, the potential role of mold exposure should be further explored by evaluating information from experimental studies elucidating possible mechanistic links. Such studies show that exposure to spores and hyphal fragments may act as allergens and pro-inflammatory mediators, and that they may damage airways by the production of toxins, enzymes and volatile organic compounds.

In the present review, we hypothesizes that continuous exposure to mold particles may result in chronic low-grade pro-inflammatory responses contributing to respiratory diseases. We summarize some of the main methods for detection and characterization of fungal aerosols and highlight *in vitro* research elucidating how molds may induce toxicity and pro-inflammatory reactions in human cell models relevant for airway exposure. Data suggest that the fraction of fungal hyphal fragments in indoor air is much higher than that of airborne spores, and the hyphal fragments often have a higher pro-inflammatory potential. Thus, hyphal fragments of prevalent mold species with strong pro-inflammatory potential may be particularly relevant candidates for respiratory diseases associated with damp/mold-contaminated indoor air. Future studies linking of indoor air dampness with health effects should assess the toxicity and pro-inflammatory potential of indoor air particulate matter, and combined this information with a better characterization of biological components including hyphal fragments from both pathogenic and non-pathogenic mold species. Such studies may increase our understanding of the potential role of mold exposure.

Practical implications:

Indoor dampness and mold have been associated with allergic and non-allergic inflammatory reactions that may be linked to various health outcomes. The present review summarize some of the main methods for detection and characterization of fungal aerosols and highlight experimental research elucidating how molds may induce toxicity and pro-inflammatory reactions in human lung. Recent studies find that a substantial part of the mold particles in indoor air are hyphal

fragments, which possess different and often much stronger pro-inflammatory effects than spores. The inflammatory potential differs highly between various mold species and some of the highly prevalent non-pathogenic species may give strong pro-inflammatory responses at low concentrations. Continuous exposure to mold particles may result in chronic low-grade pro-inflammatory responses thereby contributing to respiratory diseases. Thus, we suggest that future epidemiological studies should perform better characterization and quantification of the air samples, and include hyphal fragments from both pathogenic and non-pathogenic species and more direct analysis of pro-inflammatory responses.

Abbreviations

ALI, air-liquid interface; *A. fumigatus, Aspergillus fumigatus; A. versicolor, Aspergillus versicolor;* BAL, bronchoalveolar lavage; CLR, C-type lectin receptor; COPD, chronic obstructive pulmonary disease; CFU, colony forming units; DC-SIGN, dendritic cell receptor-SIGN; DUOX1, dual oxidase 1; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte-macrophage colony–stimulating factor; IL, interleukin; LAL, Limulus amoebocyte lysates; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; MVOC; microbial volatile organic compounds; MIP-1α, (CCL3), macrophage inflammatory protein 1-alpha, MIP-1β, (CCL4), macrophage inflammatory protein 1-β, MCP-1, (CCL2), monocyte chemoattractant protein 1; N-acetyl-β-D-glucosaminidase (NAGAse); N-acetyl- hexosaminidase (NAHA); NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; NLR, NOD-like receptor; NO, nitric oxide; PAMP, pathogen associated molecular pattern; PARs, protease-activated receptors; *P. chrysogenum, Penicillium chrysogenum;* PM, particulate matter; PI3K, phosphatidylinositol 3-kinases; PMA, phorbol 12-myristate 13-acetate; PRR, pattern recognition receptor; PLFA, phospholipid-derived fatty acids; QPCR, quantitative polymerase chain reaction; ROS, reactive oxygen species; RNS, reactive nitrogen species; *S. chartarum, Stachybotrys chartarum;* SP, surfactant proteins; TACE, TNF-α converting enzyme; THP-1, Human leukemia monocyte cell line; TER, trans-epithelial electrical resistance; TSLP, thymic stromal lymphopoietin; TNF-α, tumor necrosis factor-alpha.

1. Introduction

Indoor dampness and mold have been associated with allergic and non-allergic inflammatory reactions that may be linked to various health outcomes: wheezing and cough, allergies, development and exacerbation of asthma and allergic rhinitis (1-8). Moisture damage and mold may also link to higher risk of respiratory tract infections (3, 5, 8). Associations between these health outcomes and molds are mainly with visible mycelial growth and a characteristic musty smell of mold. The role of mold is difficult to disentangle from other dampness related exposure including other microbes as well as non-biological particle matter (PM) and chemical pollutants.

Air pollution is among the leading environmental health risk factors (9). Strong causal associations have been reported between exposure to PM and development or exacerbation of various respiratory symptoms and disease (9). Although people in the industrialized world spend approximately 90% indoor (2, 10), most information has come from outdoor PM studies. These studies have shown that PM toxicity and their potential to induce oxidative stress and inflammation are depending on factors including size, shape, structure, surface reactivity, lipophilic and hydrophilic chemicals as well as biological components adhered (11). The differences in PM composition are highly depending on sources and seasonal variation (12, 13).

Important air pollution components indoor are PM and nitrogen dioxide from outdoor sources or generated by cooking and biomass burning (14). Other indoor pollutants include gases released from building material and solvents (15), house dust mites and other indoor allergens (16). In developing countries, provision of vented stoves have led to a reduction in the levels of PM from cooking (17). Although environmental tobacco smoke indoors still is considered to be an important PM source in dwellings, exposure has been dramatically reduced the last decades in western world (18). The relative importance of outdoor versus indoor contribution on respiratory health problems are also much depending on the ventilation system in buildings (19).

Health problems related to indoor air are often linked to dampness (3, 5). As microbes and non-infectious bio-aerosols are known to give pro-inflammatory effects (12, 13), they are often characterized in indoor air and linked to respiratory symptoms and disease (15). Health problems associated with building-dampness often coincide with mold growth (3, 5); and a decrease in prevalence of symptoms has been reported following renovation of mold damage (20, 21). In studies of working populations, where exposure levels are considerably higher, associations

between exposure to fungal spores measured by personal monitoring and multiple respiratory effects are frequently reported (22). Sensitization rates to fungi typically exceed 5% of the general public with higher rates among the atopic population (23). However, there is no strong associations between the total occurrence of spores in mold-contaminated indoor environments and adverse health outcomes unless patients have underlying responses, are compromised host, or have hypersensitivity disorders to mold (23). Thus, these associations need to be further supported by experimental studies.

Experimental studies show that exposure to spores and hyphal fragments may act as allergens (24) and pro-inflammatory mediators, and that they may damage airways by the production of toxins (25), proteases (26) and microbial volatile organic compounds (MVOC) (27). In the present review, we hypothesize that continuous exposure to mold particles may result in chronic low-grade pro-inflammatory responses that can contribute to respiratory diseases development. We summarize some of the main methods for detection and characterization of fungal aerosols and highlight experimental research elucidating how molds may induce toxicity and pro-inflammatory reactions in human lung.

1.1 Mold in indoor air

Water damage in buildings is common and is often associated with mold problems (28-30). Mold can grow on virtually all organic building materials if there is enough moisture accessible. Buildings damaged by flooding or leakage (from plumbing, pipes, roofs or windows), or where temperature and humidity levels are generally high due to poor ventilation are particularly at risk (5). Growth can start on material containing 12–15% water content per weight and even at lower water content if the air relative humidity is above 85%. In Norway, it has recently been estimated that around 30% of buildings have signs of moisture damage (31), which is consistent with studies from other countries (6, 32-35). In the coming years, global climate change may increase the prevalence of mold-induced sensitization and allergic airway diseases as mold colonization and sporulation increases at higher temperatures and carbon dioxide levels (36) and the frequency of extreme weather may increase. Notably, during baseline and intervention, mold and endotoxin levels in mold-contaminated houses in New Orleans after Hurricane Katrina were similar to those found in agricultural environments (37).

Fungal components from both outdoor and indoor air may be regarded as relevant sources for respiratory exposure. As their relative importance is highly dependent on climate patterns, no established acceptable indoor levels have been established. However, the indoor levels of fungal aerosols should preferentially be lower than outdoor levels (38).

