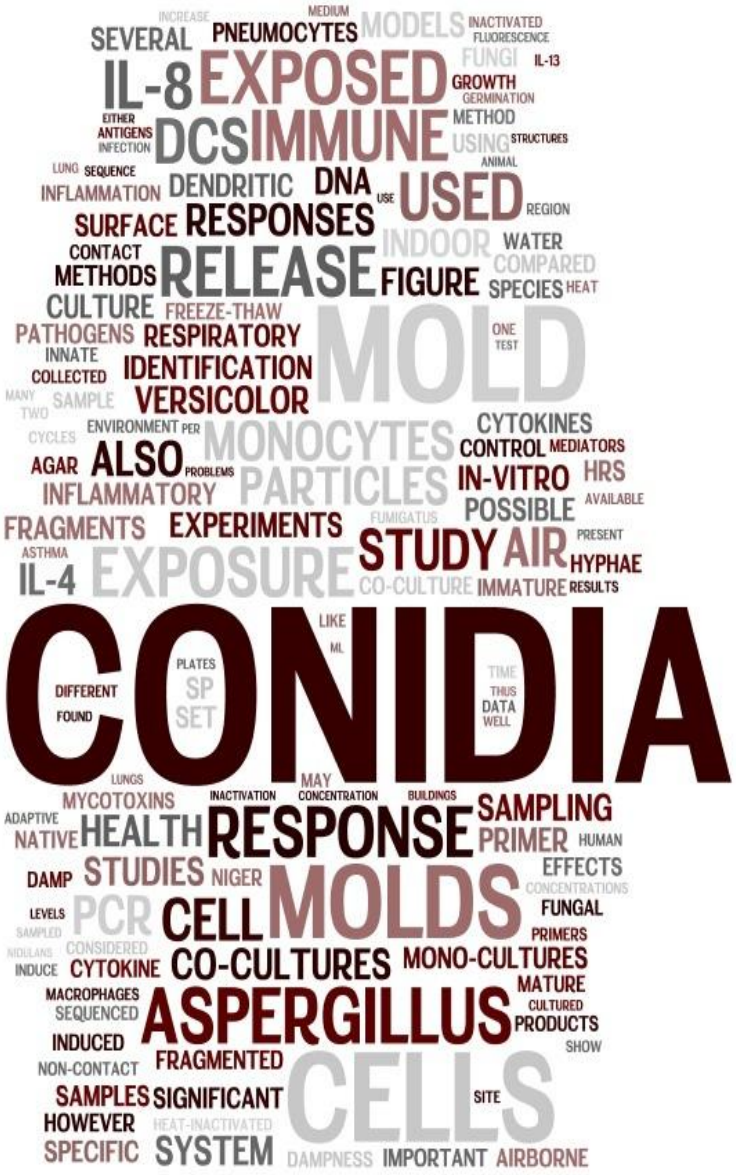

Characterization of Mold in Indoor Air and Development of Respiratory Illness:

Developing Co-Culture Techniques to Reveal How Fungal Conidia in Norwegian Indoor Air Might Cause Respiratory Health Problems

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By

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Anders Benteson Nygaard

Abstract

Living in damp or moldy homes has shown consistent associations with respiratory or allergic health effects. However, the causal link for these associations remains unclear. In this study we wished to establish a model in which it is possible to assess damp and moldy indoor environments and then study isolated molds from these environments in in-vitro models to understand how they potentially may cause respiratory health problems.

This study collected air samples from damp/water damaged rooms and used polymerase chain reaction (PCR) sequencing along with other tests to determine the identity isolated molds from damp environments. Subsequently, selected mold isolates were selected to be used for in-vitro exposure experiments with cell mono-cultures and co-cultures (THP-1 monocytes (M), THP-1 derived dendritic cells (DCs), A549 pneumocytes (P)). These cell cultures were exposed to mold conidia that were native, heat inactivated or freeze-thaw fragmented. After exposure, the release of cytokines was evaluated with enzyme-linked immunosorbent assay (ELISA).

With the use of PCR, identification of isolated molds was in general only possible at the genus level. Some isolates were identified at the species level. Of 12 molds isolated from damp environments, 9 were found to be *Aspergillus* species. In-vitro models were exposed to conidia from several species of molds. In contact co-cultures with M in contact with P and non-contact with DCs, *Aspergillus versicolor* was found to induce response of IL-8 whereas in monocultures, several species were found to induce IL-8 release.

With use of methods tested and developed in this study, it is possible to study actual isolated molds from damp environments. When combining methods for identification of molds in damp environments with in-vitro methods to study the molds immunological affects, the understanding of damp/moldy indoor environments in relation to respiratory health problems can be improved.

Sammendrag

Å leve i fukt- eller muggskadede hjem har vist en konsekvent assosiasjon til luftveis eller allergiske helse effekter. Men de avgjørende faktorene som utløser disse assosiasjonene er fremdeles ukjente. I denne studien ønsket vi å etablere en modell som gjøre det mulig å vurdere fukt og mugg skadede miljøer, og studere isolere muggsopper fra disse miljøene, for å så bruke isolerte muggsopp i in-vitro forsøks modeller så man kan forstå hvordan de kan skape luftveishelseplager.

Denne studien samlet luftprøver fra fuktige og vannskadede rom og brukte polymerase chain reaction (PCR) sekvensering sammen med andre analyser for å identifisere muggsopp isolert fra fuktige rom. Deretter ble utvalgte muggsopp brukt videre i in-vitro eksponering forsøk med celle mono-kulturer og co-kulturer (THP-1 monocytter (M), THP-1 deriverte dendrittiske celler (DCs), A549 pneumocytter (P)). Disse cellekulturene ble eksponert for muggsoppsporer som var naturlige, varmeinaktiverte eller fryse-tine fragmenterte. Etter eksponering ble nivåer at cytokiner målt med enzyme-linked immunosorbent assay (ELISA).

Når PCR ble brukt for identifisering av isolerte mugsopp, så var det som regel bare mulig å identifisere til slektsnivå. Noen muggsoppisolater ble identifisert til artsnivå. Av 12 muggsopp isolert fra fuktige innemiljø ble 9 funnet til å være av arten *Aspergillus*. In-vitro modeller ble eksponert for sporer fra muggsopp av flere arter. I kontakt co-kulturer, med M i kontakt med P og ikke-kontakt med DCs ble det funnet at *Aspergillus versicolor* induserte utslipp av IL-8, mens i monokulturer fant vi at flere arter av muggsopp induserte utslipp av IL-8.

Med metodene som har blitt testet og utviklet i denne studien, så er det mulig å studere muggsopp som er isolert fra steder med fuktskade. Når man kombinerer metodene brukt for å identifisere muggsopp i fuktskadde miljøer med in-vitro metoder for å utforske immunologiske effekter, så kan man øke forståelsen av hvordan fukt eller muggskadede hjem relaterer seg til luftveisplager.

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Abbreviations

bp	Basepair
CFU	Colony forming units
CSF	Colony stimulating factor
DCs	Dendritic cells
DG18	Dichloran glycerol 18 agar
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
HIV	Human immunodeficiency virus
IL	Interleukin
IMDC	Immature dendritic cells
IOM	Institute of Medicine
M	Monocytes
M+P/IMDC	Contact co-culture
MDC	Mature dendritic cells
MEA	Malt extract agar
MHC	Major histocompatibility complex
NOPL	Nasopharyngeal region
P	Pneumocytes
P/IMDC	Non-contact co-culture
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PRR	Pattern recognition receptors
RBA	Rose Bengal agar
SAB	Sabouraud Dextrose Agar
SEM	Standard error of mean
sp.	Specie
spp.	Species
TB	Tracheobronchial region
Th	T helper cells
TLR	Toll-Like receptors
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
UV	Ultraviolet
WHO	World Health Organization

1 Introduction

The importance of a healthy indoor environment is becoming ever more recognized. In the modern-day society more time is being spent indoors than ever before. In North America the average adult spends almost 90% of their time indoors [1], a number that probably is applicable to most European and industrialized nations. During the course of an average day most people sleep, eat, and recreate at home. In addition, most people work indoors. As a result of this, the exposure to airborne substances in the indoor environment becomes an important factor when health effects are measured and investigated. An unhealthy indoor environment may result in long-term exposures to unhealthy substances and cause health problems, if the problem is not identified and handled.

1.1 Damp buildings and visible mold

Sometimes, inhabitants will notice a moldy odor in their home, perhaps a dark spot in the ceiling, or maybe the wallpaper bulging off the wall. It could possibly be after a particularly heavy rainfall, maybe there was a severe pipe leak several years ago, or a new sink was just recently installed. Excessive building dampness may come about in a variety of ways, and many housing units and buildings experience excessive dampness at some time. A study conducted in Denmark, Sweden, Norway, Iceland, and Estonia estimated a prevalence of indoor dampness in 18% of housing units [2]. This estimate agrees with several studies conducted in other countries as well, whereas some studies in the United States have estimated the prevalence of dampness and mold to 50% of houses [3].

Dampness and moisture problems in buildings are closely connected to observed mold and other microbial growths. A damp indoor environment is problematic because it provides the moisture to support the growth of molds, bacteria and insects [4]. Dampness in buildings may have many different sources. It may stem from external sources, such as seepage of rainwater, groundwater spreading into the foundation, or flooding. Internals sources of dampness could be leaking water pipes, high relative humidity and condensation, or the use of wet construction material at the time of construction. If dampness and moisture problems are allowed to fester, either because the problem is ignored or it is out of sight, it can lead to growth of molds which sometimes may go undetected for a long period of time. Dampness and exposure to mold is a fairly common problem, and reported estimates state that 18% to 50% of buildings suffer some sort of dampness or mold related problems [5].

Figure 1. Examples of dampness related mold. A: Mold on roof beams due to condensation, B: Mold behind a skirting board, C: Mold infestation on a roof hatch due to condensation, D: Mold infestation behind wallboards in basement. All photos are courtesy of Mycoteam. Photos are obtained from www.mycoteam.no/bilder/#1.

In the later years a large number of studies conducted in several geographical regions have reported associations between indoor dampness/ molds and respiratory or allergic health effects. A handful of recent reviews and meta-analyses have summarized the findings and epidemiological evidence from over a 100 studies [6], and concluded that there is sufficient evidence to of an association between inhabiting a damp or moldy building and the prevalence of a range of respiratory and allergic health problems. This particularly relates to children but also adolescents and adults can be affected. The comprehensive reviews have been conducted by organizations like the Institute of Medicine (IOM) in 2004 [4] and the World Health Organization (WHO) in 2009 [3]. In addition, co-worker studies like those conducted by Bornehag et al. in 2001 [7] and Antova et al. in 2008 [8] and Mendell et al. in 2011 [5], as well as the meta-analysis conducted by Fisk et al. in 2007 [9] are some of the published works that all have confirmed the association of health problems with dampness/mold.

Table 1. The findings presented by the review of the Institute of Medicine in the 2004

Level of confidence for association	**Health outcomes in relation to**	
	Exposure to damp indoor environment	**Exposure to damp indoor environment, with presence of mold or other agents**
Sufficient Evidence of an Causal Relationship	No outcomes met this definition	No outcomes met this definition
Sufficient Evidence of an Association	-Upper respiratory tract symptoms -Wheeze -Cough -Asthma symptoms in sensitized asthmatic people	-Upper respiratory tract symptoms -Wheeze -Cough -Asthma symptoms in sensitized asthmatic people -Hypersensitivity pneumonitis in susceptible people
Limited or Suggestive Evidence of an Association	-Lower respiratory illness in otherwise-healthy children -Asthma development -Dyspnea	-Lower respiratory illness in otherwise-healthy children
Inadequate or Insufficient Evidence to Determine Whether an Association Exists	-All other health outcomes considered	-All other health outcomes considered

Modified from WHO Dampness and Mould review (2009)[3].

Even though the epidemiological studies have established an association between dampness/mold and health effects, there is still much to be learned: what are the specific agents causing health problems, by which mechanisms do these exposures cause health problems, and how can we better assess an unhealthy indoor environment. These are just some of the questions that still need to be answered. In order to develop preventive strategies for improving the indoor environment, a unified method for assessing indoor climate is neccesary [3, 4].

The IOM suggests a broad-based approach to gathering and evaluating information about exposures and other related factors. This way it could be possible to identify which agents are causing health problems, and once these are identified it will possible to evaluate these in depth and develop specific quantifiable measures. This in turn could lead to more effective prevention strategies [4].

1.2 Fungi and molds

Fungi are eukaryotic organisms and are classified as a separate kingdom apart from animals, plants, and bacteria. Molds are fungi that grow in multicellular filaments called hyphae. Also in the fungal kingdom are yeasts and mushrooms [10]. Unlike molds, yeasts are unicellular organisms that are incapable of forming true hyphae, however some yeasts can form pseudohyphae by consisting of a chain of connected budding cells [11]. The web of hyphae that forms when molds germinate is referred to as mycelium and this is the vegetative visible part of a mold. The cells in the hyphae that make up the mycelium are genetically identical, and thus a mold colony is considered a single organism [12].

1.2.1 Conidia

Conidia are a reproductive structure of molds. Most fungal conidia are 2-20 μm in size and come in a variety of shapes and colors, depending on the specie of the mold [10]. They are highly adapted for survival, and are resistant to heat, drought, and mechanical and chemical attacks [13]. Conidia form on specialized hyphae called conidiophores. Conidiophores have many distinct shapes and usually contain a high number of conidia that are ready to be released.

Conidia are highly adapted for dispersal. Their small size allows for them to be carried by air and they can be spread over large areas. Conidia from outdoor molds can easily travel into houses and buildings by air, or on the surface of new building materials or clothes. Once inside, usually only the presence of moisture is required and fungi can readily germinate on most surfaces.

1.2.2 General growth conditions

Most indoor fungi germinate at temperatures of 10-35^{o}C, so temperature is rarely a limiting factor in the establishment of molds indoors. As long as there is sufficient humidity and accessibility to nutrients, most molds will readily germinate. However, fungi is capable of extracting nutrients from many sources and can live off house dust, food, animal or plant material, paint, glue, and nutrients dissolved in water. Since molds are efficient in finding sources of nutrients, humidity is the limiting factor in the establishment of molds. If water is accessible, then most molds will be able to establish colonies, and thus it becomes clear why indoor dampness or water damage is so closely related to molds in homes.

1.2.3 Mycotoxins

Mycotoxins are low-mass secondary metabolite biomolecules that are produced by fungi. Some of these may be toxic to humans, for example by interfering with protein synthesis or causing DNA damage. There are many different types of mycotoxins; aflatoxins which are carcinogenic, trichothecenes that inhibit RNA and protein synthesis, and penicillins which are an important pharmaceutical antibiotic [14]. Mycotoxins are usually considered an agricultural problem, as

foods contaminated with mycotoxins can pose a big threat to both livestock and humans [15]. Several mycotoxic molds thrive in crops such as nuts, grains and seeds, thus mycotoxins have long been considered an important factor in food safety [3, 4, 16]. To become a factor for respiratory health, mycotoxins must become airborne. Mycotoxins can be found on the surface of conidia and hyphal fragments from toxin producing molds, and have been detected in air and dust samples of damp and water damaged buildings [17, 18].

The mycotoxins that perhaps have received the most attention in relation to indoor air are trichothecenes, which can be produced by the mold *Stachybotrys chartarum*, which is often found in water damaged buildings. These are potent mycotoxins that are suspected of being involved in pulmonary hemorrhage, a potentially fatal condition caused even by low doses. Studies have measured the presence of trichothecenes in air and dust samples from damp and water damaged buildings, and some have found that presence of trichothecenes can be associated to dampness and water damage [3].

Aflatoxins are produced by several species of *Aspergillus* and *Penicillium*, which also are common in damp and water damaged buildings. It has been speculated that inhalation of aflatoxins may cause cancers, especially in the cases of factory workers or farmers who handle large quantities of nuts or livestock feed. However, a direct association with cancer has not been established [3].

Most studies of exposures to mycotoxins have relied on data from animal studies. By comparison with the ingestion pathway, few studies have been done exposure via the respiratory pathway. However, the dose of exposure in most studies has been in the range of ½ lethal dose of mycotoxin, and it still remains unclear whether the levels of airborne mycotoxins in natural conditions are sufficiently high to cause health problems [3]. Another hypothesis is that a slow clearance of particles carrying mycotoxins could lead to longer residence time in the airways, thus increasing the time for absorption of mycotoxins from molds or fragments [4].