1.1.1 Species and growth stages

It is estimated that there are at least 1.5 million fungal species, although only approximately 80,000 have been described so far (39). Filamentous fungi found are called molds. Their spores may germinate into multicellular filamentous structures (hyphae), which can grow further into complex networks known as mycelium (40). Airborne molds take the form of single, aggregates or fragments of spores as well as hyphal and mycelial fragments (41). However, not all species produce spores that are easily aerosolized. Spores from *S. chartarum* are large and sticky, and are dispersed by water droplets and may thus become liberated into air after secondary dispersion (22).

Species that commonly occur in damp indoor environments include *Penicillium chrysogenum*, *Aspergillus versicolor*, *Stachybotrys chartarum* and *Aspergillus fumigatus* (30, 42, 43). The level of pathogenic *A. fumigatus,* which is associated with invasive lung diseases, is generally low (44). Even though the toxic black mold *S. charatrum* is often linked to damp building-related illnesses, concentrations of *S. chartarum* have been very low among the mycoflora identified in water-damaged and mold-contaminated buildings compared to the more common *Penicillium* and *Aspergillus* species (45, 46).

1.1.2 Mold composition

The basic fungal cell wall structural organization of both spores and hyphae is composed of a fibrillary skeleton, which consists of a matrix containing glucans (mainly β-1,3-glucan), galactomannans, glycoproteins, enzymes and lipids, reinforced by chitin fibrils (47). However, the composition and three-dimensional structure of the cell wall differ between mold species and change during their life cycle (48).

The outer surface of resting spores most often have a layer of hydrophobic RodA protein and pigments such as melanin which are lost upon swelling and germination of spores (49, 50). This may result in appearance of polysaccharide moieties such as β-glucans (51). Under certain growth

conditions, some molds may synthesize and excrete mycotoxins (52) and various proteases (26). The types and amounts of toxin and proteases produced depend on the species, the strain as well as on the substrate and growth conditions.

1.2 Mold-induced effects in the respiratory system

1.2.1 Mold deposition and clearance

On a daily basis humans continuously inhale mold. Mold bio-aerosols include three main groups based on their shape: spherical particles represented by single spores; oblong particles that comprise the submicronic, larger fragments and spore aggregates, and fibrous particles such as spore aggregates and larger fragments (53). The diversity in physical properties of inhaled fungal aerosol influence their aerodynamic behavior (54), and are of great importance for their fate in the respiratory system (28, 55). In general, large particles primarily deposit in the upper airways (nose and throat), whereas smaller particles deposit more in the lower part of the respiratory system (bronchial and alveolar regions) (56). The majority of the mold particles will be cleared out by the mucociliary escalator (45) while those in the alveoli will be first phagocytosed by macrophages (57). Notably, the geometry and function of the respiratory airways may have been remodeled in individuals with lung diseases such as asthma, leading to a several fold increased particles deposition and reduced clearance (58).

Investigations of fungal exposure in field studies indicate that the ratio of fragment number versus spores may be large; more specifically the fragment to spore number ratio was 10^3 for fragments of size 0.3 μm and 10^6 for fragment size 0.03 μm (59, 60). The smaller mold particles/fragments that deposit deeper into the lungs are not as rapidly removed and may thus trigger more pro-inflammatory and immunological signals. They may interact with and even be translocated across the epithelial barrier into the bloodstream and to secondary organs (61). Thus, a proper physical characterization of particles in the fungal aerosols is important in order to provide good prediction of their deposition and effects in the respiratory tract.

1.2.2 Immune responses

The respiratory system is constantly exposed to various microbes including molds (62). The epithelium layer representing a structural barrier combined with the innate immune responses by in particular the lung macrophages are crucial to maintain normal lung function (63).

Pattern Recognition Receptors (PRRs) expressed on immune cells and respiratory epithelial cells recognize various mold products excreted as well as components on the mold particle surface. Important classes of PRRs in antifungal immunity are the toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-oligomerization domain (NOD)-like receptors (NLRs) and protease-activated receptors (PARs) (64). Human TLRs comprise both extracellular and intracellular receptors (65). TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 on the cellular surface, bind to extracellular pathogens/ligands. In contrast, pathogens/ligands taken up by phagocytosis may fuse with cytosolic endosomes and bind to TLR3, TLR7, TLR8, and TLR9. The specific receptor location has been reported to partly depend on cell types and tissues (66, 67). Expression of TLRs modulates rapidly in response to environmental stresses including pathogens and cytokines (68). Furthermore, TLRs may interact with each other as well as CLRs, protease activated receptors (PARs) or cofactor/adapter proteins to mediate mold-associated inflammation (69). TLR2 and TLR4 have both been suggested to have a crucial role in mold recognition (70-73).

Several cell wall components such as β-glucans, chitins, and mannans act as ligands that are readily recognized by CLRs like dectin-1, dectin-2 and mannose receptor (MR). Dectin-1 is the major PRR involved in the recognition of β-glucans (74). CLRs collaborate with each other as well as other PRRs in mounting a mold-specific immune response (64). The lung surfactant proteins (SP) SP-A and SP-D also contribute to mold detection by trapping/agglutination of mold particles which enhance their phagocytosis by pulmonary alveolar macrophages (75). NLRs are a family of intracellular receptors that recognize internalized microbial components including mold (64), and they can cooperate with TLRs to activate pro-inflammatory cytokine production (76). They can be divided into the NOD subfamily including NOD1 and NOD2, and the leucine-rich repeat and pyrin domain-containing protein (NLRP) subfamily (77). Proteases from mold can be recognized directly by cell surface receptors on innate immune cells (epithelial and macrophages) like protease-activated receptors (PARs) which comprise a family of four 7-transmembrane G protein–coupled receptors (78). Secreted fungal proteases induce inflammatory responses by changing the permeability of epithelial barrier and by induction of pro-inflammatory mediators through PARs (26).

After a pathogen challenge, neutrophils are the first responders to be recruited and they have a higher phagocytic activity than macrophages; whereas monocytes are recruited later to the infected site within 24 to 48 hours (79). Freshly recruited monocytes display a pro-inflammatory phenotype

with high phagocytic activity, but during a few days they are differentiated into macrophages in the alveolar environment (80). The more specific adaptive immune responses develop slower, but crosstalk with the less specific innate cells (81).

A number of cell types may participate in the immune responses following inhalation of mold particles. The bronchial epithelium is pseudostratified and made up of a variety of cell types such as basal, ciliated, mucous-secreting goblet and secretory non-ciliated club cells many responding with cytokine secretion (82, 83). Type II cells recognize mold particles through membrane receptors and secrete pulmonary surfactant and cytokines. Moreover, these cells are involved in alveolar repair, by functioning as self-renewing cells and precursors of type I cells, which is primarily involved in gas exchange (83).

Airway macrophages play an essential role in innate immunity by secretion of a range of inflammatory mediators like cytokines and growth factors, crucial for the maintenance of healthy tissues (84, 85). Macrophages recognize and engulf mold into phagosomes, which subsequently fuse with lysosomes containing various antimicrobial effectors including proteases, antimicrobial peptides, and lysozymal enzymes. Increased formation of reactive oxygen (ROS) and nitrogen species (RNS), often called “oxidative burst”, are also important part in the defense against mold (86).

Tissue-resident macrophages may arise from embryonic origin and self-maintain locally (87, 88), or be recruited from circulating monocytes which in response to pro-inflammatory stimuli will be recruited to the lung (89, 90). Monocytes also participate in the surveillance of the lung, as they may enter tissues during steady state without differentiating to macrophages, and transport antigens from tissue to lymphoid organs (91, 92). Thus, thereby contributing both to adaptive immune responses and long-term immunity (93). Whereas blood monocyte-derived cells are pro-inflammatory, alveolar macrophages seems to have more anti-inflammatory functions and thus preventing excessive inflammation in response to environmental microbes (94). In response to tissue damage resident macrophages seems to become polarized toward a pro-inflammatory phenotype (95). However, the roles of monocyte-derived macrophages versus tissue-resident macrophages in the lung are still not fully understood (96).

1.2.3 Role of inflammation in disease linked to mold exposure

Mold may cause irritation or adversely affect human health through three specific mechanisms: direct infection by the organism, generation of allergic and non-allergic immune responses, or toxic effects from exposure to very high levels of mold byproducts such as mycotoxins and proteases (97-99). In severe cases, mold exposure may pose a life-threatening risk for invasive pneumonia in immunocompromised individuals (100). Allergic and non-allergic respiratory-related disease outcomes such as shortness of breath, coughing, wheezing, development and worsening of existing asthma, chronic bronchitis, allergic rhinitis, and other symptoms of upper respiratory tract are more often reported (3, 5, 6). Deposition on the mucous membrane initiates irritation that may result in conjunctivitis, allergy or asthma that are IgE mediated. Hypersensitivity pneumonitis, chronic rhino-sinusitis and allergic fungal sinusitis, are mediated by IgG and T-cell immune response. Between 3 and 10% of the world population is estimated to have IgE mediated allergy against mold (101), and 5% are predicted to develop clinical allergy, making mold exposure an important public health issue in many countries (99).

Whereas inflammatory reactions often are needed to destroy and remove intruders like mold, prolonged duration pro-inflammatory responses may lead to tissue damage and airway disease. Chronic inflammation is often found to be a key factor in mold-induced pulmonary diseases (Figure 1). There is a positive association between mold exposure and elevated levels of pro-inflammatory markers in the airways (102-104), which are linked to several respiratory diseases.

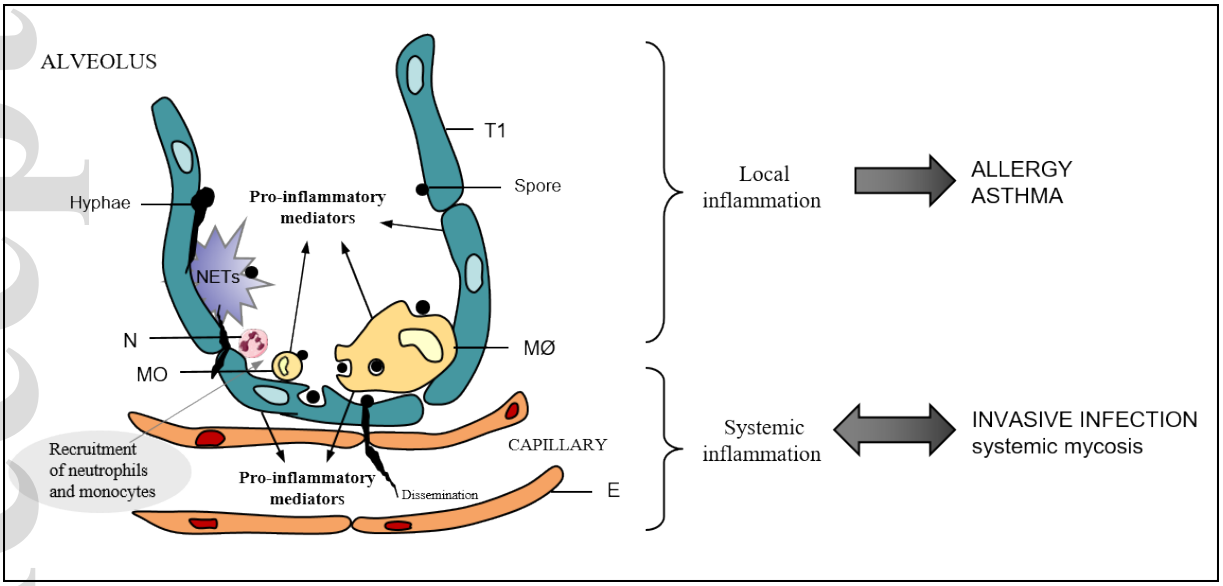

Figure 1: Illustration of how mold-induced inflammation may cause health effects. T1= Type 1 pneumocyte, MO = monocyte, MØ =

macrophage, E = endothelial cell, N = neutrophil, NETs = neutrophils extracellular traps. Modified from A, K. Bolling, PhD-thesis, Oslo, Norway2008.

2. Characterization and pro-inflammatory potential of mold particles

Many reviews on various aspects of indoor mold have been published (29), some focusing on characterization of exposure (105), others on health effects associated with indoor mold exposure (3). Here, we summarize some of the main methods for detection and characterization of fungal aerosols and highlight *in vitro* and *in vivo* research elucidating how molds may induce toxicity and pro-inflammatory reactions in human cell models relevant for airway exposure.

2.1 Methods for sampling of mold material

Experimental sampling of mold is most often done by sampling from specific mold species grown on culture substrate, or on gypsum board which is commonly used as model for indoor building material. Mold are cultured for various time periods which allow for more specific production of spores, hyphal fragments with or without mycotoxins (22).

By using cellophane covered semi solid enriched agar media, mycelial biomass free of spores can be grown on a solid substrate so to mimic the natural growth characteristics of molds. Depending on the experimental exposure studies, different approaches of sampling are used among which gently scraping of mold spores after flooding with an appropriate medium. Alternatively, mold particles including both spores and fragments may be aerosolized using a particle generator (53), so that the collected particles represent airborne particles. This technique is efficient for collecting spores but not mycelial fragments that were collected in too low amount for in vitro exposure studies. This sampling challenge can be efficiently overcome by grinding freeze-dried cellophane grown mycelial biomass followed by aspiration and size-fractionation with a cyclone, so to obtain the thoracic mold particle fraction.

Indoor sampling of mold particles from moisture-damaged buildings can be done from contaminated surfaces, house dust and air. Detection and quantification of mold particles are the preferred method for characterizing mold exposure indoor. However, as this requires more resources and time due to practical constraints, sampling of settled particles and microbial growth on surfaces are often used as an approximation. Airborne mold particles are most often collected with instruments specifically developed for sampling of biological material (106). Airborne

samples indoors will be a mixture of particle mater from various sources indoors as well as outdoors and need to be further characterized, never the less, this is the best approach for associating mold exposure with health outcome. Airborne sampling is too time consuming and costly to be used in large population studies. Thus, specific sampling from surfaces or house dust is most often used, although a quantitation of mold exposure is difficult. House dust consists mainly of textile fibers, and includes biological material from animals and plants as well as bacteria besides mold (107).

2.2 Methods for detection and characterization of mold aerosols

Different approaches has been taken in order to characterize experimental obtained mold material and identify mold in moisture-damaged buildings. Air samples can be collected on filters and on semi-solid nutrient media by impactors or in liquid impingers.

The sampling method is highly dependent on the method for subsequent analysis. Analytical methods can be divided in essentially different methods that quantify culturable organisms and methods that do not depend on culturability. As the proportion of culturable organisms is highly dependent on species, sampling strain and culture conditions the culture-based methods underestimate the collected microorganisms substantially and with great variability, and provide at best semi-quantitative results. On the other hand, when viability and species is important these methods are essential. In indoor air viability is rarely important and are the non-culture-based methods preferred.

Several comprehensive reviews of measurement methods of microorganisms have been published (108, 109). These are shortly described below and extended with recent developments (29, 110). Filter samples as well as material harvested from fungal cultures can be suspended into a liquid for further quantification and analysis.

2.2.1 Culture

Culturing is a selective method, which provides qualitative and quantitative estimate about fungi present in the sample that favor colony growth under the given conditions. By applying various growth conditions, analysis may target specific mold types. The results are reported as colony forming units (CFU) per volume of air sampled (108, 111). Culturing methods have been extensively used in the characterization of mold concentrations and mycobiota in indoor air. The

advantage of culture method is the possibility to assess phenotypic characteristics of fungal species for identification. Research from such investigations suggest that the composition of mold particle in indoor air shifts in the presence of dampness and can be dominated by certain 'indicator organisms'. Species indicative of increased dampness include *Penicillium chrysogenum, Penicillium expansum, Aspergillus versicolor, Aspergillus penicillioides* and also *Stachybotrys chartarum* (112, 113). Yet, the method is limited to detection and quantification of fungal spores and fragments that can germinate on synthetic media. One CFU may contain many viable cells. Not germinated fungal particles are overlooked, although these may be potential carriers of allergens, antigens or toxins, and the total fungal bioaerosol can be substantially underestimated (108). The sensitivity of the culture method has been estimated to be below 10 CFU per m^3 of air samples (114).