1.3 Characterization of Fungal Exposure in Homes

A central role of epidemiological studies is the assessment of exposure to environmental agents in indoor air.

1.3.1 Methods for detecting and sampling fungi in indoor environments

There are several sampling methods developed for detecting fungi in both healthy and damp/moldy buildings. Most sampling methods can be placed in one of two categories: surface sampling and air sampling. Surface sampling can be performed by, for example taking a sample of the construction material infected with mold or by pressing a piece of tape on the mold

infected surface. Both these samples can then be examined under a light microscope and give clues to which species of mold are present. A culture based method is simply involves pressing an agar plate on the affected surface, and mold conidia and hyphae fragments will stick to the agar plate. The sample can then be incubated allowing the study of the molds more in depth, perhaps allowing more accurate identification and characterization of the molds. The method also enables quantification of the mold [19].

The second category, air sampling, usually involves drawing air through a sampling machine, and trapping airborne particles and microorganisms on for example a filter. The trapped particles can then be extracted from the filter for further analysis. Another air sampling example is impingement into liquids, where sample air is drawn through a liquid, causing airborne particles to become suspended. Probably the most established method is air sampling with an impactor, where the sample air is directed onto an adhesive or agar surface, thus causing airborne particles such as conidia, to be bound to the surface. The agar plates can then be incubated directly without further treatment [20, 21]. By enumerating how many viable conidia were collected, this method can be used to calculate colony forming units (CFU) per cubic meter of air. This can give a quantifiable measure as to how much mold is present in an environment, and higher CFU/m^3 has been associated with water damage and dampness [20, 22].

1.3.2 Phenotypic identification of molds

The classical method for identifying molds and fungi is identification based on phenotypic traits. This is a culture method that involves growing the mold and observing its traits. It is possible to study the reproductive structures of the mold, like the shape of conidia or conidiophores, under a light microscope. Recognition of growth patterns, size, height, coloration of conidia, and rate of growth are all macroscopic traits that can be used to identify molds. It is also possible to select for growth at specific temperatures or detect for presence of mycotoxins or other biochemical compounds. Several textbook atlases have been published to help systematically assess morphological traits to identify molds [23].

It is not always possible to determine the identity of molds with culture methods, as some molds may have atypical morphology. Sometimes cultivation of molds requires specialized growth media that might not be readily available. The biggest problem is that different species can bear high resemblance to each other. With molecular identification, molds that have been thought to be identical have been determined to be separate species [23].

1.3.3 Molecular identification of molds

Polymerase chain reaction (PCR)/sequencing is a well-established molecular method for identification based on amplification of specific segments of DNA. It has become one of the

preferred methods for determining the identity of indoor biological contaminants, and techniques for extracting and amplifying fungal DNA from air, water and surface samples have been developed. The benefits from using PCR and DNA for identification compared to cultivation methods is that PCR is fast, accurate and can provide objective data. PCR is also capable of detecting dead and fragmented organisms as long as there is DNA present; whereas culture methods of identification rely on the presence of viable conidia and will not detect dead fungal fragments [24-26].

By knowing a sequence of DNA from whatever mold you are searching for, it is possible to design specific DNA-primers that amplify that specific sequence of DNA. After 30-40 cycles of amplification it will be possible to visualize on an agarose gel the presence DNA amplified from the targeted mold in the sample. It is possible to desing primers that are specific to single species of mold, or primers that can detect molds of several genera. For example FF2/FR1 is a primer set designed to only amplify DNA of some genera of common indoor molds, while EUK1209/UNI1392 is a primer set designed to amplify all eukaryotic DNAs [26, 27]. Even if different species give PCR products with the same primer sets, their separate PCR products may vary, both in terms of basepair (bp) length or base composition. Therefore, it is possible to identify separate molds using the same PCR primer set. If the PCR products vary greatly in size an agarose gel can be used. If they differ only in composition the technique Denaturing Gradient Gel Electrophoresis is preferred. Alternatively, after DNA sequencing, the sequence data can be entered into DNA databases such as the GenBank sequence database (http://blast.ncbi.nlm.nih.gov/) and compare to other available sequences [23, 26]. Based on other reported sequences it could be possible to determine the identity of the mold from which the PCR product came.

1.4 Respiratory Health Effects of Exposure to Molds

1.4.1 Respiratory system

The function of the respiratory system is uptake of oxygen and removal of carbon dioxide in the body. The surface area of the respiratory system when laid out amounts to 150 m^2 and the average adult circulates about 10 000 liters of air through the respiratory system in a day [28, 29]. On a given day the respiratory system inhales a variety of airborne particles, ranging from road dust, tobacco smoke, organic dust, and mold particles. The upper respiratory system, made up of the nasopharyngeal region (NOPL), functions to moisture the inhaled air, adjust the temperature, and filter the air before it reaches the lower respiratory system. The tracheobronchial region (TB) carries the air down to the lungs. The bronchioalveolar region

(BAL) of the lungs is the respirable area of the lungs (Figure 2). This is where the exchange of oxygen and carbon dioxide occurs, and it is the part that makes up the biggest surface area of the lungs [29, 30].

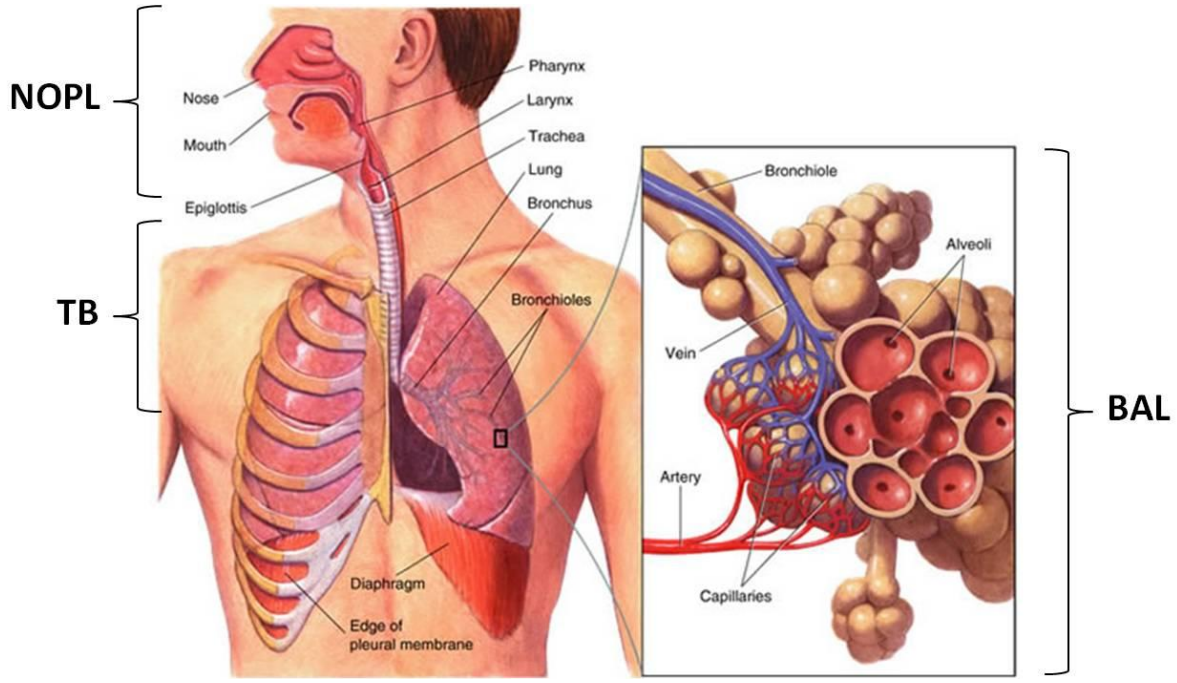

Figure 2. Schematic illustration of the human respiratory system separated into the nasopharyngeal region (NOPL), tracheobronchial region (TB) and the bronchioalveolar region (BAL). (modified from **http://www.goldiesroom.org/Note%20Packets/13%20Human%20Other/00%20Human%20Other%20Systems--WHOLE.htm)**

1.4.2 Deposition of mold particles in the lung

The deposition of particles in the lungs is determined by the aerodynamic properties of the particles. The shape and size of mold particles will determine the probability of where they will deposit in the lungs. In general, smaller particles are capable migrating further down in the respiratory system. Large particles, bigger than 2.5µm, will normally be deposited in the nasopharyngeal region. These particles are usually cleared by mucus that gets transported to the throat and swallowed. Particles that are smaller than 2.5 µm will pass through the upper respiratory system, and deposit in the tracheobronchial region or in the bronchioalveolar region [30]. Most mold conidia are between 2 and 20 µm in size, so the deposition of molds can vary greatly according to the genus of the mold. Particles that are smaller than 10 µm are generally considered to be respirable [30]. However, several molds are capable of reaching the bronchioalveolar region of the respiratory system where they can trigger immune responses (Figure 3). Mold fragments are also inhaled. These are usually much smaller in size than conidia and will also be deposited deep in the lungs [31]. The concentration of airborne fragments is

often much larger than airborne conidia [32]. The particles that penetrate deep down in the lungs takes longer time to clear out from the respiratory system.

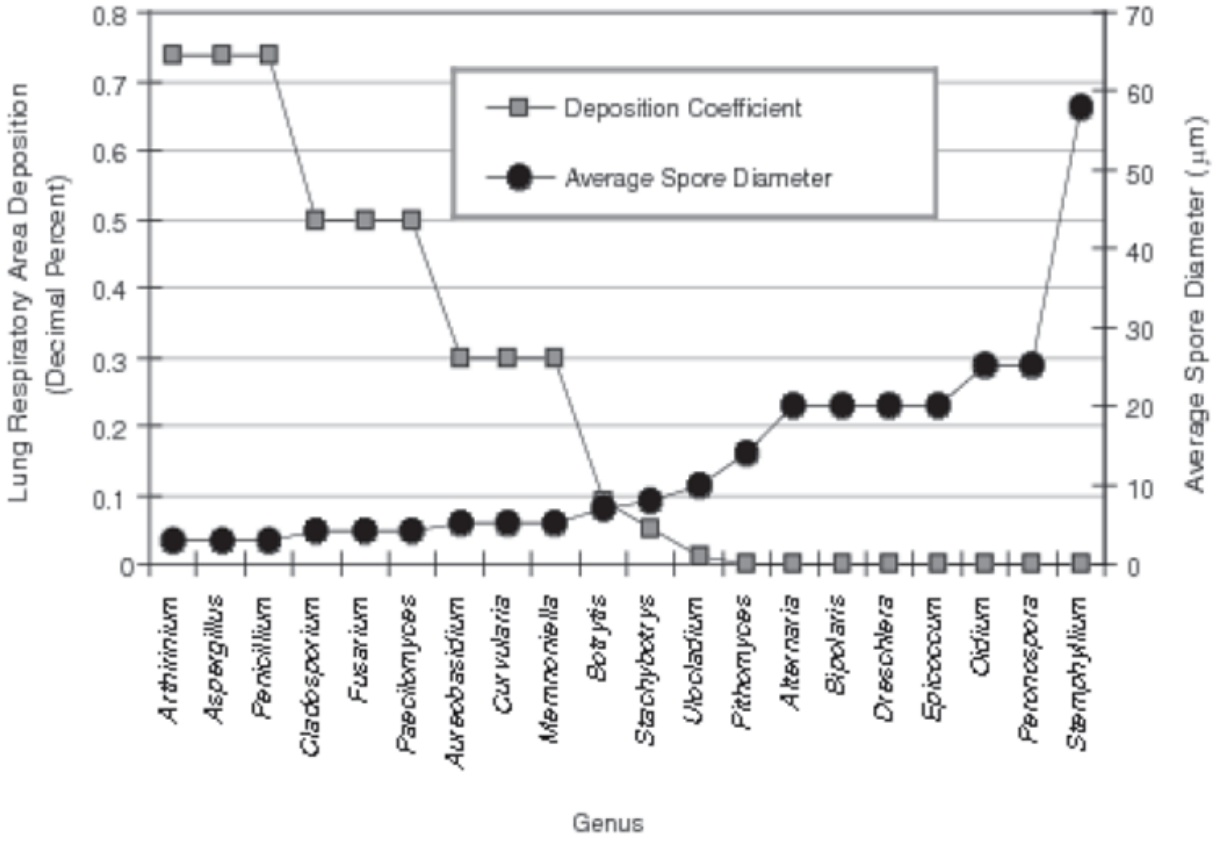

Figure 3. Conidial sizes for different genera of molds, and percentage of conidial deposition in the bronchioalveolar region of the respiratory system. IOM (2004) [4]

Deposition of mold particles does not only occur in the bronchioalveolar region of the lungs. Also small mold particles will deposit in the tracheobronchial region or in the nose or mouth (Figure 4). Humans are constantly exposed to mold conidia and fragments [10], and the body is normally capable of clearing out the majority of these particles before they reach deep into the lungs [32].

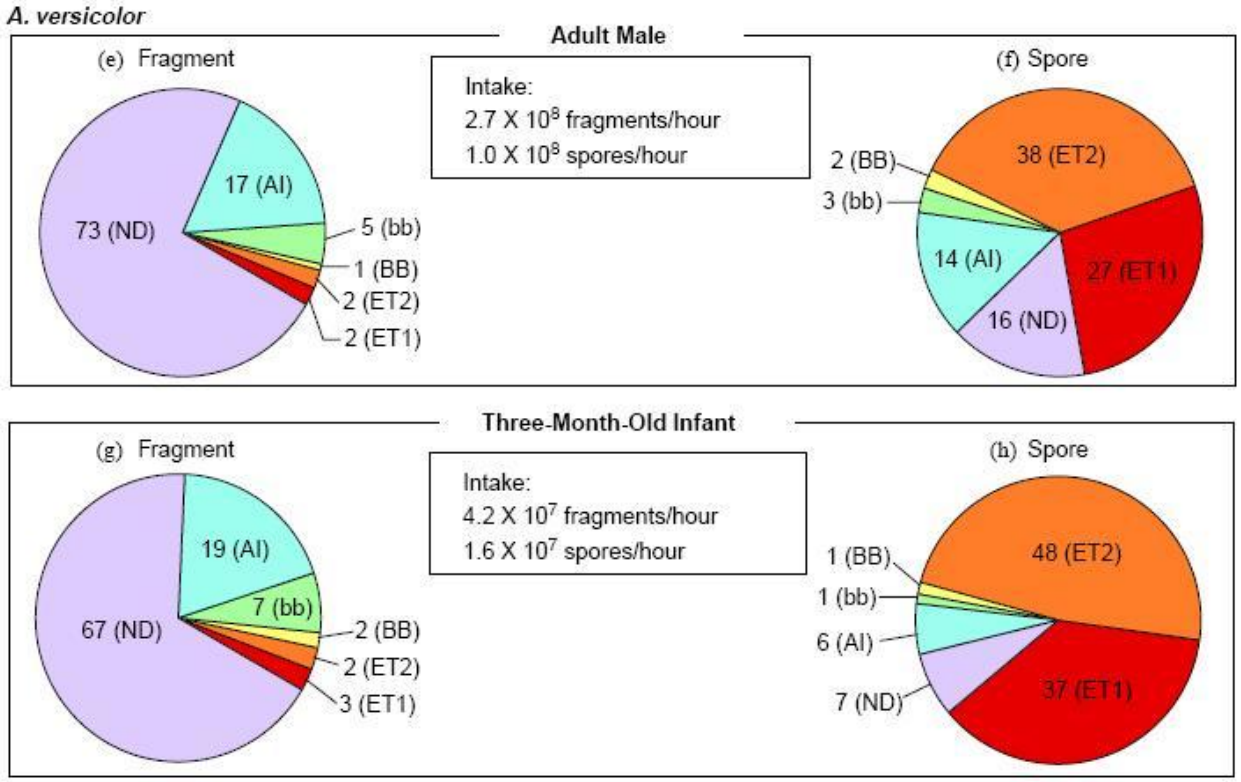

Figure 4. Modeling the deposition of fragments and conidia (spores) (%) of *Aspergillus versicolor* into different regions of the respiratory tract for 1 h of exposure. ET1 indicates the anterior nasal region; ET2 indicates the main extrathoracic region (ET1 and ET2 corresponds to NOPL); BB indicates the bronchial region (BB corresponds to TB); bb indicates the bronchiolar region; AI indicates the alveolar interstitial region (bb and AI corresponds to BAL); ND indicates not deposited particles. The total and fractional depositions were calculated for an adult male and a 3-month-old infant. Obtained from Cho et al. [32].