2.2.1 Microscopic methods

Microscopic analyses allow detection, morphological characterization and enumeration of fungal particles, regardless of their viability. However, although some spore types can be identified and quantified directly from samples, in general microscopic counting does not provide taxonomic discrimination. There are three major microscopic techniques commonly used in the detection and characterization of fungal aerosols: light microscopy, fluorescence microscopy and scanning electron microscopy (115). For indoor environments, direct microscopy is one of the methods that can be used in "Indoor Air Quality-related building" investigations.

Light microscopy is routinely used for detection and quantification of fungal aerosols. Fungal spores present in indoor or outdoor air samples can be recognized and quantified in light microscope, but staining with fuchsine acid improves visibility of hyaline spores (116). Shapes, sizes, colors and surface ornamentation of spores can be described to some extent, but classification to species level is difficult to achieve (108).

Fluorescence microscopy including *confocal microscopy* can be used for identification and quantification of fungal aerosols after staining with fluorochromes. The staining can be done either by means of specific proteins like antibodies or lectins, or fluorescing compounds that bind to chemical structures in the cells; e.g. acridine orange is commonly used to non-specifically stain nucleic acids in airborne microorganisms including fungi (114, 117) and fluorescein diacetate to

stain proteins. However, many fungal spores are not stained by fluorochromes, or if they do, their fluorescence from the dye can be masked by their natural pigmentation (118).

Scanning electron microscopy has been used for identification and quantification of spores and spore aggregates and hyphal fragments (108, 119, 120). It has also been used to confirm the occurrence of submicronic fragments in *in vitro* aerosolization experiments (121, 122). Detailed characteristics of surface morphology, shape and size can be revealed by scanning electron microscopy. However species recognition is not possible.

Immunolabelling of surface antigens for microscopic visualization is an efficient technique for detection and characterization of cells or microorganisms in a complex matrix. Polyclonal primary antibodies conjugated with detection probes are required in direct immunostaining. Spores from *Alternaria* sp. *Cladosporium herbarum, A. versicolor* and *P. chrysogenum* have been successfully stained with antibodies and gold particles that were resolved in scanning electron microscopy (55). Monoclonal or polyclonal primary antibodies and secondary antibodies with detection probes are required in indirect immunostaining of fungal or other microbial surface antigens (123). The choice of detection probe is dependent on the type of microscope. For fluorescence and confocal microscopy fluorochromes are used where excitation, emission wave length and bleaching characteristics of these compounds are important. Various fluorochromes have been tested on spores and large hyphal fragments with success (124, 125). For detection by high resolution in electron microscopy, electron-dense probes such as gold and silver are required (126, 127).

Microscopy is one of the best techniques to describe morphological characteristics of particles. *The performance of quantification* is dependent on the magnification, the size of the view field, the number of counted fields and exposed filter area (for filter sampling). Some spore types can be identified and quantified directly from samples, but in general, microscopic counting does not provide taxonomic discrimination. Despite the fact that microscopy is time consuming, direct microscopy is commonly used in indoor air quality investigations.

2.2.2 Flow cytometry

Flow cytometry has also been successfully used for quantification of fungal spores following appropriate staining with fluorochrome or dyes (128, 129). As an example, the presence of 8 - 260x10^3/m^3 fungal spores in outdoor air samples (Beijing China) was successfully demonstrated using flow cytometry following staining with Calcofluor-White M2R fluorochrome (129).

However, flow cytometry does not reveal the shape characteristics and the fungal source of quantified particles.

2.2.3 Chemical and biochemical methods for detection of fungal aerosols

Different fungal constituents have been used as biomarkers for detection of fungal aerosols in indoor or outdoor air. Use of membrane constituents such as ergosterol, phospholipid-derived fatty acids (PLFA) and β-glucans have been reported Also fungal allergens, antigens, fungal alcohols (mannitol and arabitol) and mycotoxins have been used to demonstrate occurrence of airborne fungal particles. Moreover, molecular techniques based on quantitative polymerase chain reaction (QPCR) have also been used to reveal the presence of fungi in indoor bioaerosols. These methods are suitable for quantification of fungal biomass in complex matrix, but do not provide any information on fungal particle morphology.

Ergosterol is the main sterol of the fungal membrane and is considered as a robust indicator of total fungal biomass (111, 130). Ergosterol has been used as a biomarker of fungal biomass in settled house dust (131), in materials from moldy buildings in outdoor particulate matter with size <2.5 μm ($PM_{2.5}$) and particulate matter with size between 2.5 and 10 μm ($PM_{2.5-10}$) samples (132, 133). Analysis of ergosterol requires high performance liquid chromatography (HPLC) (limit of detection: 500 pg/sample) or gas chromatography/tandem mass spectrometry (GC/MS) (detection limit: 10 pg/sample) (130). The average range of 0.68-1.89/ pg spore versus 1 μg/mg mycelia have been reported (130, 133). Although ergosterol is a highly specific estimator of fungal biomass, it is labile and degrades quickly after cell death (134), suggesting that non-viable and older fungal fragments will be underestimated.

Fungal phospholipid-derived fatty acids (PLFA). There are two membrane PLFA (18:2ω6 and 18:3ω3) that are fungi specific (135). The 18:2ω6 has been used for viable fungal biomass quantification in complex matrix like soil (136). Both PLFA have been quantified in air samples as surrogate for fungal biomass (137). The concentration of PLFA in *Cladosporium* spp. ranged between 0.15 and 1.75 pg/spore. However, the use of PLFA is limited to detection of fresh or viable fungal biomass due to their fast degradation after fungal cell death (136).

Beta-glucans are polymers of glucose molecules linked through β-(1→3); β-(1→4) and β-(1→6) linkages (138). They can be quantified in environmental samples by Limulus amoebocyte lysates (LAL) or antibody based assays (139-141). In environmental samples, the LAL assay is more

efficient with detection limit of 20 pg/mL (142, 143) as compared to the antibody based assay (800 pg/mL and 42600 pg/mL with monoclonal and polyclonal antibodies, respectively) (140, 144). Recently, Rivera-Mariani et al reported a halogen immunoassay adapted to microscopic quantification of (1→3)-β-D-glucan-bearing airborne particles (145).

The presence of (1→3)-β-D-glucans in plant materials, algae and bacteria reduces their specificity as biomarkers of fungal biomass (146). However, β-glucans independently from their origin have been shown to be potent activators of different immune cells including macrophages and neutrophils (141, 147).

Other fungal alcohols. Arabitol and mannitol have been suggested and used as specific tracers for fungal spores in environmental samples (132, 148). Mean values of 1.2 (range: 0.8 – 1.8) and 1.7 (range: 1.2 – 2.4) pg were estimated per spore for arabitol and mannitol, respectively. High correlations were found between the concentrations of arabitol (R^2=0.75) or mannitol (R^2=0.70) in outdoor air and fungal spores measured by flow cytometry (129).

Immunoassay for enzymes, antigens and allergens. Different enzymes have been used as molecular tracers for airborne fungal biomass. N-acetyl-β-D-glucosaminidase (NAGAse) and N-acetyl- hexosaminidase (NAHA) have been associated with the presence of fungal biomass in airborne or settled dust in moldy indoor environments (59, 149-151). This method is commercially available as Mycometer Air™. Antigens and allergens can be detected by immunoassays based on monoclonal or polyclonal antibodies against specific antigens or allergens present in the fungal particles, usually by enzyme-linked immunosorbent assay (ELISA) techniques (152).

The polymerase chain reaction (PCR) is a molecular technique based on the extraction of fungal genomic DNA followed by exponential amplification of a given DNA fragment (primer) *in vitro* using the polymerase chain reaction. For fungal species detection, primers of the internal transcribed spacer (ITS) region of the nuclear ribosomal coding cistron are used. This region has been proposed as an universal DNA barcode for fungi (153) and has been sequenced for fungal identification in indoor airborne or settled dust (154). There are two consensus primers (ITS4 and ITS5) that have been used to amplify the region between 3'18S-like and 5'28S-like genes including the 5.8S gene and 2 internal transcribed spacers (155). Other species specific primers can be used for that purpose (156).

Furthermore, fungal aerosols can be quantified using quantitative PCR (QPCR) of genomic DNA in spores from targeted species. QPCR uses a fluorescence reporter whose signal increases proportionally with the quantity of the PCR products. To date mold specific QPCR assays have been developed at EPA (United States Environmental Protection Agency) with probes and primers for about 100 fungal species (157). Results are reported as equivalent DNA per spore unit in bioaerosol samples. The advantage of this method is that identification to species level is possible, which is important for species profile characterization in indoor air. However, genomic DNA extraction of fungi is challenging because of varying efficiency, and the number of nuclei per cell/spore. It is not known whether submicronic fragments contain genomic DNA.

2.3 Mold induced toxicity and pro-inflammatory reactions in vitro

A lot of important information useful for approaches for testing of complex indoor samples is obtained from experimental research on clean and more well-defined mold preparations in different experimental models.

2.3.1 Mold samples

Many studies are conducted on viable spores (102, 158), some studies distinguish between inactivated spores, germinating spores and hyphae (71, 103, 159, 160), whereas others have studied the effects of mold extract (161, 162). As mold do not need to be intact or viable to have a significant health impact (40), various methods to inactivate/"kill" the mold are often used to study cellular responses triggered by specific mold stages (71, 163, 164). Treatment by X-ray are often recommended, as this method for inactivation of mold samples does not alter enzymatic activity or surface molecules important for the immune responses (71, 163, 164).

Resting spores are often found to be more inert toward recognition by the immune system when compared to swelling spores and hyphae (102, 165-168). This is partly due to pigments such as melanin and the rodlet layer composed of the hydrophobic RodA protein on the outer surface of spores. These protective layers are lost upon swelling and germination (49, 50), resulting in appearance of polysaccharide moieties and other more immunological active surface components (47, 51). The core component β-glucan is consider to be an important factor as it has been found to trigger strong inflammatory responses (169). Chitin which contribute to the rigidity and integrity of the cell wall (48), may interact with immune cells and trigger both pro- and anti-inflammatory immune responses (170). Galactosaminogalactan has pivotal roles in the adherence of hyphae to

surfaces and the formation of an extracellular matrix that glues hyphae together (171). It is consider to protect the fungal cell wall from immune recognition, as well as to modulate the immune responses (172). Using extracts, many immunological important mold components have been detected. However, the method may also have unrevealed masked elements in the mold that cells not necessarily will be exposed to. The cytotoxicity of mold species are highly dependent on growth conditions, due to the fact that this will influence their capacity to synthesize and excrete mycotoxins as well as various proteases (52, 173). Mycotoxins may have a variety of different biological effects; the immune modulating are often related to its capacity to induce cell death (52). Dust sampled for mycotoxins have shown less than 1–43 pg/mg of dust (174, 175). Thus, indoor air samples most often reveal very low concentrations of mycotoxins, and a causal link between inhalation of mycotoxins and health problems is yet to be established (29, 98, 176, 177). Proteases, mostly linked to the hyphal stage, have been suggested to compromising mucociliary clearance as well as altering the permeability of epithelial barrier and thereby activating innate immune responses (178). Obviously, it very difficult to determine "the most" important immune triggering component of mold, as this will be highly dependent on its natural environment as well as the host and immune response under investigation.

Several studies have reported that hyphal fragments induce more inflammatory reactions than spores (42, 103, 179-181). In a recent study, mold spores and hyphal fragments from *A. fumigatus*, *P. chrysogenum*, *A. versicolor* and *S. chartarum* were isolated and inactivated by X-ray. They were characterized by number and morphology, mycotoxins, β-glucan and protease content/activity as well as pro-inflammatory properties (182). As expected hyphal preparation had a higher total level of β-glucans than spores. However, only the hyphal preparations of *A. fumigatus* and *P. chrysogenum* were more potent than the respective spore preparations, showing that swelling and germination not necessarily result in appearance of immune active surfaces in all mold species. Most interestingly, none of the characteristics of the mold preparations alone could explain the differences in their pro-inflammatory potential, illustrating that the response is a result of multiple factors as discussed above.

Table 1: Species differences in inflammatory responses

Mold species	Inflammatory responses *	
	Hyphal fragments	Spores
A. fumigatus	↑ ↑ ↑ ↑	↑ (↑)
P. chrysogenum	↑ ↑ ↑ (↑)	↑
A. versicolor	↑	↑
S. chartarum	↑	↑

*A rough simplification of the relative inflammatory responses (cytokine release) based on studies in various macrophage models (THP-1 macrophages and human monocyte derived macrophages; IL-1β, IL-6 and -8) and epithelial cells (IL-6 and -8) based on studies (167, 168, 182); illustrating that the inflammatory responses are highly depending on the mold species.

The pro-inflammatory responses between mold species vary substantially, and growth conditions may change their potency (173, 182, 183), as exemplified in Table 1. In a study in human macrophage models hyphal fragments of the pathogenic *A. fumigatus* were found to induce cytokines at concentrations that were in the order 10^3-10^4 lower than those from *A. fumigatus* spores and from less pathogenic *A. versicolor* hyphal fragments, however, a rather similar cytokine profiles were seen (167). Previous studies have reported similar differences between the two *Aspergillus spp.* in mouse dendritic cells (104). In accordance, no production of inflammatory mediators was induced by *A. versicolor* in mouse RAW264.7, human 28SC macrophages or A549 lung epithelial cell lines (184).

Variation in pathogenic potential may in principle be determined by the molds' capacity to form hyphae and/or the immune cells' capacity to recognize and destroy certain mold species. Pathogens like *A. fumigatus* have been reported to escape recognition by the immune system (185). However, there are no apparent correlation between the pathogenicity of *A. fumigatus* versus *P. chrysogenum* and *A. versicolor* their pro-inflammatory effects (168). Actually, the inflammatory potential of *A. fumigatus* hyphal fragments were higher than the two less pathogenic species tested. In strong contrast to *A. fumigatus*, spores of *P. chrysogenum* germinated poorly *in vitro* (168). A mouse study found that resting *A. fumigatus* spores formed germ tubes in the lung tissue rapidly after inhalation, whereas no signs of germination of *A. versicolor* spores were seen

(158). Thus, the spores' ability to germinate *in situ* may be of more importance for pathogenic properties than the molds potential to escape the immune system.

Although *A. fumigatus* is a pathogen, it poses no severe threat, as its relative levels are commonly low in indoor air (186). Since chronic inflammation is linked to various health outcomes, sustained release of pro-inflammatory cytokines due to continuous exposure to prevalent mold species with strong pro-inflammatory potential may be more critical to health. Furthermore, substantial higher levels of mold fragments compared to spores are often found in indoor air samples (28, 60, 187). Thus, hyphal fragments of more prevalent species with strong pro-inflammatory potential including *P. chrysogenum* (168, 182) could be pertinent candidates for respiratory diseases associated with mold exposure.

From experimental studies it seems important to not only quantify the total amount of mold, but also the specific mold species and the relative proportion of hyphal fragments versus spores. As the growth conditions may affect the molds pro-inflammatory potential (188), this type of testing should preferentially be done on environmental samples. Although individuals show characteristic allergy linked IgE immune responses for different mold (23), the cytokine profiles obtained after exposure to different mold species, as well as to either spores or hyphal fragments, seems to be rather similar (167, 168). Thus, pattern of cytokine response in experimental systems or in human studies does not necessarily give a straight answer to the type of immune triggering causal agent.

2.3.2 Experimental systems in vitro

In general, *in vitro* studies may provide information regarding cellular mechanisms linked to mold-induced disease. They may also be useful for rapid screening of larger number of samples for several endpoints under well-defined exposure conditions. Although macrophages, neutrophils and epithelial cells are known as primary producers of inflammatory mediators in the lung after exposure to fungi (184), monocytes and dendritic cells are also recruited to the lung during inflammation (80).