1.4.3 Cells of the alveolus

Pneumocytes are epithelial cells found inside the alveolar space. They allow transport of gases between the inhaled air and the network of capillaries in the lung. Type I pneumocytes are the most prominent cells, and make up more than 93% of the alveolar surface. They are approximately 0.1 mm thick, providing short diffusion distances for gases [33]. Type II pneumocytes are much more numerous, but only make up about 7% of the alveolar surface. They produce surfactant, and in cases of damage and repair they serve as progenitor cells to Type I pneumocytes [29].

Alveolar macrophages are important since they eliminate pathogens and particles that deposit in the alveoli. They are phagocytes that contribute to the maintenance and remodeling of lung tissue, and are together with Type II pneumocytes responsible for the repair and remodeling of tissue injury in the lungs. Macrophages are thought to be essential in regulating the immune system by secreting a variety of mediators upon infection. During inflammation, monocytes will migrate to the infected alveoli to help clear particles and coordinate the immune responses [30].

Endothelial cells are cells that make up the lining of blood vessels and capillaries. They are very abundant and make up approximately 40% of the cell population in the lungs. These cells, like the Type I pneumocytes are very thin, so that diffusion of gasses can take place over short distances [30].

1.4.4 Health effects of mold

Molds and fungi can affect human health by three different processes: 1, by infection; 2, by toxic effects; and 3, by causing allergies or chronic inflammations [34, 35]. Infections are rarely a problem for most people with a healthy immune system. However, for immune compromised people infections are a much more common problem. In hospitalized patients taking immunosuppressants or patients with Human Immunodeficiency Virus (HIV), invasive fungi can cause severe health effects [20, 36, 37]. The toxic effects of fungi are due to mycotoxins. Most reports of poisoning by mycotoxins are usually attributed to ingestion of toxins [15]. Toxins can also be inhaled, but usually only become a health factor when inhaled in large quantities, as in the case of some farmers and factory workers [34]. When the body develops antibodies to inhaled fungi, symptoms like allergic asthma or allergic rhinitis are likely to occur. People can become sensitized to molds and experience several adverse health effects because of this [38, 39]. In cases of chronic exposure to mold particles, chronic inflammation of the lungs can occur. The innate immune system will respond to inhaled particles by causing inflammation. However, this may cause tissue damage to the lungs [40, 41].

1.5 Immune response

The first line of defense against inhaled mold particles is the innate immune system, where the response is primarily orchestrated by cells such as monocytes, macrophages and neutrophils [42]. The response of the innate immune system is highly unspecific and generalized and will respond to a range of pathogens and work to eradicate and incapacitate these as soon as possible. The result of this response is most often inflammation at the site of injury or infection.

If immune responses are insufficient, as in cases of immunocompromised individuals, inhalation of mold conidia can lead to mold infections. The conidia will be able to germinate when immune system cannot incapacitate or remove the conidia. Deep tissue mold infections rarely pose a problem to immunocompetent individuals [34]. On the other hand, if immune responses are exaggerated, exposure to mold conidia may lead to several adverse health reactions such as allergy, hypersensitivity, asthma, and inflammatory tissue damage [34, 42, 43]. Consequently, it is essential to have a well-balanced immune response to mold conidia and other pathogens; otherwise one is susceptible to a range of different health problems.

The immune response of the body is a result of the interaction between wide varieties of signaling molecules such as cytokines released by tissues and immune cells at the site of infection. These signaling molecules play an important role when it comes to orchestrating the inflammatory response, as well as the allergic response to invading pathogens [44].

1.5.1 Innate immune response

The innate immune system is the body's first line of defense to invading micro-organisms and pathogens. If pathogens are able to pass the physical barriers of the body, such as the skin or the mucous membranes, the innate immune system provides an immediate response. The response by the innate immune system is an abrupt non-specific one, meaning it will respond to a wide range of pathogens in a generalized manner. Cells of the innate immune system make use of Pattern Recognition Receptors (PRRs), such as Toll-Like receptors (TLRs) and Dectin-1 receptors, to recognize and respond to a wide variety of pathogens. PRRs recognize pathogens by identifying molecules on the pathogen surface that carry patterns common to a broad range of pathogens. As soon as foreign pathogens are recognized by PRRs, the innate immune system has a number of ways for neutralizing them.

One of the primary responses of the innate immune system is inflammation; this is a generalized response in which pathogens are engulfed by phagocytosis, primarily by neutrophils but also to some extent by monocytes and macrophages. Inflammation is often marked by pain, redness, heat, and swelling at the site of infection. Cells involved in inflammatory response are neutrophils, and later also monocytes, macrophages and lymphocytes. When PRRs have been activated at the site of infection, the inflammatory response is mediated by a variety of pro-inflammatory signaling molecules.

The innate immune system is mediated by a number of cells. Monocytes play an important role as progenitor cells that circulate in the body. They can be referred to as immature macrophages, as monocytes will circulate in the bloodstream for about 36 hours before they mature into macrophages. In some conditions, in the presence of specific cytokines, monocytes can also differentiate into dendritic cells (DCs). Monocytes also migrate to sites of infection, where they differentiate into macrophages or DCs, and release a number of cytokines to mediate the inflammatory response.

Neutrophils make up the biggest share of leukocytes, about 60 - 70 %. They have a relatively short lifespan of about 8 -12 hours and are constantly renewed from the bone marrow. These cells are essential in the inflammatory response. They are the first to react to infections, and neutrophils will rapidly accumulate at the site of infection [45]. There they actively engage in

phagocytosis of pathogens. Once pathogens are internalized, neutrophils will undergo programmed cell death (apoptosis). This is essential to prevent damage to healthy tissue [46].

Macrophages are the tissue-counterparts to monocytes. They are phagocytes, and will phagocytose pathogens and dead cell debris. The latter function is important during inflammation. Neutrophils that undergo apoptosis must be cleared out from the tissues to allow for tissue repair. Macrophages contain lysosomes which fuse with engulfed pathogens or cellular debris. Lysosomes contain enzymes that break down and "digest" waste material, so that the waste material can be dispersed out of the macrophages after being broken down. Macrophages also release mediators that will recruit other immune cells to the site of inflammation [47].

1.5.2 Important mediators involved in innate immune responses

Some of the key cytokines when it comes to mediating the acute inflammatory response are interleukins (IL), Tumor Necrosis Factor (TNF) and chemokines, as well as glycoproteins such as Colony stimulating factors (CSF). Most of these mediators have important autocrine and paracrine functions at sites of infection, but in some cases of chronic inflammation they can also have major systemic effects [44].

IL-8 is a chemokine that earlier was classified as a cytokine [44, 48]. It was given the cytokine name IL-8, but has later been given the chemokine name CXCL8. In this study it is referred to with its cytokine name, IL-8 [49]. IL-8 is one of the key mediators in the acute inflammatory response due to its highly potent chemotactic potential [50]. This chemokine is produced by a variety of cells such as stimulated monocytes, macrophages and endothelial cells. It is chemotactic, and responsible for the migration and activation of neutrophils and other leukocytes at the site of inflammation [45]. IL-8 was before also known as Neutrophil Activating Protein (NAP-1).Thus the inflammatory impact resides in the ability of IL-8 to facilitate diapedesis of leukocytes, like neutrophils, through vessel walls and leukocyte activation at the site of inflammation. The release of IL-8 is strongly influenced by increased levels of TNF-α and IL-1 [44, 48, 50]. Chemokines like IL-8 have been implicated in several inflammatory conditions such as acute respiratory distress syndrome, allergic asthma, and chronic inflammatory disorders [44].

The pro-inflammatory cytokine IL-6 is produced by a range of cells, but the main sources of this cytokine are stimulated monocytes, fibroblasts, and endothelial cells. Like IL-8, the synthesis of IL-6 is strongly stimulated by TNF-α and IL-1 in vivo. IL-6 has been described as a pleiotropic cytokine due to its ability to induce both B-cell differentiation and T-cell proliferation, as well as playing a key role in inflammatory responses [45, 51]. IL-6 is an important inducer of many

factors involved in the inflammation process at sites of inflammation [52]. IL-6 is also released systemically, thus stimulating the synthesis of acute phase proteins by the liver [44].

TNF-α plays a key role when it comes to initiating the inflammatory response of the innate immune system. Along with IL-1 and TNF-α are early pro-inflammatory cytokines responsible for mediating several inflammatory responses as well as systemic responses, e.g. inducing fever and the release of acute phase proteins from the liver [30, 48].

1.5.3 Adaptive immune response

The second line of defense against inhaled particles is the adaptive immune system. In contrast to the innate immune system this defense is highly pathogen specific, and the immune system will develop responses and antibodies that are specific to antigens that are specific for pathogens. Once the body is exposed to a pathogen, antigen presenting cells, such as dendritic cells (DCs), will engulf the pathogens at the site of infection and migrate to a peripheral lymphoid organ. Once there, the DCs will present the antigen structures on the surface of the cell to lymphocytes, which in turn will activate an antigen specific response by T-cells. Once the lymphocytes have been activated, they will react to the specific pathogens at the site of infection, either by attracting neutrophils and macrophages or by marking the pathogens for destruction.

When the adaptive immune system starts responding to pathogens in an excessive manner, it may lead to health problems such as asthma or hypersensitivity rhinitis. The immune system starts to respond to antigens that normally would be considered harmless and initiates an inflammatory response against these antigens. If this inflammatory response persists, it may in turn lead to chronic inflammation and possibly tissue damage [53].

DCs are widely distributed in most tissues and organs of vertebrates. DCs are usually closely associated with epithelial cells, and provide a crucial link between the innate immune system and the adaptive system. DCs are antigen presenting cells; after capturing pathogens by phagocytosis in peripheral tissues, DCs cleave proteins of the pathogen and bind them to major histocompatibility complexes (MHC). Thereafter, the processed MHC-antigen complex is carried to the cell surface. Activated DCs then migrate to lymphoid organs where they present the processed MHC-antigen complex to T-cells. In this way they initiate an antigen-specific T-cell response [54]. Depending on the antigen and the cytokine environment, naïve T-cells differentiate into different types of effector T-cells. At the site of infection, T helper 1 cells (Th1) work with macrophages to maximize their ability to attack the invading pathogens. T helper 2 cells (Th2) respond by stimulating B-cells to proliferate and produce antigen-specific antibodies. This response is marked by increased levels of IL-4, IL-5 and IL-13, and Th2 responses are often involved in asthma pathogenesis [28]. Th1 responses are usually considered beneficial and

protective, whereas Th2 responses are non-protective and can potentially lead to allergy and asthma. Studies have shown that DCs exposed to conidia from *Aspergillus fumigatus* will stimulate a Th1 response while hyphae induce a Th2 response [36]. If the innate immune system is not able to clear conidia of *A. fumigatus*, as in the case of immunocompromised patients, and conidia manage to germinate in the lungs, they will induce an adaptive but disadvantageous Th2 response [41]. A model of the activation of T-cells by DCs is showed in Figure 5.

In vivo, DCs arise from either CD34 positive bone marrow progenitor cells or CD14 positive monocytes. DCs migrate from the bone marrow to tissues and organs as immature DCs. In the peripheral tissues, DCs will mature in response to signals generated by local inflammation caused by infection [55]. DCs play an important role in mediating immune responses due to their ability to detect and present modified antigens. By presenting processed antigens to T-cells in peripheral lymphoid organs and secreting a plethora of mediators that activate other cell types, they activate antigen-specific immune responses. Many different variations of DCs exist in the body and DCs are often referred to as a group of leukocytes with functional differences rather than distinct cell types. DCs are often marked by high expression levels of MHC class 2 antigens, and they also express adhesion molecules which are usually upregulated during activation and maturation [45].

In tissues DCs exists in two states, immature and mature. Immature DCs have a maximal ability to detect, ingest and process inhaled antigens. Upon ingestion of pathogens, the DCs activate and start to undergo maturation. Once matured the DCs ability to detect and ingest pathogens attenuates, and their ability to stimulate and activate T-cells increases as they begin to present the processed antigen fragments on MHC molecules on their surface. Mature DCs then migrate to peripheral lymphoid organs where they stimulate antigen-specific naïve T-cell proliferation.

The term monocyte-derived DCs refer to DCs obtained by in-vitro maturation from monocytes. All DCs used in this study were monocyte-derived DCs.

T-cells reside in the lymphoid organs, and become activated when they are presented with MHC antigen complexes from DCs. Depending on the signaling from the DCs, they can develop into several types of T helper cells (Th). Their stimulation of B-cells, and production of antibodies, is a cause of allergies and allergic asthma [56].

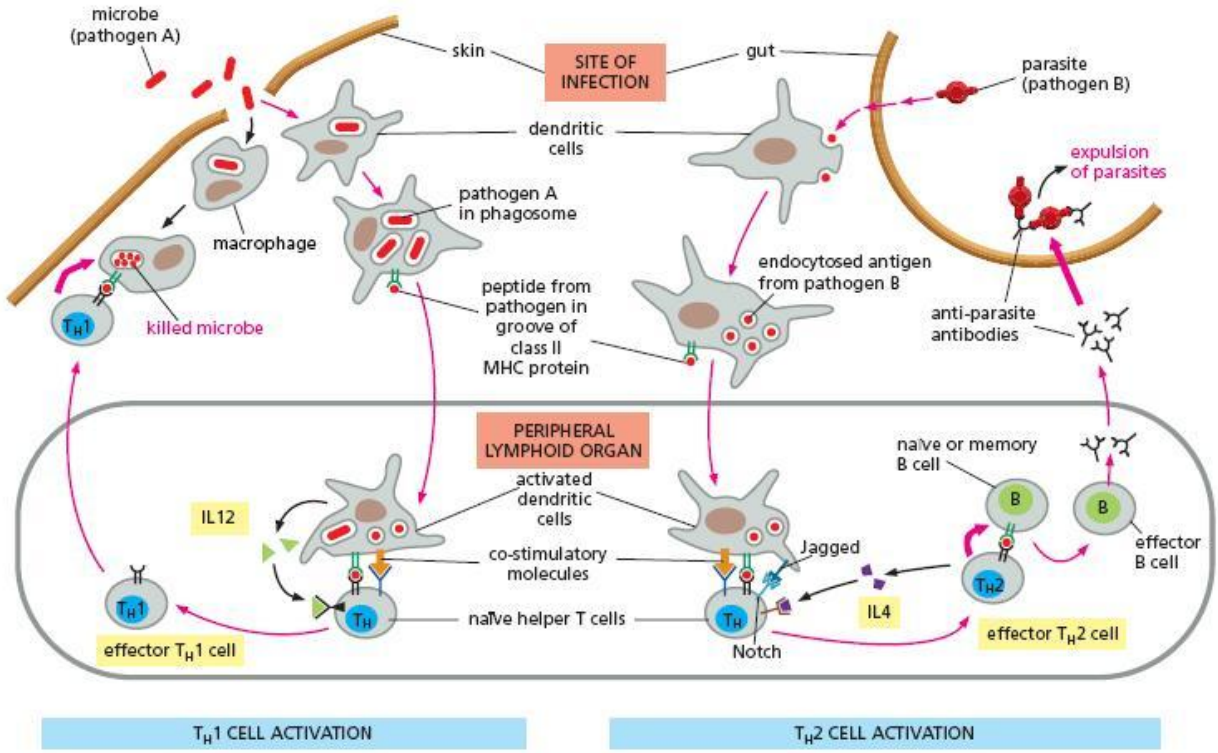

Figure 5. Model for activation of T-cells and potential development of allergy by activated DCs **[54]**

1.5.4 Important mediators involved in adaptive immunity responses

The cytokine IL-4 is mainly produced by a subset of activated T-cells and by mast cells. It is also suggested that DCs can produce IL-4, but due to autocrine stimulation and cell binding, it is hard to measure IL-4 release from DCs [57]. IL-4 promotes the differentiation and proliferation of B-cells and T-cells, and increases the expression of MHC class 2 antigens by B-cells and DCs [44]. This promotes the capacity of B-cells to respond to stimuli and present antigens to T-cells. By having B-cells presenting antigens it is then possible for the innate immune system to respond to low concentrations of antigens [45]. IL-4 is also an anti-inflammatory cytokine due to its ability to inhibit the production of several pro-inflammatory cytokines and chemokines such as IL-1, IL-6, IL-8, and TNF-α [44, 45].

IL-13 has functions very similar to those of IL-4 and is also produced by DCs and airway epithelial cells. Airway epithelia is thought to be an important source of IL-13 [58, 59].This cytokine induces proliferation and differentiation, and increases presentation of MHC class 2 antigens on B-cells. However, IL-13 also induces proliferation and differentiation of monocytes. Like IL-4, IL-13 also has anti-inflammatory properties and inhibits the expression of IL-1, IL-6, IL-8, and TNF-α [44, 45]

TSLP is a cytokine that is mainly produced by epithelial cells. It functions to induce the release of T-cell attracting chemokines from monocytes [60], as well as stimulating the activation and maturation of DCs. It will also cause naïve T-cells to differentiate into effector cells that can produce B-cell stimulating IL-5 and IL-13. Along with IL-13, TSLP promotes the Th2 response. Studies have established that high levels of TSLP and IL-13 are associated with airway inflammatory disease in both humans and mice [58, 61].

1.6 In vitro research models

Several different methods have been used to study the health effects from inhaling airborne particles. In-vivo animal models can be used, and can provide a good system for studying exposure to particles. However, it is problematic to quantify and administer a dose-specific exposure in such systems. Intubation, can introduce high levels of stress in the animal and some models have been regarded to not represent actual events. This could possibly limit the significance of the results [29].

In the past years there have been efforts to replace animal models with in-vitro cell models. The advantage of such models is that it is easier to study specific cellular and molecular pathways, thus making it possible to study specific mechanisms of interest. Studies with cultured cells are easier to standardize and such systems are simpler reproduce in other laboratories.

Alveolar cell-culture systems have been established to learn about the immune effects of a variety of airborne particles, such as road dust and exhaust particles [62, 63]. However, there is limited information available on the effects of airborne mold particles on alveolar cell-culture systems. By adapting established in-vitro research models to fungal studies, they can be used to learn about specific immune responses to indoor fungi.

1.6.1 Cell cultures

In-vitro cell mono-cultures have been used in a number of studies to examine the response of specific cells to particles. It is common to use various cells of the immune system, which makes it is possible to learn about specific immune responses. The advantage of mono-culture systems is that they are specific and involves fewer factors than animal models or co-culture models, thus leaving less room for error and increasing the reproducibility of such studies [33].

In this study, mono-cultures of monocytes and monocyte-derived mature DCs were used in exposure experiments. Monocytes play an important role in the innate immune response, by coordinating the release of several inflammatory mediators like cytokines and chemokines [30]. They work to recruit cells and activate inflammatory reactions at the site of infection, and by

studying monocytes exposed to particles it is possible to learn about inflammation and tissue damage. As already indicated, DCs provide an important link between the innate immune system and the adaptive immune system, since they are responsible for processing and presenting antigens to cells of the adaptive immune system, thus facilitating the adaptive immune response [54, 64]. By studying the response of DCs to various particles, it could be possible to understand more about the role of those particles in the development of for example allergic immune responses.

In-vitro cell co-cultures can function as a very simplified model of biological systems. In a co-culture model, two or more cells types are cultured and exposed separately or together. Unlike a mono-culture system, a co-culture system is likely to be a much more physiologically relevant model for studying immune responses [33]. In lungs, epithelial cells monocytes and DCs continuously communicate through intracellular signaling to maintain homeostasis. When stimulated they collectively coordinate the immune response [29]. Studies have shown that the release of cytokines in co-cultures far exceeds the release of cytokines from the respective mono-cultures, thus exemplifying the importance of cell interplay in immune responses [65, 66]. In-vitro co-culture systems could provide a better representation of actual physiological events than mono-culture systems.

Many different co-culture designs have been used in studies, as they can designed in a variety of ways. Usually two or three types of cells are cultured together, and the types of cells can vary from study to study [33, 50]. Cell types can also be cultured in contact with each other or non-contact by separating them with a membrane in the culture dish. It is also important to consider which cells are to be exposed to particles, as this will be pivotal in the design of the co-culture.

2 Aim of Study

The association between dampness/mold growth and the development of respiratory health problems such as asthma, hypersensitivities, wheeze, and cough has been established by several epidemiological studies. Still much needs to be learned about the specific mechanisms by which damp and moldy environments cause health problems. With a better understanding of which factors are triggering health problems, and how they trigger them, we can better develop routines and methods for dealing with the problems.

With this study we hoped to establish techniques to assess and characterize damp and moldy indoor environments. Thereafter our goal was to study isolated molds from these environments in in-vitro models, in order to gain understanding of how they potentially may cause respiratory health problems.

By employing a systemized approach for assessing damp and moldy indoor environments, it is possible to learn which molds are relevant in a Norwegian climate. Once relevant molds have been identified, we wished to study these for their effects on the respiratory system. With the help of the in-vitro cell models we may learn how these molds potentially influence the respiratory immune system, and if they should be investigated further in more complete systems, like animal models.

To our knowledge, this is the first study that employs lung co-culture models with dendritic cells to study how molds affect the respiratory immune system.

3 Materials and Methods

3.1 Sampling of Mold Conidia

Four housing units with reports of water damage or dampness, visible mold or both were selected for air sampling. At each housing unit, air samples were taken at three sites; first site was in the room with the reported water damage or visible mold, the second site was a room in the same housing unit with no reports dampness or mold, and the third site was outdoors.

3.1.1 Air Sampling

For the collection of airborne conidia, a SAS Super Iso 180 (PBI) microbiological air sampler was used. SAS Super Iso is an impaction air sampler, which impacts the sample air directly onto a 9 cm nutrient agar dish. The rate of airflow was 180 l per min. To sample airborne conidia, three types of nutrient agar was used, Malt Extract agar (MEA), Rose Bengal agar (RBA) and Dichloran glycerol-18 agar (DG18)(appendix 3-5), which only allows for molds and yeasts to grow. The sampling procedure is listed in appendix 1. and was modified from Morris et al. [20].

3.2 Mold Identification

Once air samples had incubated for 96 hours, the colonies were counted and each mold colony was streaked out in individual MEA dishes and cultured at 37^{o}C for 96 hrs. to culture each individual sample mold as pure colonies. The colonies were described according to their morphological traits after 48 hrs. and 96 hrs.

3.2.1 DNA Primers

Two sets of primers were selected to use for identification of the sampled molds. Primer set 1 is described as indoor fungi specific primers, with the forward primer FF2 and the reverse FR1 [26]. Primer set 2 is described as generalized eukaryotic primes with forward primer EUK1209 and reverse UNI1392 [27]. All primers were ordered from Eurofins MWG Operon (Ebersberg, Germany).

Table 2. PCR primers used to identify airborne molds

	Primer name (direction)	Source	Primer sequence	Specificity	PCR product size [a]
Primer Set 1	FF2 (Forward)	Zhou et al. 2000 [26]	5′-GGTTCTATTTTGTTGGTTTCTA-3′	Fungal 18S rRNA	425 bp
	FR1 (Reverse)	Zhou et al. 2000 [26]	5′-CTCTCAATCTGTCAATCCTTATT-3′	Fungal 18S rRNA	425 bp
Primer Set 2	EUK1209 (forward)	Diez et al. 2001 [27]	5′-CAGGTCTGTGATGCCC-3′	Eukarya 18S rRNA	210 bp
	UNI1392 (reverse)	Diez et al. 2001 [27]	5′- ACGGGCGGTGTGTRC -3′	Universal 18S rRNA	210 bp
Primer Set 3[b]	EUK1A (forward)	Diez et al. 2001 [27]	5′- CTGGTTGATCCTGCCAG -3′	Eukarya 18S rRNA	540 bp
	EUK516 (reverse)	Diez et al. 2001 [27]	5′- ACCAGACTTGCCCTCC -3′	Eukarya 18S rRNA	540 bp

[a] According to source
[b] Primer set 3 was only used to sequence two sampled molds

3.2.2 DNA extraction and PCR amplification

To extract fungal DNA from the molds to be identified, conidia and hyphae samples were harvested by pipetting 100µl of sterile H_2O onto the mold culture and dissolving a small fraction of conidia and hyphae in the water using a sterile inoculation loop. Water containing conidia and hyphae was then transferred to a microbead-beating tube containing sterile water and silica beads. The tube was then placed in a MiniBeadbeater (Biospec products, Inc., Bartlesville, OK, USA) and homogenized for 4 minutes, to release DNA from fragmented conidia and hyphae.

To amplify the fungal DNA, 2µl of supernatant was transferred to PCR-tubes along with 10pmol of each primer in either primer set 1 or 2, Hot Start Polymerase (1.25 U; Promega, Madison, WI, USA), dNTPs (200 µM; Sigma-Aldrich, Oslo, Norway), $MgCl_2$ (1.5 mM; Promega) in 50µl of reaction buffer. Tubes were placed in a PCR Thermal cycler (Palmcycler, Corbett Life Science, Sydney, Australia) and the conditions for PCR were as follows: initial DNA denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, primer annealing at 52°C for 1 minute, and extension at 72°C for 2 minutes. At the end of 35 cycles, samples were allowed to extend at 72°C for an additional 10 minutes.

10µl of PCR product was mixed with loading buffer and loaded onto a 2% agarose gel to control for PCR products. All PCR amplifications that yielded PCR products were then sequenced.

3.2.3 DNA Sequencing and identification

In order to produce good PCR sequences, the PCR products were purified to remove inhibitors and leftover primers using a DNA extraction kit (GenElute Plant Genomic DNA Miniprep Kit. Sigma-Aldrich, Oslo, Norway). Purified PCR products were diluted 1/10 by transferring 1µl of PCR product to PCR tube strips along with 9µl of RNAfree water. Then 1µM primer (either forward or reverse primer) was added to the tube. Sequencing reactions was performed by a commercial laboratory (ABI-Lab, Blindern, Oslo) and samples were sequenced up to five times in order to derive a coherent genomic sequence for each sample. Once a genomic sequence had been determined, the sequence was entered into NCBI Standard Nucleotide BLAST and the Nucleotide collection database was searched for matching sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.2.4 Mycotoxin test

As a majority of the molds that were sampled in a damp room were identified to be of the genus *Aspergillus,* a test for the carcinogenic mycotoxin aflatoxin, produced by several species of *Aspergillus,* was included. The culture based procedure for identifying molds producing the mycotoxin Aflatoxin was scored on three criteria to test for aflatoxin: 1) fluorescence on β-cyclodextrin supplemented agar (β-cyclodextrin will enhance the natural fluorescence of aflatoxin); 2) formation of yellow pigmentation; and 3) color change in response to ammonium hydroxide vapor. The method is adapted from Abbas et al. 2004 [16].

First, all collected molds were grown on dishes containing potato dextrose agar (PDA) supplemented with β-cyclodextrin, which enhances the fluorescence of aflatoxin. Colonies were grown in the dark at 37°C for 96 hours. To determine formation of yellow colony pigmentation, the dishes were examined in natural light for bright yellow pigmentation on the underside of the colony. Presence of fluorescence was determined by looking for blue fluorescence under long wavelength ultraviolet light (UV, 365 nm). Finally, 500µl of ammonium hydroxide was placed in the lid, and the colony dish was inverted over the lid. A positive ammonium hydroxide test was detected by color-change on the underside of the colonies, from yellow to plum-red.

3.3 Mold Conidia Preparation

Two molds were isolated from air samples, *Emericella nidulans* and sampled *Aspergillus specie (sp.)*, and three molds, *Aspergillus fumigatus, Aspergillus niger* and *Aspergillus versicolor* obtained from (DSMZ, GmBH, Braunscweig, Germany). To prepare mold conidia for in-vitro exposure experiments, molds were grown on Sabouraud Dextrose Agar (SAB) slants at 37°C (except for *A. versicolor*, which was grown at 22°C) for 10-12 days. Conidia were dislodged from colonies by

adding 9 ml of phosphate-buffered saline (PBS) containing 0.025% Tween 20, and tapping the slants on a hard surface and gently scraping the medium. PBS and conidia solution was transferred to a 50 ml centrifuge tube and vortexed for 20-30 seconds to overcome surface tension of the conidia. Conidia suspension was then filtered twice through a 40 µm nylon cell strainer (BD Biosciences, Aalst, Belgium), and the conidia concentration was counted using a Bürker chamber. The conidia stock was immediately stored at -20°C. To make a combined conidia and hyphae stock, the straining step was omitted and the stock counted directly with a Bürker chamber and the conidia and hyphae stock was stored at -20°C until required.

Conidia used in in-vitro exposure models were transferred from conidia stocks to 1.5 ml centrifuge tubes containing the amount of conidia required for one in-vitro exposure experiment. These tubes were then subjected to various treatments to inactivate fungal growth. Two different treatments were used: heat inactivation and freeze-thaw fragmentation. Some in-vitro exposure experiments also used live native conidia that did not undergo any inactivation treatments.

3.3.1 Heat inactivation

To inactivate mold conidia at 60°C, 1.5 ml centrifuge tubes containing selected conidia was placed in an incubator set at 60°C for 30 min. After incubation, conidia were ready to be used in in-vitro exposure experiments, but could be stored at -20°C until later use. Before use, the tubes were centrifuged at 1.6 x 10^4 g for 10 minutes. The supernatant was discarded and the conidia pellet was resuspended in culture medium.

3.3.2 Freeze-thaw fragmentation

The process of freeze-thaw fragmentation of the mold conidia involved repeated cycles of shock-freezing in liquid nitrogen (-196°C) and thawing at 40°C. First, selected mold conidia were transferred from the 1.5 ml centrifuge tubes to cryotubes. Then the tubes were submerged in liquid nitrogen for 45 seconds, until completely frozen. The tubes were immediately transferred to a water bath measuring 40°C for approximately 2 minutes, until completely thawed. This represented one complete cycle of freeze-thawing. Depending on the in-vitro exposure protocol the conidia went through either 15 or 30 cycles of freeze-thawing.

After completing the freeze-thaw cycles, conidia were transferred to bead-beating tubes containing silicabeads and placed in a MiniBeadbeater ((Biospec) and homogenized for 4 minutes. This was to disrupt the conidia (and hyphae) into smaller fragments. After bead-beating the silicabeads were allowed to sediment, and supernatant with conidia fragments was transferred back to 1.5 ml centrifuge. 500 µl of sterile water was added to the silicabeads and the tube was shaken by hand to extract remaining conidia fragments. Silicabeads were allowed

to sediment, and supernatant was transferred to the centrifuge tube containing fragmented conidia. Rinsing repeated one time. Fragmented conidia was now ready to be used in in-vitro exposure experiments, and could be stored at -20^{o}C until later use. Before use, the tubes were centrifuged at 1.6 x 10^4 g for 10 minutes. The supernatant was discarded and the conidia pellet was resuspended in culture medium.

3.4 Cell Cultivation

For this study, primarily two types of cells were used, THP-1 monocytes (M) and A549 alveolar pneumocytes (P). THP-1 cells were also differentiated in to immature (IMDC) and mature dendritic cells (MDCs). Procedure for differentiation of DCs from THP-1 was performed as described by Berges et al. 2005 [55]. All cells were cultured in RPMI 1640 culture medium supplemented with 2 mM L-Glutamine (Cambrex Bio Science Verviers, Belgium), modified by adding 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM pyruvic acid (Sigma-Aldrich St. Louis, MO, USA), 0.1% gentamicin (GIBCO. UK), and 10% heat-inactivated fetal calf serum (FCS) (Euroclone, Italy). Some cell culture procedures also involved culturing cells in culture medium without 10% FCS according to the appropriate procedures [55]. All cells were cultured in NUNC 75 cm^2 cell culture flasks (Fisher Scientific, Oslo, Norway) in a humidified incubator set at 37^{o}C and 5 % CO_2, and all cell culture preparations were performed using sterile technique in a laminar flow hood.