Monocytes. Monocytes are often directly involved in the very early effector phase of infection and seen as the major producers of interleukin (IL)-1β during inflammation (189). The monocytic cell line THP-1 (human peripheral blood, leukemia) is commonly used as a simplified model for human blood monocytes to study cytokine production following immune stimuli including various mold (162, 182, 190, 191). THP-1 monocytes has been reported to be far more sensitive than

epithelial cells, and slightly less than THP-1 macrophages with regard to IL1-β and tumor necrosis factor (TNF)-α release following exposure to mold (182, 190). There have been well-conducted studies using both THP-1 cells and primary human peripheral blood monocytes strongly suggesting that the THP-1 model does not seem to be a very representative (192, 193). In fact these studies showed that THP-1 cells are less responsive to lipopolysaccharide (LPS), than human peripheral blood monocytes due to low levels of CD14, an important part of TLR4 signal transducing protein complex (192). Nevertheless, studies on THP-1 may give interesting information.

Test system using whole human blood or monocytes (fresh or frozen) has been used broadly to assess cytokine release for diverse immune stimuli also including spores from various fungi. Fungal spores from various mold species may induce the release of IL-1β and TNF-α by human whole blood (194, 195). Although the absolute response of the donors are found to differ, the ranking of different stimuli in response of individual blood donors was found to be the same (194). Monocytes have also been successfully used in mechanistic studies. However, as monocytes are not exposed to mold unless infection, they are not often used to test mold samples typical for indoor air.

Macrophages. Typically, macrophage *in situ* seems to require a double stimulation to induce IL-1β release, while this is not required in blood monocytes (196).

The RAW 264.7 cells are a monocyte/macrophage-like cells originating from Abelson leukemia virus transformed cell line derived from BALB/c mice, which are often used to initially screen natural products for bioactivity and to predict their immunological potential *in vivo* (197). Exposure to living *A. fumigatus* spores increase the expression of TNFα, MIP-1α, MIP-1β, and MCP-1 in RAW 264.7 (102). Even though there were no differences between the phagocytosis of live and heat-killed spores, heat-killed spores were found to be inactive in the expression of chemokines and cytokines. Fungal spores of *S. chartarum, A. versicolor*,or *P. spinulosum* are reported to be weak inducers of inflammatory mediators in RAW 264.7 (184, 188). Despite apparently giving a rather low response to mold spores, RAW 264.7 cells has been described as an appropriate model for studying phagocytosis (198). Upon LPS stimulation RAW 264.7 cells increase nitric oxide (NO) production and enhance phagocytosis. Furthermore, these cells are able to kill target cells by antibody dependent cytotoxicity. An advantage is that it is easy to use as it need no further differentiating to be used as a macrophage model.

In contrast to RAW 264.7 cells, human THP-1 monocytes need to be differentiated in order to be used as a macrophage model, often by treatment with phorbol-12-myristate-13-acetate (PMA) (199-202). The THP-1 macrophages differentiation process provide several changes in phenotypes including loss of proliferation, increased adherence and changed expression of receptors (200). The THP-1 macrophages model is very sensitive to both X-ray killed spores and hyphal fragments and respond with a broad cytokine response seen at very low doses (167). However, PMA treatment also interfere with essential mechanisms of the cytokine production (203). Accordingly, the treatment have been found to give a high cytokine baseline (167). Furthermore, in strong contrast to what is seen in monocyte derived human macrophages, THP-1 macrophages give high release of IL-1β following exposure to low concentrations of LPS (196) and mold (167, 190). Besides the high release of IL-1β, the response seems to be rather similar to that seen in human monocyte derived macrophage. Nevertheless, it should be pointed out that the often used THP-1 macrophages are not always a reliable model for human macrophages *in situ,* due to its high release of IL-1β.

Human primary macrophages derived from peripheral blood from healthy volunteers represent a practical and interesting model as they also may be derived from frozen monocytes. *In vivo* and *ex vivo* macrophage-differentiation from human blood monocytes is driven by human granulocyte-macrophage colony–stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF), which may induce a pro-inflammatory or an anti-inflammatory phenotype respectively (204). Both states of differentiation are reversible as macrophages may adapt and change according to stimuli (200). Monocytes may also be differentiated into a type of dendritic cells (205). The various models have successfully been used in various advanced mechanistic studies.

Monocyte derived macrophages differentiation with GM-CSF for studying pro-inflammatory has been suggested as a model relevant for lung with an ongoing infection (206, 207). In a recent study, testing various mold samples relevant for indoor air monocyte derived macrophages were markedly less sensitive to mold exposures compared to THP-1 macrophages, as the significant responses started at 100x higher concentrations (167). Interestingly, human monocyte derived macrophages had a marked upregulation in the IL-1β expression which was not reflected in a correspondingly high release of protein. This may indicate that these cells are primed in an alert state, in which IL-1β may be rapidly released if exposed for signal triggering the second step in the cytokine release process. The cytokine response observed besides IL-1β and the relative potency

between the various mold species, spores versus hyphal fragments tested were rather similar in the two different models.

Macrophages isolated from sputum with an adequate macrophage purity and viability and has been characterized and suggested as a primary airway macrophage model (208). Although indeed being a potential interesting model, the sputum macrophage model still needs to be refined as it was found to have high background and give a very low cytokine/chemokine response following exposure to mold particles (167).

Neutrophil granulocytes also play a major role in the early immune defense against IA, as they are able to prevent germination and kill fungal hyphae through the release of ROS, phagocytosis, or formation of neutrophil extracellular traps. Increased ROS as well as pro- and anti-inflammatory cytokine release has been reported to be strongly induced in both murine and human macrophages upon co-culture with *A. fumigatus* germ tubes, while resting spores were mostly inactive (209). However, human and murine neurophiles differed in their pattern of cytokine response, emphasizing the advantage of using human cell models. Neutrophils greatly enhanced the upregulation of co-stimulatory molecules on dendritic cells exposed to *A. fumigatus in vitro*, a process that was dependent on cell contact and the dendritic cell receptor DC-SIGN (210). Thus, there may be an immunomodulatory cross talk between neutrophils and dendritic cells in response to *A. fumigatus* that promotes the maturation and efflux of lung dendritic cells.

Dendritic cells represent an important bridge between innate and adaptive immunity as they process fungal antigens and subsequently stimulate specific T-cells *via* antigen-presentation. Human monocyte-derived dendritic cells are generated from leukocyte concentrates from healthy human donors by the treatment with GM-CSF and IL-4 (211), but may also be derived from murine bone marrow (209). Mechanistic studies done *in vitro* with such cells have shown that they secrete various pro- and anti-inflammatory cytokines after exposure to *A. fumigatus,* however, the responses seen in murine and human immune cells are different (209).

Epithelial cells are a major target of inhaled mold particles as they make up the interface between the external environment and the internal milieu. Alveolar and bronchiolar cell lines and primary cells from various species has been used, although not so often as macrophages models. The human bronchial epithelial cell line BEAS-2B has been reported to have great homology in gene expression to primary human cells compared to other cell lines (212). Conidia from *A. fumigatus*

in studies with BEAS-2B suggest that only a small fraction of conidia are internalized (119), while the majority stay adherent to the surface of cells or are washed away during sample processing. *A. fumigatus* conidia are also taken up by alveolar epithelial A549 cells (213). They stay alive for longer periods when compared to macrophage, and although their germination are reduced, these cells may serve as a kind of reservoirs for immune cell evasion and dissemination throughout the host (213).

While exposure to living spores or mold extract may give cytokine secretion responses in epithelial cells, exposure to resting spores give almost no pro-inflammatory responses (168, 182). While X-ray treated hyphal fragments *A. fumigatus* and *P. chrysogenum* did not induce release of IL-1β in BEAS-2B cells, increased secretion of other cytokines such as IL-6 and IL-8 are seen. The responses in epithelial cells are, however, seen first at much higher concentration in comparison to most of the human macrophage models (168, 190).