3.4.1 Monocyte Cell Cultivation

THP-1 monocyte cells obtained from American Tissue Type Culture Collection (ATCC, Rockville, MD, USA) were used in both mono-culture models and co-culture models. This cell-line was cultured from the blood of an Asian 1-year old infant male suffering with acute monocytic leukemia. These cells are phagocytic, and lack surface and cytoplasmic immunoglobulins. THP-1 are spherical cells that grow in suspension, and the cells have a doubling time of approximately 26 hours.

The cells were cultured in culture medium, and was partially subcultured every 2-3 days and completely subcultured by centrifugation every 7 days. Cell concentrations were counted using a Bürker chamber, and concentration was adjusted to between 5 x 10^5 cells/ml to 6 x 10^5 cells per ml every time cells were subcultured. In this study the THP-1 cells used were in the range of 7 to 34 passages. Cells were obtained from frozen cultures in passage 3 to 5.

3.4.2 Dendritic Cell Cultivation

The derivation of dendritic cells from monocytes was performed in accordance to the protocol developed by Berges et al. 2005. [55]

To produce IMDC that were used in co-culture models, THP-1 cells were centrifuged down, resuspended in culture medium with 10% FCS to a concentration of 2 x 10^5 cells/ml, and a volume of 12 ml was transferred to culture flasks. To induce differentiation rhIL-4 (1500 IU/ml) (Immuno Tools GmbH, Altenoyther Str.Friesoythe; Germany) and rhGM-CSF (1500 IU/ml) (Immuno Tools GmbH) was added to the culture medium. The cells matured for 5 days with this cytokine cocktail to acquire properties as IMDC. Cells were subcultured with a fresh cytokine cocktail and cell concentration adjusted to 2 x 10^5 cells/ml every 2 days.

To produce MDC to be used in mono-culture models, IMDC were centrifuged and resuspended in 12 ml serum free culture medium (without FCS) at a concentration of 2 x 10^5 cells/ml. To the serum free medium was added a cytokine cocktail of rhIL-4 (3000 IU/ml), rhGM-CSF (1500 IU/ml), rhTNFα (4000 IU/ml)(Immuno Tools GmbH) and Ionomycin (200ng/µl) (Sigma Aldrich, Oslo, Norge). The appropriate quantity of cells (2 x 10^5 cells/well for 12-well plates, and 4 x 10^5 cells/well for 6-well plates) was then transferred to wells in either 6 or 12 well plates (NUNC), and cultured for 2 to 3 days in preparation for exposure experiments. Since matured DCs change morphology from suspended spherical cells to fibroblast-like adherent cells final maturation was done in 6- or 12-well plates.

3.4.3 Pneumocyte cultivation

A549 epithelial cells were obtained from American Tissue Type Culture Collection (ATCC, Rockville, MD, USA), and for this study the cells were used in all the co-cultures and some mono-cultures. The cell-line was established from culturing lung carsinomatous tissue from a 58-year-old Caucasian male. A549 cells are alveolar basal epithelial cells, their morphology is squamous adherent cells, and their doubling time is approximately 22 hours.

The cells were cultured in culture medium, and were subcultured every 2-3 days with trypsination (Sigma Aldrich, Oslo, Norge) and centrifugation. Cell concentrations were counted using a Bürker chamber, and cell numbers were adjusted to between 3.2 x 10^4 cells/ml to 4 x 10^4 cells/ml in a culture flask every time cells were subcultured.

3.5 Mono-Culture Models

Cell-types used to prepare mono-culture models to be exposed to mold-particles, were prepared differently depending on whether the cells were adherent or suspended. Monocytes and

immature DCs derived by differentiation from THP-1 cells grow suspended in culture medium, so these cells were transferred to culture wells approximately 30 minutes prior to particle exposure. Prior to transfer, cells were centrifuged and resuspended in fresh culture medium. Monocytes were transferred at a concentration of 1 x 10^6 cells/ml, and IMDC at a concentration of 2.8 x 10^5 cells/ml to either 6- or 12-well plates (Nunc).

To seed pneumocytes, 8-9 x 10^4 cells/ml was seeded in 12-well plates and left to incubate for about 20 hrs. prior to experiment. In 12-well plates culture medium volume was adjusted to 800 µl and to 1.6 ml in 6-well plates during exposure.

3.6 Lung Co-Culture Models

Cells were cultured in non-contact co-cultures with pneumocytes in non-contact with immature DCs (P/IMDC)(Figure 6) and contact co-cultures with monocytes in contact with pneumocytes cultured in non-contact with immature DCs (M+P/IMDC)(Figure 7).

Both P/IMDC and M+P/IMDC were established by seeding A549 pneumocytes in inserts with a microporous permeable membrane (pores size 0.4µm) (Transwell – clear inserts for optimal visibility from Corning Incorporated Costar, NY, USA). Cells were added to inserts at 0.9 x 10^6 cells/insert, and inserts were incubated for 20-24 hours. After incubation, inserts were transferred to 36 mm 6-wells culture dishes (Corning Incorporated Costar, NY, USA). Below inserts IMDC was seeded with 0.4 x 10^6 cells/well. In contact co-cultures, 1.6 x 10^6 cells/insert of monocytes were added in the inserts. Total volume of culture medium was adjusted to 2.2 ml. Cells were now ready for particle exposure.

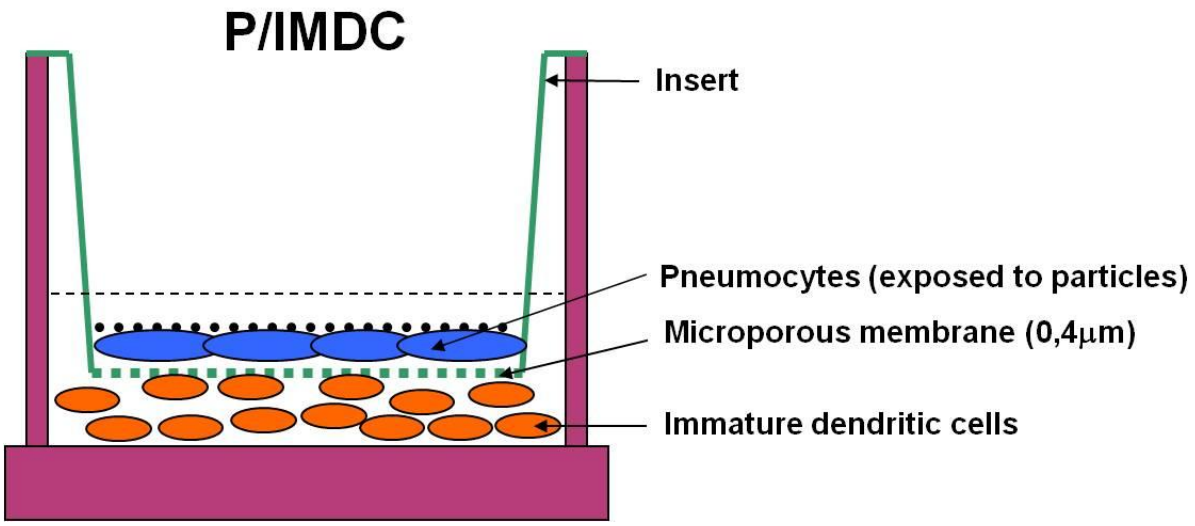

Figure 6. Illustration showing the distribution of pneumocytes (P) and immature dendritic cells (IMDC) in the non-contact co-culture design used in this study. (modified from Herseth 2008 [62]).

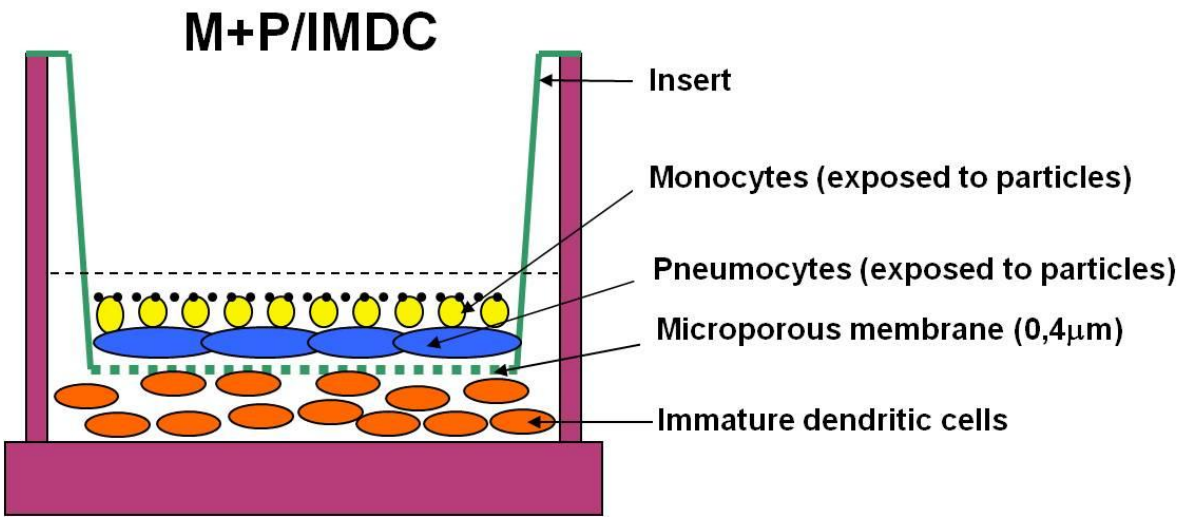

Figure 7. Illustration showing the distribution of monocytes (M), pneumocytes (P) and immature dendritic cells (IMDC) in the contact co-culture design used in this study, with monocytes and pneumocytes being cultured in contact, and in non-contact with IMDC. (modified from Herseth 2008 [62]).

3.7 In-vitro Particle Exposure Experiments

All in-vitro exposure experiments were conducted for 8 hrs. Preliminary studies (data not shown) indicated that excessive germination and sporulation from native conidia would occur at longer exposure times.

3.7.1 Conidia concentrations

Mono-cultures of monocytes and mature DCs were exposed to either native conidia, heat inactivated conidia or freeze-thaw fragmented conidia from *Aspergillus niger, A. versicolor, Emericella nidulans,* and sampled *Aspergillus sp.* All conidia were exposed at a quantity of either 5 or 15 conidia per cell. For freeze-thaw fragmented conidia, the quantity of fragments used for exposure was equal to the quantity of conidia prior to freeze-thaw fragmentation.

All co-cultures were exposed to heat inactivated *A. niger* or *A. versicolor.* Co-cultures were exposed with conidia in concentrations of 10.9×10^6 conidia/ml.

3.8 Particle Binding of Cytokines

Many cytokines and chemokines have been shown to bind to various particles [67-69]. If cytokines and chemokines are bound to particles in in-vitro experiments they will be removed from the supernatant when particles are removed by centrifugation, thus potentially altering the analysis of cytokines and chemokines since only the remaining levels are detected. To document

particle binding, a cell-free model system was used to estimate the amount of IL-8 and IL-4 bound by mold conidia.

Wells of 12-well plates (NUNC) were supplied with 800 µl of culture-medium. Stock solutions of ELISA standard IL-4 (Invitrogen) or ELISA standard IL-8 (Invitrogen) were added to the wells to give concentrations of 500pg/ml or 1000pg/ml. Then conidia from *A. niger* or *A. versicolor* were added to the wells in a quantity of 12 x 10^6 conidia/well. The conidia were native, heat-inactivated and freeze-thaw fragmented. One well for each concentration of IL-4 and IL-8 was left to incubate without conidia, as a control. After conidia had been added to the wells, plates were incubated in a humidified incubator set to 37°C and 5% CO^2 for 2 hrs.

3.9 ELISA Analysis

Enzyme-linked immunosorbent assay (ELISA) kits were used to determine the release of immunity mediators in supernatants. Assays were performed on cell-free supernatants that had been recovered by centrifugation, first at 300 G for 10 min. to remove cells, followed by 10 min at 16.000 G to remove conidia. Supernatants were stored at -20°C until they were assayed. IL-8 and IL-4 was determined using Invitrogen ELISA kits (Camarillo, CA USA). TSLP and IL-13 was determined using DuoSet ELISA kits from R&D Systems Inc. (Minneapolis, MN, USA). All cytokines were analyzed according to the manufacturer's manuals for the ELISA kits, except for Invitrogen where half concentrations of coating antibody and detection antibody were used. Color intensity of ELISA was quantified in a Victor Multiplate reader with software (Perkin Elmer, Waltham, MA, USA).

3.10 Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 5.0 for Windows, GraphPad Software, San Diego, California, USA). One-way analysis of variance (ANOVA) coupled with Dunnet's test of significance was used on all datasets to test for significant difference from control group. ANOVA is a test of variance; ANOVA will presume a Gaussian distribution when given a large sample size, so the data was not tested for Gaussian distribution. As indicated in the figure legends, some data were log transformed before performing ANOVA to fulfill assumption of equal standard variation for all sets of replicates [70]. P values < 0.05 were considered to be statistically significant. In all figures, bars represent mean ± standard error of mean (SEM).

4 Results

4.1 Identification and characterization of indoor molds

After initial sampling of molds, several methods were used to identify and characterize these for subsequent analysis.

4.1.1 Air sampling

Air sampling was done in 4 water damaged or damp rooms, 4 non-damp rooms, and at 3 outdoor sites. 25 molds were collected at all three sites combined. Table 3 shows how many molds were collected separated by sample site.

Table 3. The number of molds collected by air sampling on a site-by-site basis.

Sample site	Total collected molds
Damp / water damaged rooms (n=4)	12
Control rooms (n=4)	6
Outdoors (n=3)	8
Total	25

4.1.2 Identification of molds through bead-beating and PCR/sequencing

The bead-beating procedure to release fungal DNA from conidia and hyphae was highly efficient. After bead-beating conidia and hyphae samples from all collected molds, we were able to successfully extract DNA for PCR amplification from 24 of 25 mold samples. Observing of treated samples with a microscope showed that bead-beating was efficient at breaking and fractioning the mold conidia. (Figure 8)

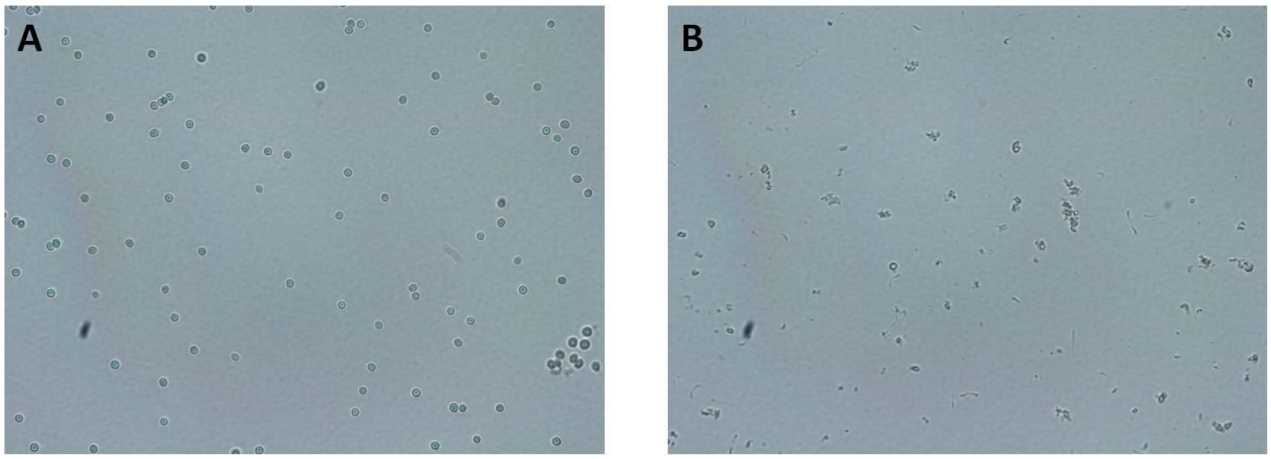

Figure 8. Fragmentation of conidia achieved by bead-beating method. A: shows native conidia prior to any treatment. B: after 4 minutes of bead-beating, conidia appear to be fragmented and disrupted.

4.1.3 DNA identification of airborne molds

Sequencing was performed on all PCR products to derive sequences for genetic identification of the molds. All products were sequenced between 1 to 5 times to derive a coherent sequence based on the forward and reverse PCR products. Listed in Table 4 are the results from sequencing using primer pairs FF2/FR1 and EUK1209/UNI1392. Sequencing reactions was performed by a commercial laboratory (ABI-Lab, Blindern, Oslo). Sequences were compared with those in the nucleotide BLAST database [71] for similarity. All the derived sequences are listed in appendix 2. On average, PCR products for primer set 1, FF2/FR1 were approximately 400 bp in length, while primer set 2, EUK1209/UNI1392 yielded products of approximately 200 bp in length. Nine out of 24 sampled molds gave PCR products with primer set 1, while 23 out of 24 molds gave PCR products with primer set 2.

Table 4. Identification of sampled molds by searching the NCBI Nucleotide BLAST database with sequenced DNA using primer set 1, FF2/FR1 indoor mold specific primers, and primer set 2, EUK1209/UNI1392 general eukaryotic primers.

#	Closest relative[a] Accession number (BLAST)	Primer Set 1[b] Sequence Length (bp)	Primer Set 2[c] Sequence Length (bp)	Max Identity (%)
1*	*Rhizomucor pusillus* HQ845298.1	*Not sequenced*	358	100
2*	*Aspergillus sp.*	*Not sequenced*	204	100
3*	*Aspergillus sp.* *Aspergillus sp.* *Aphanoascus fulvescens* JN941603.1	399	 151	100 99
4*	*Rhizopus microsporus* FN182240.1	*Not sequenced*	192	100
5*	*Aspergillus sp.* *Rhizomucor pusillus* HQ845298.1	315	 188	100 100
6*	*Aspergillus sp.* *Penicillium sp.* *Aspergillus sp.*	396	 50	100 99
7	*Aspergillus terreus* JN831364.1 *Aspergillus sp.*	406	 192	100 100
8	*Aspergillus sp.*	*Not sequenced*	209	100

9*	*Aspergillus sp.*	*Not sequenced*	211	100
10	*Paecilomyces variotii* JF416647.1	412		100
	Aspergillus sp.			100
	Paecilomyces variotii JF416647.1		211	100
11	*Aspergillus sp.*	*Not sequenced*	195	100
12*	*Aspergillus sp.*	399	*Not sequenced*	100
13*	*Aspergillus sp.*	*Not sequenced*	192	100
14*	*Emericella nidulans* AB008403.1	368		100
	Aspergillus sp.		222	100
15	*Lichtheimia corymbifera* JQ004931.1	*Not sequenced*	202	99
16	*Absidia corymbifera* AF113407.1 *Lichtheimia corymbifera* JQ004931.1	*Not sequenced*	143	100
17	*Engyodontium album* JF797223.1 *Halophytophthora vesicula* HQ161089.1 *Artomyces pyxidatus* JQ086388.1	*Not sequenced*	105	97
18	*Tritirachium oryzae* JF797224.1 *Paratritirachium cylindroconium* JF797220.1 *Hyphodermella corrugata* JN940192.1	*Not sequenced*	73	97
19*	*Aspergillus sp.*	401		100
	Penicillium sp.		22	100
	Aspergillus sp.			
20*	*Aspergillus sp.*	*Not sequenced*	157	100
21	*Hyphodermella corrugata* JN940192.1 *Hyphodermella rosae* JN940191.1	*Not sequenced*	194	100

22	*Aspergillus sp.*	*Not sequenced*	222	100
23	*Aspergillus terreus* JN831364.1	413		100
	Aspergillus sp.		222	100
24	*Aspergillus sp.*	*Not sequenced*	187	100

*Mold samples collected from damp or water damaged rooms
[a]Based on homology with previously reported sequences. Top three corresponding species listed in search results were included. If more than three species of the same genus had the same Max Identity score, the mold was only identified at the genus level.
[b]Primer Set 1 used PCR primers FF2/FR1, indoor mold specific primer set.
[c]Primer Set 2 used PCR primers EUK1209/UNI1392, general eukaryotic primer set.

4.1.4 Mycotoxin test

As earlier described, all collected molds were cultivated on Potato Dextrose agar supplemented with β-cyclodextrin. Under these conditions, aflatoxin production is recognized by: 1) fluorescence in the agar 2) the formation of yellow pigmentation in the hyphal mass and 3) a color change to plum red in response to ammonium hydroxide vapor. None of the collected molds were identified as aflatoxigenic species. Two molds displayed strong fluorescence after 48 hrs. of incubation, but the fluorescence declined rapidly after 96 hrs. of incubation. After 96 hrs. of incubation, 12 out of 24 molds displayed faint fluorescence when compared to the strong fluorescence of the aflatoxin positive control under 365-nm UV light (Figure 9).

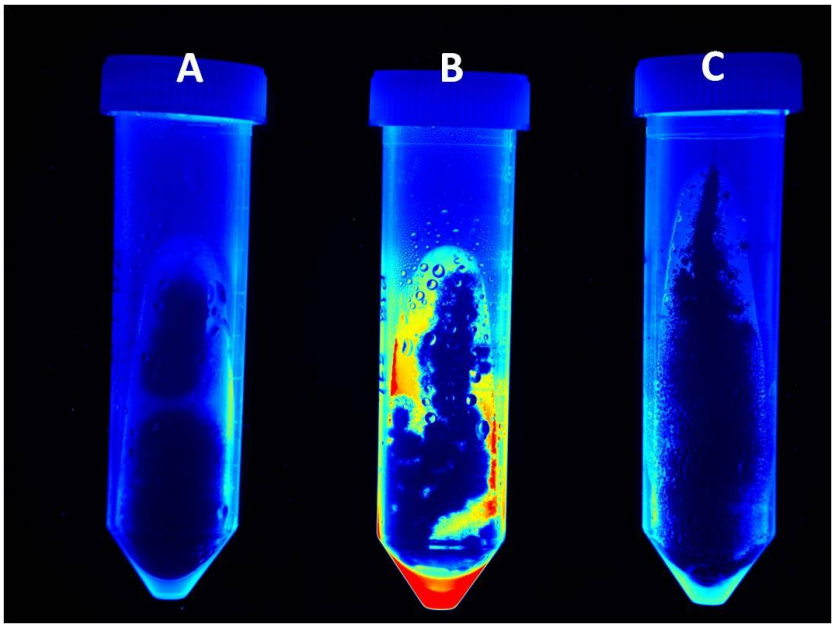

Figure 9. Three mold samples after 96 hrs. of cultivation on PDA supplemented with β-cyclodextrin. A: Collected *Aspergillus sp.* which displayed strong fluorescence at 48 hrs. but none at 96 hrs.. B: Positive control, *Aspergillus parasiticus*, known aflatoxigenic strain. B: Negative control, *Aspergillus niger,* known aflatoxin-negative strain. Note: Faint reflection in sample A and C is probably due to reflection from sample B.

4.1.5 In depth specie identification

Two molds were selected for in depth identification, and in-vitro cell exposure experiments. These were molds identified as *Aspergillus sp.* (#3 in Table 4) and as *Emericella nidulans* (#14 in Table 4). These were observed under a light microscope and conidiophore structures were compared to reference photos in the atlas "Microfungi" [10]. When compared to the atlas, structures of mold #14 showed a high resemblance to reported structures of *Emericella nidulans* (Figure 10B). The structures of mold #3 showed high resemblance to several species of *Aspergillus*, and no assumptions about the specie of this mold could be made (Figure 10A).

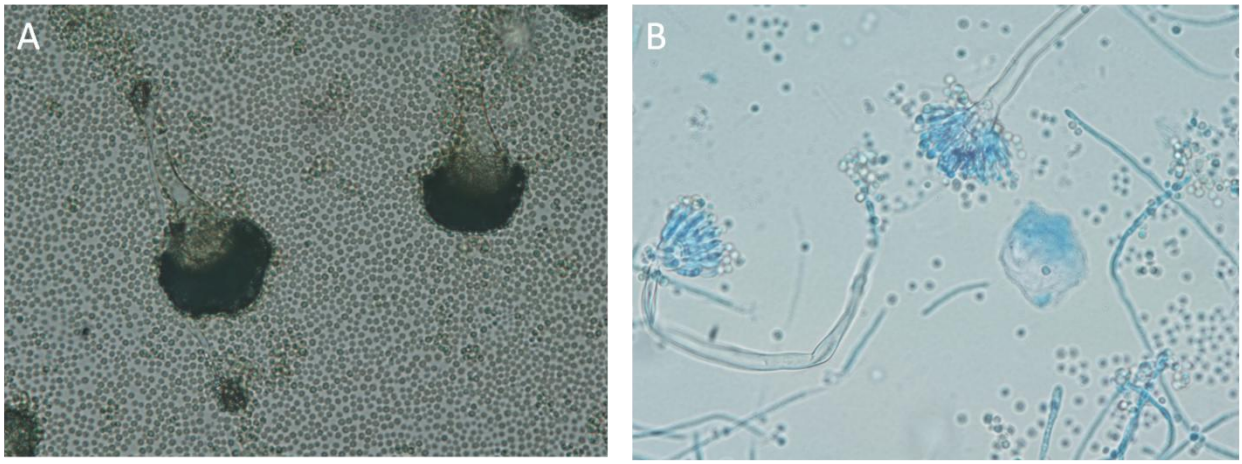

Figure 10. Mold A is #3 and mold B is #14 in Table 4. Based on morhpholgy, A has structures that resemble the structures of various species of *Aspergillus*. The structures of B resemble structures commonly seen in *Emericella nidulans.*

Just prior to the deadline for this thesis, these two molds were sequenced with an additional eukaryote specific primer set (EUK1A/EUK516). This primer set confirmed mold #14 as *Emericella nidulans*, and identified mold #3 as *Aspergillus fumigatus* (Table 5). Although this primer set gave additional information about the identity of mold #3, it is still referred to as collected *Aspergillus sp.* for the remainder of this thesis.

Table 5. Identification of two sampled molds by searching the NCBI Nucleotide BLAST database with sequenced DNA using primer set 3, EUK1A/EUK516 general eukaryotic primers.

#	Closest relative[a] Accession number (BLAST)	Primer Set 3[b] Sequence Length (bp)	Max Identity (%)
14*	*Emericella nidulans* U77377.1	534	99
3*	*Aspergillus fumigatus* M60300.1	529	100

*Mold samples collected from damp or water damaged rooms
[a]Based on homology with previously reported sequences.
[b]Primer Set 3 used PCR primers EUK1A/EUK516, general eukaryotic primer set.

4.2 In vitro cell models

In-vitro cell cultures were exposed to conidia and fragments of mold. The immune system response by the cell-cultures was measured by measuring the release of important immune system mediators. Results that are presented here are not corrected for particle binding of cytokines.

4.2.1 Effect of mold conidia on the release of IL-8 and IL-4 from mono-cultures

Mono-cultures of monocytes (THP-1) and monocyte derived mature DCs were exposed to conidia from various molds for 8 hrs. before cells were harvested. The conidia used for exposure were either native viable conidia, freeze-thaw fragmented conidia, or heat-inactivated conidia. Cells were exposed to concentrations of either 5 conidia or 15 conidia per cell.

4.2.1.1 Collected Aspergillus sp.

Figure 11A shows results from monocytes exposed to conidia from a collected *Aspergillus sp*. Cells exposed to native conidia showed a significant increase in the release of IL-8 when compared to non-exposed controls. Exposure of conidia freeze-thaw fragmented for 15 cycles did not yield a significant response of IL-8. There was visual evidence of germination and formation of hyphae when cells were exposed to freeze-thaw fragmented conidia at a concentration of 15 conidia per cell, thus 15 cycles of freeze-thaw fragmentation was not sufficient to inhibit fungal growth. To ensure full inactivation of fragmented conidia, the procedure was adjusted to 30 cycles of freeze-thaw fragmentation. As seen in Figure 12, the induction of IL-8 by conidia freeze-thaw fragmented 30 cycles did not alter from the induction by conidia freeze-thaw fragmented 15 cycles, and there were no signs of germination. Silica used to bead-beat conidia did not induce release of IL-8, and was only tested for immunological response in monocytes.

Mono-cultures of mature DCs exposed to a collected *Aspergillus sp.* did show a slight increase in the release of IL-4 in response to native conidia at a concentration of 15 conidia per cell, however it was not enough to be significant, hence mature DCs did not show any statistically significant changes compared to non-exposed controls in response to exposure to any variety of conidia. (Figure 11B)

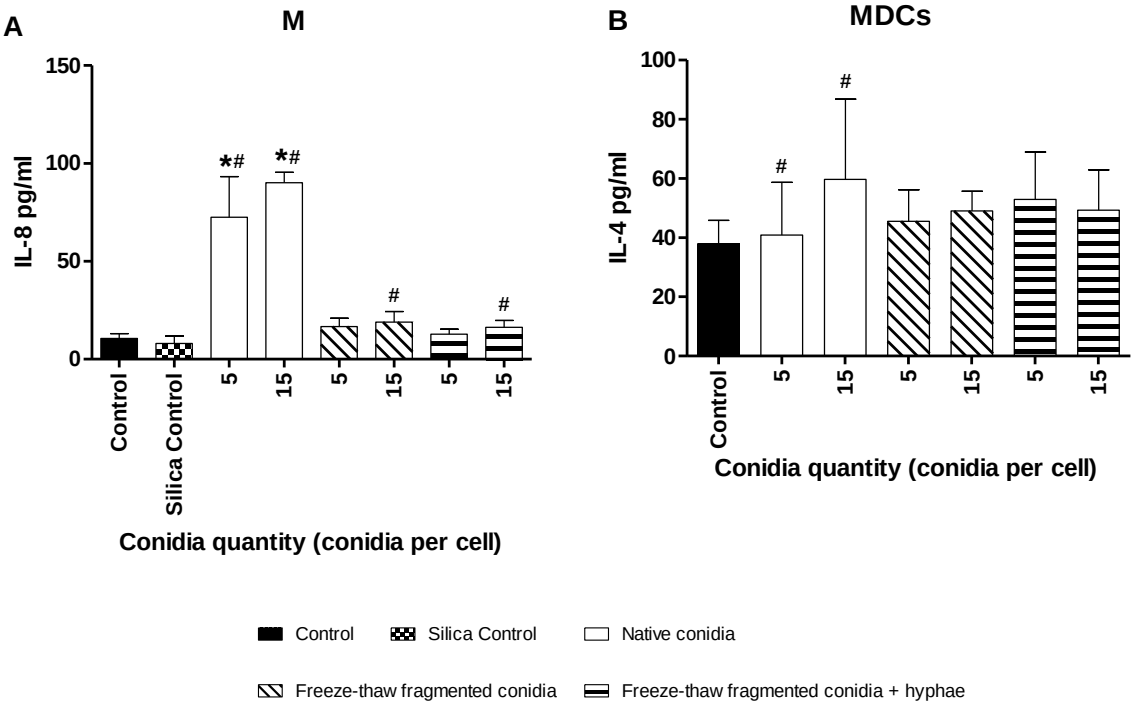

Figure 11. Induced release of IL-8 and IL-4 from mono-cultures when exposed to sampled *Aspergillus sp*. (8 hrs.). Monocytes (A) and mature DCs (B) were exposed to native conidia, conidia fragmented with 15 cycles of freeze-thawing, and conidia + hyphae fragmented with 15 cycles of freeze-thawing. Monocytes were also exposed to medium containing bead-beating silica. Data represent mean ± SEM for n = 3 (IL-8), n = 4 (IL-4) separate experiments. * $P < 0.05$ shows statistical difference of exposed culture compared to un-exposed control. # Indicates cell-cultures where conidia had germinated and formed hyphae after 8 hrs. of incubation. One-way ANOVA with Dunnett's compare to control tests was used on log transformed data to show significant differences.