The IL-1 family member IL-33 plays a critical role in type 2 innate immune responses to mold allergens (214, 215) and is an important mediator of allergic asthma (216). Although the release of IL1α, IL-1β, IL-33 and TNF-α was not significantly increased in BEAS-2B cells after mold exposure, notably these cytokines/alarmins were markedly increased at gene level (168). Apparently, the secondary proteolytic cleavage of their pro-forms by caspase-1, calpain and TACE which is required for release, was not initiated by hyphal fragments or spores. Such steps may, however, be activated by exposure to living mold and extracts of mold components. Indeed, studies on cultured human airway epithelial cells subjected to acute challenge with *A. alternata* have increased secretion of IL-33, which depended critically on DUOX1-mediated activation of epithelial epidermal growth factor (217). Most interestingly, DUOX1 expression and allergen responses were found to be enhanced in cells from asthmatic patients. A serine protease from *A. alternata* filtrate applied to human bronchial epithelial cells (16HBE14o-) induced changes in intracellular Ca^{2+} concentration, by cleaving PAR-2 (218).

Despite the apparently low pro-inflammatory responses in BEAS-2B cells, the epithelial cells communicate with immune cells and *vice versa,* and even low mediator levels may contribute to modify responses *in situ*. Airway epithelial cells may regulate innate and adaptive immunity in the lung by expressing various molecules that can modulate immune cells. Moreover, alveolar macrophages coordinate the recruitment of neutrophils and monocytes to the lung through the release of IL-1β, which induces IL-8 production by the respiratory epithelium (219).

Recently more advanced experimental *in vitro* models mimicking various part of the human respiratory system has been developed including lung-on-a chip (220), stem cell-derived models (221), and primary cells obtained from various parts of the human respiratory system (222). These respiratory primary cells can be further differentiated to human lung tissue consisting of multiple cell types. Host infection were studied by adding conidia and germ tubes from *A. fumigatus* into a trans-well membrane model of the alveolus with human alveolar epithelial cells (A549,) and endothelial cells (HPAEC) (223). They found a relatively low number of differentially expressed genes in A549 upon contact with *A. fumigatus,* and an even lower and very limited differential gene expression profile in HPAECT. The addition of dendritic cells to simulate the spatial and cellular complexity in the alveolus greatly increased the number of differentially expressed immune genes (223). On the other hand, the monocyte-derived dendritic cells added were also proven to reduce the mold induced toxic responses (224).

Human bronchial epithelial cells on insert with or without macrophages may also differentiate and form cilia and start mucus secretion (225). In such polarized air-liquid interface (ALI) culture with 16HBE cells, *A. alternata* extracts stimulated release of IL-8 and TNF-α, leading to a concomitant reduction in trans-epithelial electrical resistance (TER) (225). A further examination of these effects with protease inhibitors suggested that serine proteases mediated the effects. ALI cultures from asthmatic donors exhibited a reduced IL-8 response to *A. alternata* extracts, as compared to those from healthy controls, while neither responded with increased thymic stromal lymphopoietin (TSLP) release. However, only cultures from asthmatic donors were susceptible to the barrier-weakening effects of *A. alternata* (reduced TER), suggesting a more susceptible bronchial epithelium of severely asthmatic individuals (225).

Table 2: Relative inflammatory responses in experimental models

Experimental system	Hyphal fragments IL-1β*	Hyphal fragments IL-6 and -8	Spores IL-1β	Spores IL-6 and -8
THP-1 macrophages	↑↑↑↑	↑↑↑↑	↑	↑
Monocyte derived macrophages	↑	↑↑↑↑	-	↑
Sputum derived macrophages	↑	-	-	-
Epithelial cells (BEAS-2B)	-	↑↑↑	-	-

*A rough simplification of the relative inflammatory responses (IL-1β, IL-6 and -8 release) after exposure to *A. fumigatus* (hyphal fragments or spores) seen in various human macrophages models as well as in BEAS-2B lung epithelial cells based on studies (167, 168, 182); illustrating that the specific responses are highly depending on experimental system.

As illustrated in Table 2, the responses to mold exposure observed in the various cell models were indeed very different in terms of both quantitative and qualitative responses. Based on the current data combined with literature, human monocyte derived macrophages seems to be an interesting and relevant test system for *in vitro* studies that should be further explored for testing of more complex samples of indoor air. However, further studies are needed to clarify the most relevant experimental model for studying pro-inflammatory effects of air samples; which should balance between being simple and useful for high throughput screening, sensitive and giving responses relevant for the synthesis and release of pro-inflammatory markers *in situ*.

2.3.3 Experimental systems in vivo

For ethical and cost reason, the use of animal studies for these type of testing should be limited, and preferentially be replaced by validated alternative *in vitro* methods. Furthermore, there are genetically as well as environmental factors to take into consideration when extrapolating from animal studies to the human situation. Thus, information from *in vitro* studies of human cells give important additional information to animal studies. As discussed in the following section, animal studies add important understanding of health effects that may be linked to mold exposure. They also illustrate that the results obtained from *in vivo* studies are highly depending on factors including type and dose of mold sample, type of exposure and time after exposure for collecting the samples.

A number of experimental studies on animals have shown that inhalation exposure may result in pro-inflammatory effects. Spores of *A. versicolor* are frequently present in the indoor air. One month after exposure to spores of *A. versicolor* via aspiration, germ-free rats had high proliferation

rate of cells in lymphoid tissues, increased foamy alveolar macrophages, increased expression of IL-1 and frequent granulomatous lesions (226). In another study, mice were exposed via intra-tracheal instillation to various single doses of *A. versicolor spores.* The highest TNF-α and IL-6 concentrations in lungs were measured at 6 hours after the dose, and they returned to the control level by 3 days (227). Some inflammatory responses were observed already at the dose level of 1×10^6 spores of *A. versicolor.* A radical increase in the non-specific inflammatory mediator were seen at higher doses, probably due to overload of the alveolar macrophages capacity to phagocytose the spores (227). TNF-α and IL-6 are also found to be important components of host defense in *A. fumigatus*-induced inflammation in mouse lungs (228). Increased production of TNF-α protein in response to inhalation of *A. fumigatus* spores have also been detected in rat bronchoalveolar lavage (BAL) cells, 95–98% of which are macrophages (229). Notably, expression of IL-β was not markedly enhanced within 24 hours following exposure to *A. fumigatus* spores, which in line with a controlled release of IL-1β in lung tissue due to continues expose to microbiota.

Inflammation-associated gene transcription and expression in mouse lungs has been reported to be induced 12 hours after intratracheally instilled single doses of low molecular weight compounds (toxins) from mold common on damp building materials (230). Expression of transcriptionally regulated genes was confirmed using immunohistochemistry that demonstrated MIP-2 and TNF-α staining in respiratory bronchiolar epithelia, alveolar macrophages and alveolar type II cells. The authors suggested that the inflammatory nature of metabolites/toxins from such fungi may contribute to the development of non-allergenic respiratory health effects.

Studies in mice and humans describe plasticity among T_H17 and transitional stage T_H1 effector populations in secreting IL-13 in response to inhalation of foreign antigens (231-233). This plasticity is suggested to be due to re-programming of T_H1 and T_H17 cells to secrete T_H2 cytokines.

A recent study on BALB/cJ mice showed that repeated inhalation of *A. fumigatus* spores can result in dysregulation of immunological mediators impacting the pulmonary architecture and function (234). In exposed mice the immune response were similarly skewed toward an allergic phenotype, resulting in significant expansion of diverse subpopulations of $CD4^+$ T cells, whereas Tc17 cells were no longer detectable. Antifungal T cell responses (IFN-γ(+) or IL-17A(+)) co-expressed IL-13, and total IgE production was augmented in animals repeatedly exposed. The study emphasize

the role of pulmonary innate recognition of inhaled fungal spores in providing composite signals for promoting fungal clearance and allergic responses. Based on the results, the authors suggested that personal exposure to low doses of fungal bioaerosols for long durations may result in the development of allergic responses involving moderate airway inflammation (234). Interestingly, mice pre-treated with vehicle of the major allergen (a serine protease) from *A. fumigatus* (*Af*a) has been reported to promote airway hyper-responsiveness by infiltrating the bronchial submucosa, and thereby disrupting interactions between airway smooth muscle and cell-extracellular matrix (235). Thus, illustrating how exposure to *A. fumigatus* may result in modification of pulmonary architecture and function as described above. Another indoor fungi with potential health issues is *S. chartarum*. In a recent sub-chronic exposure study in mice, immuno-pathological responses including Th2 biased immune responses with pulmonary arterial remodeling occurred earlier in mice exposed to strain producing more fragments as compared to the one producing more spores (236).