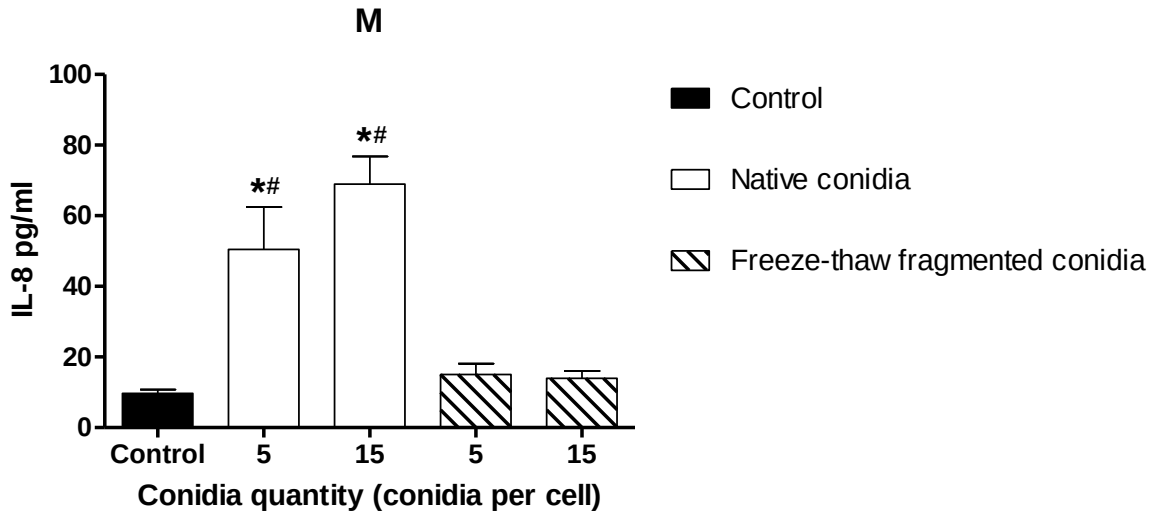

Figure 12. Induced release of IL-8 from mono-cultures of monocytes when exposed to sampled *Aspergillus sp.* (8 hrs.). Cells were exposed to native conidia, and conidia fragmented with 30 cycles of freeze-thawing. Data represent mean ± SEM for n = 4 separate experiments. * $P < 0.05$ shows statistical difference of exposed culture compared to un-exposed control. # Indicates cell-cultures where conidia had germinated and formed hyphae after 8 hrs. of incubation. One-way ANOVA with Dunnett's compare to control tests was used on log transformed data to show significant differences.

4.2.1.2 Collected Emericella nidulans

In Figure 13A release of IL-8 from monocytes increased significantly compared to non-exposed controls when exposed to all varieties of *E. nidulans* conidia, except for freeze-thaw fragmented conidia exposed at concentrations of 15 conidia per cell. The release of IL-8 was greatest when exposed to native conidia with 15 conidia per cell. Both concentrations of heat-inactivated conidia, as well as freeze-thaw fragmented conidia at a concentration of 5 conidia per cell also induced an increased release of IL-8 compared to control, but at lower levels than native conidia.

Mono-cultures of mature DCs exposed to *E. nidulans* did not show any statistically significant increase or decrease compared to non-exposed controls in response to exposure to any variety of conidia. A statistically non-significant increase in IL-4 levels was observed in cultures exposed to both heat inactivated and freeze-thaw fragmented conidia. (Figure 13B)

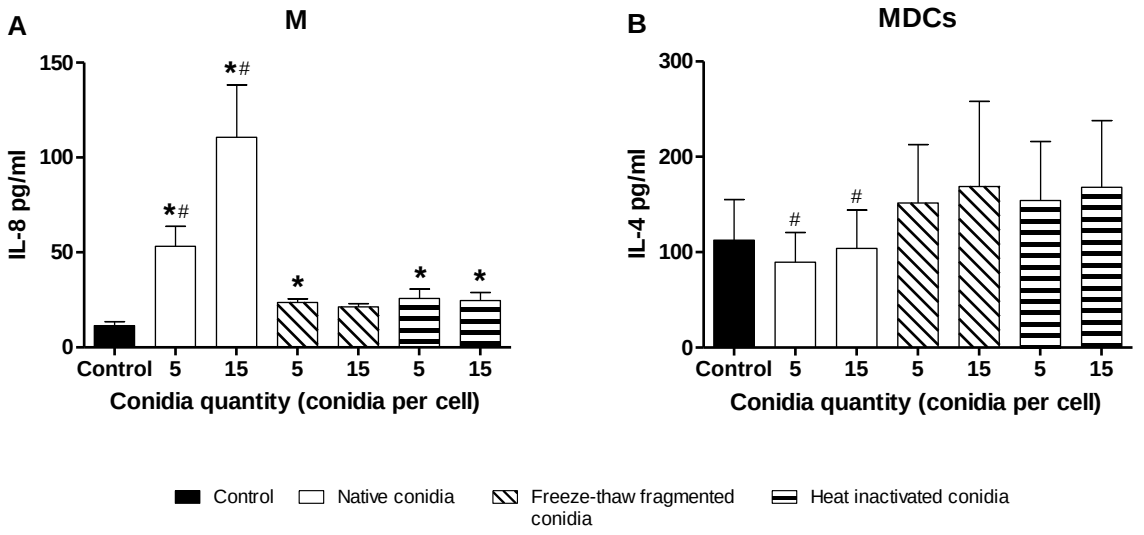

Figure 13. *Emircells nidulans* induced release of IL-8 and IL-4 from exposed mono-cultures (8 hrs.). Monocytes (A) and mature dendritic cells (B) were exposed to native conidia, conidia fragmented with 30 cycles of freeze-thawing, and conidia heat-inactivated at 60° C. Data represent mean ± SEM for n = 5 separate experiments. * $P < 0.05$ shows statistical difference of exposed culture compared to un-exposed control. # Indicates cell-cultures where conidia had germinated and formed hyphae after 8 hrs. of incubation. One-way ANOVA with Dunnett's compare to control tests was used on log transformed data to show significant differences.

4.2.1.3 Aspergillus versicolor

All monocytes showed increased release of IL-8 compared to non-exposed controls when exposed to all varieties of *A. versicolor* conidia. The release of IL-8 was greatest when cells were exposed to native conidia and heat-inactivated conidia at a concentration of 15 conidia per cell. The release of IL-8 from non-exposed controls was non-detectable. (Figure 14A)

Only mono-cultures of mature DCs exposed to heat-inactivated *A. versicolor* conidia at a concentration of 15 conidia per cell resulted in statistically significant increase of IL-4 compared to non-exposed controls, which looks similar to the response induced by heat inactivaeted conidia in monocytes (Figure 14A). Exposure to all other varieties of conidia did not yield any significant change in release of IL-4. (Figure 14B)

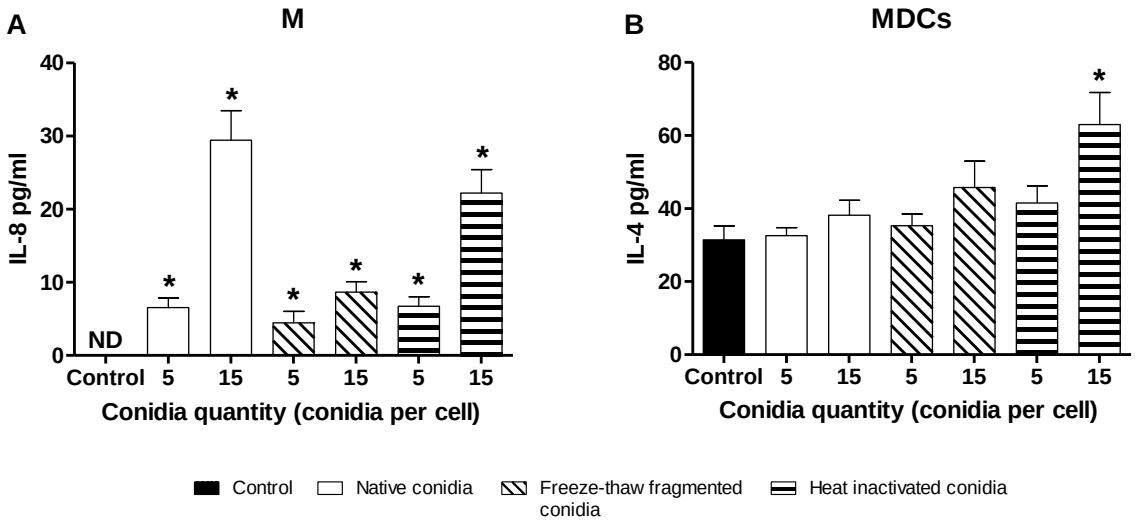

Figure 14. *Aspergillus versicolor* induced release of IL-8 and IL-4 from exposed mono-cultures (8 hrs.). Monocytes (A) and mature dendritic cells (B) were exposed to native conidia, conidia fragmented with 30 cycles of freeze-thawing, and conidia heat-inactivated at 60° C. ND= not detectable. Data represent mean ± SEM for n = 3-4 separate experiments. In order to perform statistical analysis for IL-8, values for non-detected was arbitrarily set to equal half of detection level. * $P < 0.05$ shows statistical difference of exposed culture compared to un-exposed control. One-way ANOVA with Dunnett's compare to control tests was used on log transformed data to show significant differences.

4.2.1.4 Aspergillus niger

Mono-cultures of monocytes exposed to A*. niger* did not show any statistically significant changes in IL-8 release compared to non-exposed controls in response to exposure to any variety of conidia. (Figure 15A).

Mono-cultures of mature DCs exposed to A*. niger* did not show any statistically significant changes in IL-4 release compared to non-exposed controls in response to exposure to any variety of conidia.

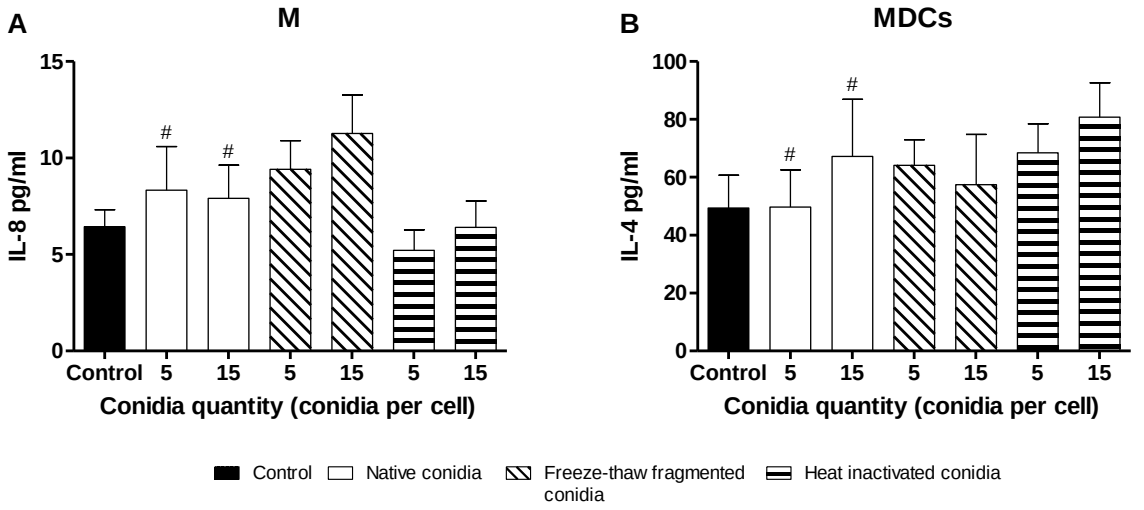

Figure 15. *Aspergillus niger* induced release of IL-8 and IL-4 from exposed mono-cultures (8 hrs.). Monocytes (A) and mature dendritic cells (B) were exposed to native conidia, conidia fragmented with 30 cycles of freeze-thawing, and conidia heat-inactivated at 60° C. # Indicates cell-cultures where conidia had germinated and formed hyphae after 8 hrs. of incubation. Data represent mean ± SEM for n = 4 separate experiments. * $P < 0.05$ shows statistical difference of exposed culture compared to un-exposed control. One-way ANOVA with Dunnett's compare to control tests was used to show significant differences, and in graph A analysis was performed on log transformed data.

4.2.2 Effect of mold conidia on the release of IL-8, IL-4, IL-13 and TSLP from co-cultures

Non-contact co-cultures of pneumocytes and immature DCs (P/IMDC) and contact co-cultures of monocytes in contact with pneumocytes, and in non-contact with immature DCs (M+P/IMDC) were exposed to heat-inactivated *Aspergillus niger* and *Aspergillus versicolor* conidia for 8 hrs. After exposure, cells were harvested and the release of cytokines was measured.

4.2.2.1 Pro-inflammatory response by IL-8

Non-contact co-cultures exposed to heat-inactivated conidia from *A. niger* or *A. versicolor* did not show any statistically significant increase or decrease in release of IL-8 compared to non-exposed controls (Figure 16A). In contrast, only contact co-cultures exposed to heat inactivated *A. versicolor* showed a statistically significant increase of IL-8 compared to controls (Figure 16B). Comparative monocultures of monocytes, pneumocytes and immature DCs showed that monocytes had a significantly increased IL-8 response to heat-inactivated *A. versicolor* conidia. Immature DCs also had an increased response to *A. versicolor* although not significant, whereas pneumocytes displayed no significant IL-8 response (Figure 17).

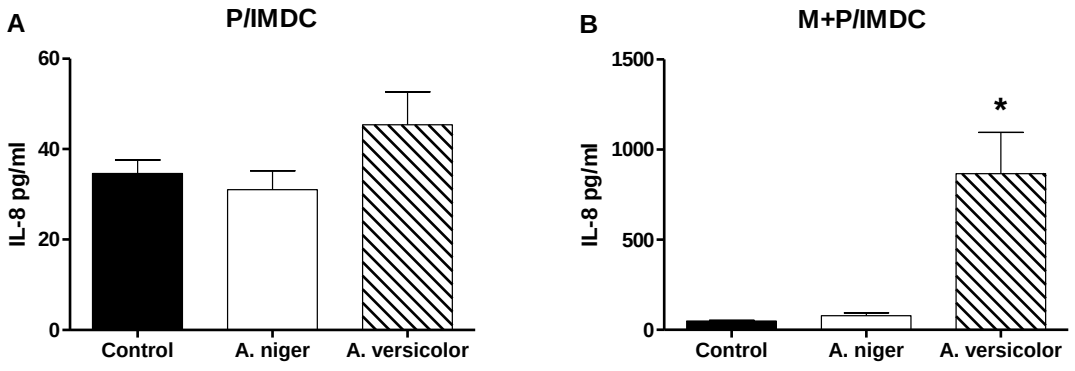

Figure 16. Release of IL-8 from exposed co-cultures (8 hrs). Non-contact co-cultures (A) of pneumocytes (P) and immature DCs (IMDC), and contact co-cultures (B) of monocytes (M) in contact with pneumocytes (P), and in non-contact with IMDC were exposed to conidia, heat-inactivated at 60° C, from *Aspergillus niger* and *Aspergillus versicolor* in concentrations of 10.9 x 10^6 conidia / ml. Data represent mean ± SEM for n = 3 (non-contact co-culture),n = 4 (contact co-culture) separate experiments. * P < 0.05 shows statistical difference of exposed culture compared to un-exposed control. One-way ANOVA with Dunnett's compare to control tests was used to show significant differences.