Epidemiological studies have reported strong correlation between *A. alternata* sensitization and an increased risk of severe and fatal asthma (237, 238). Mice exposed to *A. alternata* extract intra-nasally develop an allergic airway response characterized by increases in lung expression of the type 2 cytokines IL-4 and IL-13, eosinophil infiltration, and high levels of serum IgE (239, 240). Exposure to *A. alternata* is characterized by a predominant T_H2 response, but may also induce factors associated with T_H17 responses (e.g., CXCL8) from epithelial cells (241). Human with asthma have various distinct phenotypes, including differential expressions of T_H2 and T_H17 signatures (242, 243). IL-17A expression and neutrophils are present at high levels in patients with severe, persistent asthma (244). Studies in mice suggest that the suppression of neutrophil responses is dependent on T_H2 cytokine production by T cells and that airway neutrophilia is primarily an innate response to allergen (241), thus once more illustrating the important crosstalk between innate and adaptive immune system and the relevance of testing for pro-inflammatory responses.

2.4 Induced toxicity and pro-inflammatory reactions experimentally mixed mold samples

The microbial exposure in houses with dampness related health problems consists of a heterogeneous group of components, including live and dead bacteria and fungi. The possible interactions for cytotoxicity and inflammatory potential are huge and complex, and still little is known. A study in mouse RAW264.7 macrophages, spores of *S. chartarum* alone did not evoke

any inflammatory responses in these cells, however, when exposed in conjunction with a low dose of the gram-positive bacterium *Streptomyce californicus* they triggered a significant dose-dependent cytokine response (245). On the other hand, no other synergistic increase were seen among the tested six indoor air microbes (245).

Interestingly, studies have documented the synergistic effects of bacterial endotoxins such as LPS and abiotic factors such as mineral particles (246, 247). Recently, a similar synergistic releases of IL-1β in primary human airway macrophages, peripheral blood monocyte-derived macrophages and in BEAS-2B cells after co-exposure of *A. fumigatus* hyphal fragments and crystalline silica (190). Thus, there may be unwanted synergistic effect of between various type of microbes and other type of air pollution that are not easily revealed in epidemiological studies.

As microbiota are considered to be a natural part of the lung, and play a crucial role in developing and maintaining the pulmonary immune system (248-251). Unbalanced microbiota may cause irritation and modify the epithelial barrier, thereby increasing probability for infection, as well as allergic and non-allergic pro-inflammatory reactions which often are linked to health effects such as asthma and sinusitis (251, 252). Similarly to unbalanced microbiota in the gastrointestinal tract (253), unbalanced lung microbiota may also skewed a developing immune system towards a more allergic phenotype.

2.5 Induced toxicity and pro-inflammatory reactions of indoor samples

There have also been studies where complex indoor samples have been tested in different experimental models for markers mediating inflammatory reaction such as NO and cytokines. Such studies have shown to give relevant information when assessing the immunotoxic potential of moldy house microbes and organic dust.

In an intervention study, the effect of moisture-damage repairs on the exposure and on the upper airway inflammatory responses of the occupants were evaluated and compared with inflammatory potential of air-borne particle samples (254). After the repair of moisture-damage, concentrations of the measured airborne microbes decreased. The samples had significantly lower production of IL-6 and TNF-α in RAW264.7 macrophages and the concentration of IL-4 in the nasal lavage samples was significantly lower after the renovation. These results illustrate a correlation between various approaches taken to measure repair of the moisture damage. The immunotoxic potential (cell death, TNF-α and IL-6) of the furniture dust from moisture-damaged buildings tested in

RAW264.7 cells were also found to decrease after cleaning with steam wash and ozone (255). RAW264.7 macrophages exposed to indoor air filter sample extracts representing 'low' and 'high' exposure to viable fungi or bacteria suggested that bacterial stains induced more profound production of NO, TNF-alpha and IL-6 than the studied fungal strains as well as a decrease in cell viability (256). Altogether, these results suggest that indoor air bacterial strains are potent inducers of inflammatory responses and thus possibly related to adverse health effects of the inhabitants. However, it should also be noted that the relative immune response between bacteria and mold will be highly depending on experimental model used.

Occupational health symptoms related to bio-aerosols exposure containing microorganisms and microbial components have been observed in a variety of working environments. Personal filter samples collected from the subjects living areas homes and working places with high microbial exposure induced a significant increase in the production of cytokines in the RAW264.7 macrophages, as compared to those from the subjects with low exposure (257). Using an *in vitro* assay based on granulocyte-like cells the total inflammatory potential of bio-aerosols were found to be well-correlated with more direct method to measure microbial composition (149). High inflammatory values measured with the granulocyte assay were found to be correlate with bio-aerosol samples from areas that were highly contaminated and was found to react to multiple contaminants in the environment.

Monocytes are the major producers of IL-1β during inflammation. Memory tends to enhance the reaction of monocytes, which are the effector cells in inflammatory reactions, thereby making them more efficacious in combating the potential danger. Thus, the induction of innate memory could help increasing the host resistance to infections without causing excessive local tissue damage (189). Most interestingly, the release of IL-8 and IL-1β after stimulation of whole blood with *A. versicolor* was increased in subjects exposed to moisture damage (258). Furthermore, in the exposed subjects the IL-1β release was significantly enhanced after *in vitro* stimulation with *E. coli* endotoxin (1000 pg/mL). The authors concluded that certain features of the innate immune system (receptor expression and mediator release of monocytes) are altered in subjects exposed to moisture damage which may be a potential explanation for the increased incidence of respiratory health diseases observed in these populations.

3 Perspectives and future studies

Recent studies find that a substantial part of the mold particles in indoor air are hyphal fragments, which possess different and often much stronger pro-inflammatory effects than spores. The inflammatory potential differs highly between various mold species and some of the highly prevalent non-pathogenic species may give strong pro-inflammatory responses. Although acknowledging the fact that it is not easy to know the most relevant experimental model for human exposure, it should be noted that some these pro-inflammatory responses may be detected at very low concentrations. Thus, future epidemiological studies should perform better characterization and quantification of the air samples, and include hyphal fragments from both pathogenic and non-pathogenic species. The possibility to include more direct analysis of pro-inflammatory responses should be further explored, as this may give valuable additional information to epidemiological studies exploring adverse respiratory health effects of indoor dampness/mold.

A number of relevant *in vitro* lung cell models have been established and used to compare different inflammatory responses of mold particles. These models should be further developed and evaluated in order to find a high throughput sensitive and relevant test model (or battery) for characterizing the pro-inflammatory potential of samples from indoor environments. More complex *in vitro* models with polarized differentiated human bronchial epithelial cells grown on insert with macrophages and endothelial cells in ALI should be explored to further explore the relevance of these findings.

Recently, a connection between subtle innate immune system elements and fungal allergic disease is being revealed. Thus, further studies should address whether chronic fungal exposure may lead to changes in the innate immune system and how such changes are linked to the adaptive immunity and allergic disease such as in patients with allergies. More specifically, it would be interesting to more directly compare the effects obtained in primary cells from susceptible sub-populations (e.g. asthmatics) versus those from healthy population. Furthermore, important information with regard to the role of environmental factors may be obtained by doing similar studies on cells derived from their corresponding stem cells. Finally, there is a need for research on effects on microbiota in the lungs and linked to combined exposure with biotic as well as abiotic air pollution.

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