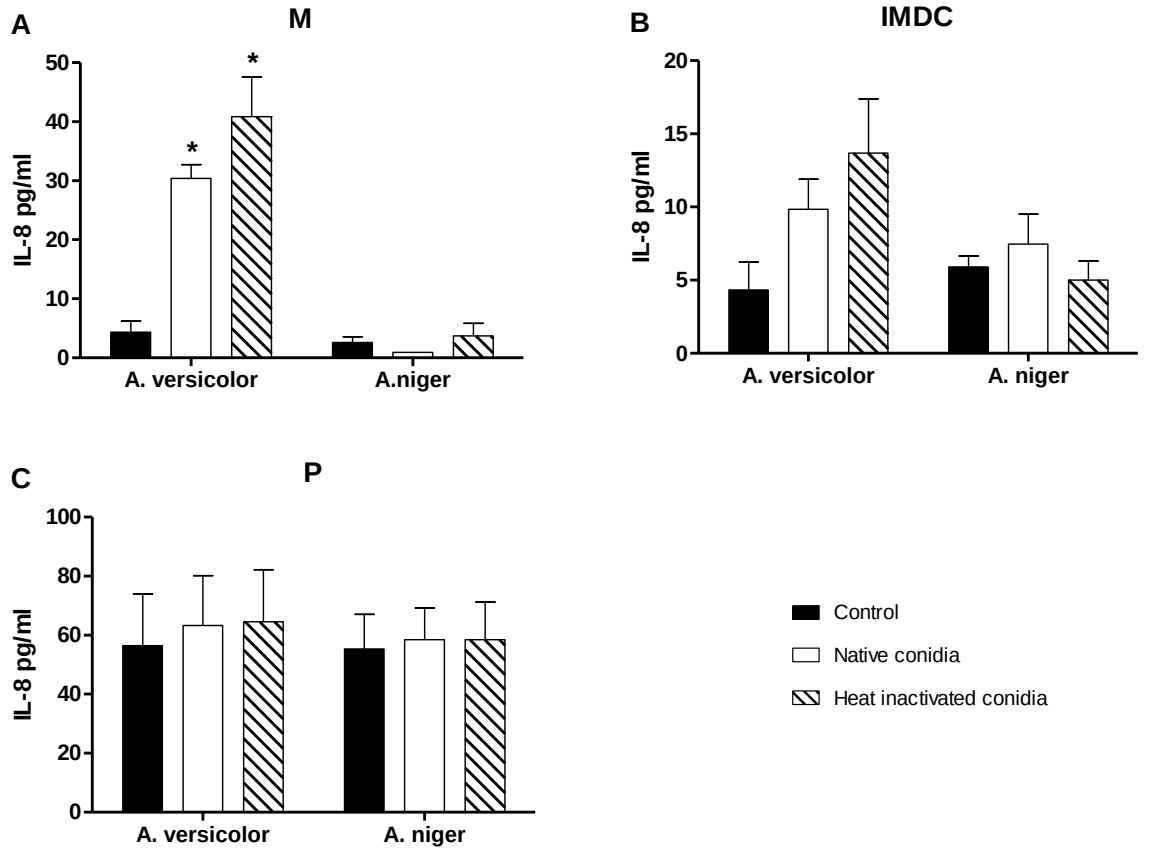

Figure 17. Release of IL-8 from exposed mono-cultures (8 hrs.). Monocytes (A), pneumocytes (B), and immature DCs (C) were exposed to native conidia and conidia heat-inactivated at 60° C, from *Aspergillus niger* and *Aspergillus versicolor* in concentrations of 15 conidia / cell. Data represent mean ± SEM for n = 4 separate experiments. * P < 0.05 shows statistical difference of exposed culture compared to un-exposed control. One-way ANOVA with Dunnett's compare to control tests was used to show significant differences.

4.2.2.2 Response by IL-4

Neither non-contact nor contact co-cultures exposed to heat-inactivated conidia from *A. niger* or *A. versicolor* did show any statistically significant changes in release of IL-4 compared to non-exposed controls

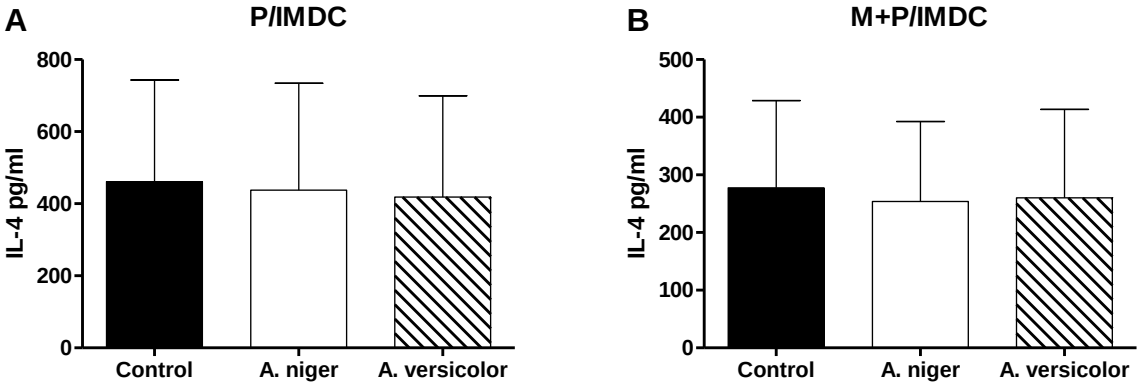

Figure 18), likewise did mono-cultures of immature DCs not show any response (Figure 19). IL-4 was not measured in monocytes or pneumocytes.

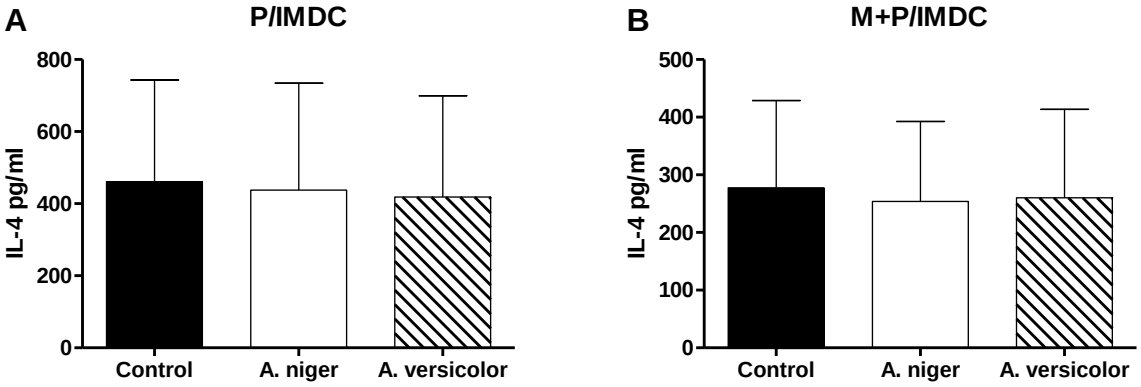

Figure 18. Release of IL-4 from exposed co-cultures (8 hrs.). Non-contact co-cultures (A) of pneumocytes (P) and immature DCs (IMDC), and contact co-cultures (B) of monocytes (M) in contact with pneumocytes (P), and in non-contact with IMDC and were exposed to conidia, heat-inactivated at 60° C, from *Aspergillus niger* and *Aspergillus versicolor* in concentrations of 10.9×10^6 conidia / ml. Data represent mean ± SEM for n = 3 (non-contact co-culture), n = 4 (contact co-culture) separate experiments. * $P < 0.05$ shows statistical difference of exposed culture compared to un-exposed control. One-way ANOVA with Dunnett's compare to control tests was used to show significant differences.

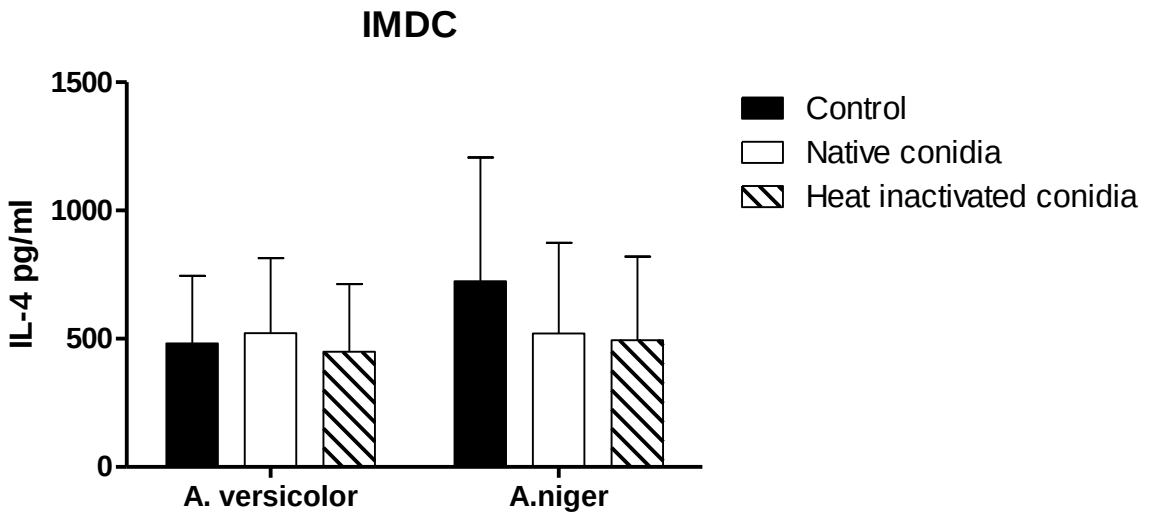

Figure 19. Release of IL-4 from exposed mono-cultures of immature dendritic cells (8 hrs.). Cells were exposed to native conidia and conidia heat-inactivated at 60° C, from *Aspergillus niger* and *Aspergillus versicolor* in concentrations of 15 conidia / cell. Data represent mean ± SEM for n = 4 separate experiments. * $P < 0.05$ shows statistical difference of exposed culture compared to un-exposed control. One-way ANOVA with Dunnett's compare to control tests was used to show significant differences.

4.2.2.3 Allergic response by IL-13 and TSLP

The non-contact and contact co-cultures did not release any detectable levels of IL-13 nor TSLP, even when exposed to heat-inactivated conidia from *A. niger* or *A. versicolor.*

4.2.3 Relative binding of cytokines to mold particles

Relative binding of cytokines to mold conidia was estimated using a cell-free model. Displayed in Figure 20 is the percent of available cytokine bound by mold conidia. With this assessment, on average, between 20-30 % of available cytokine was bound to either native conidia, freeze-thaw fragmented conidia, or heat-inactivated conidia compared to non-exposed control. *A. niger* showed various patterns of binding varying with the type of treated conidia, type of cytokine and concentration of cytokine in media. This test was only performed once, and no statistical analysis was used to analyze the data. Some of the tests were inconclusive, and only a selection of the results is presented. It is necessary to run more tests to ensure the quality.

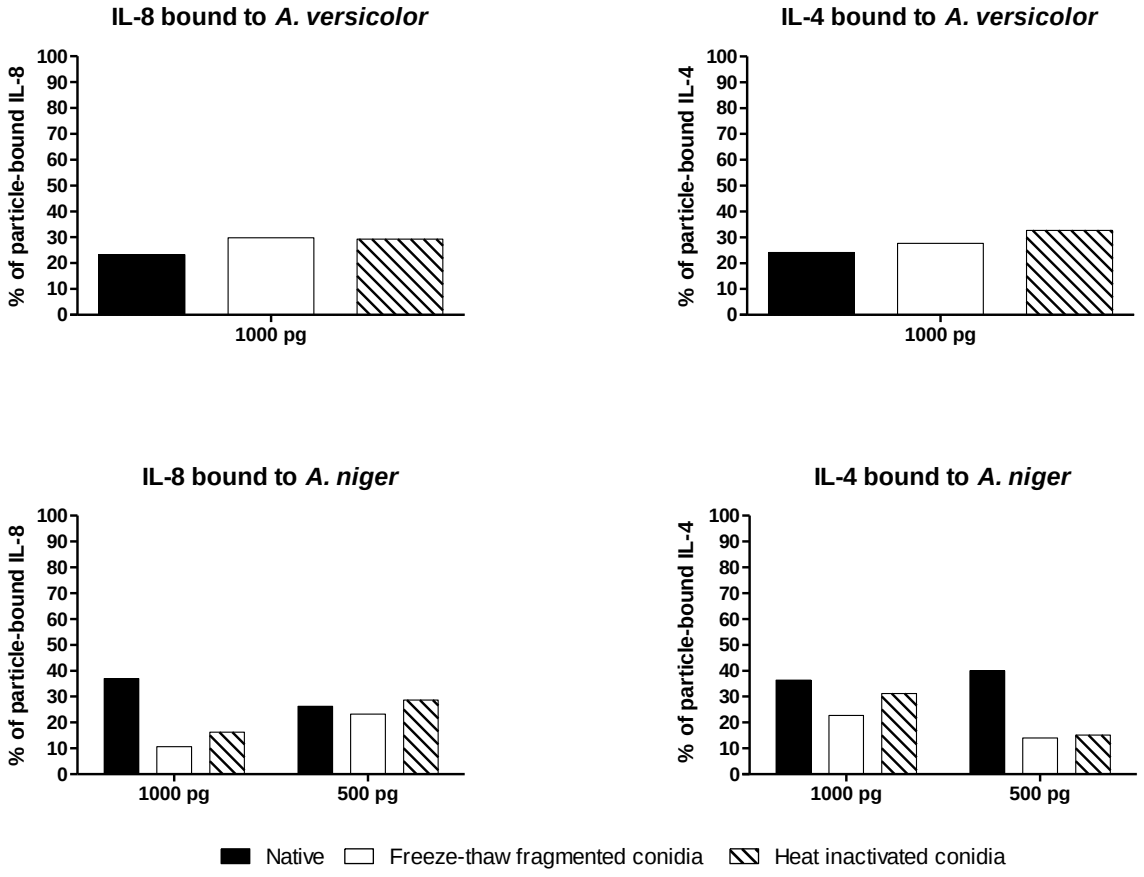

Figure 20. Percent of available cytokine absorbed by particles (mold conidia). 1000 pg / ml and 500 pg / ml of recombinant IL-8 was exposed to native conidia, conidia freeze-thaw fragmented for 30 cycles, and conidia heat-inactivated at 60° C from Aspergillus versicolor and Aspergillus niger for 2 hrs. 1000 pg / ml of IL-4 was also exposed to conidia from Aspergillus versicolor and Aspergillus niger. Conidia and fragments were exposed in concentrations of 12×10^6 conidia / well.

5 Discussion

5.1 Sampling and Identifying Molds

5.1.1 Air Sampling of Viable Conidia

Several methods for collecting mold samples were considered for this study. We decided early that surface sampling methods were not suitable for this study. The drawback of surface sampling is that it is only possible to collect samples of visible molds, or airborne conidia that have settled. There is also a problem of sampling bias. The method involves transferring surface samples to an agar plate, However, since a 9 cm agar plate pressed at one site may not give the same result as another agar plate pressed elsewhere at the same site. Because of these drawbacks, it is difficult to establish standardized sampling procedures that will eliminate bias. A suggested application of surface sampling is as quality control, after measures have been taken to decontaminate the house for molds. By doing this it is possible to control specific areas to see if mold growth reoccurs [19]. As the main focuses for this study was respiratory, it was considered more pertinent airborne mold particles rather than settled conidia or mold growth.

Air samplers such as an impactor will collect airborne viable conidia on agar plates. An advantage of most air sampling methods is that samples can be collected at a certain flow rate and volume, such as 10 or 500 liters of air. This enables quantification of the contamination. For this study we sampled 500 liters of air onto agar plates which were incubated at 37^{o}C. Preliminary experiments (not shown) suggested that this was an appropriate sample size that would yield about 1-20 mold samples on each plate when cultivated at 37^{o}C. Furthermore, sampling volumes in excess of 500 liter can cause drying of the plates.

However, air samplers will favor fungi that produce large quantities of small dry conidia, such as *Penicillium* species or *Aspergillus* species. Fungi that produce large conidia or conidia in slime, such as *Stachybotrys* species, are not as likely to be detected by air sampling as these conidia are less likely to become airborne [72]. Also, by cultivating air samples directly onto agar only viable airborne conidia are counted. Hyphal fragments and unviable components of mold growth which can have relevance for health, will not be detected using this method [72]. Studies using air impactors have tried to determine if there is a connection between CFU/m^3 air and dampness and mold associated health problems, but have failed to find such connections [22]. This would imply that a high number of airborne viable conidia do not necessarily pose a health threat.

A SAS Super Iso 180 (PBI) microbiological air sampler, which impacts airborne conidia directly onto agar plates, was used. Furthermore it was chosen to only examine molds that were able to grow at 37^{o}C. This temperature was selected for two reasons. Results in preliminary sampling

experiments (not shown) found that by incubating the plates at room temperature, the plates would be overgrown after 48 hrs. of incubation, even when only sampling 10-20 liters of air. By choosing a more selective temperature, we were able to limit the sampling material for further study to a manageable number isolates. Secondly, by selecting for molds that are capable of growing at 37°C we were also selecting for molds that could potentially germinate and grow in the respiratory system. The ability to cause infection is also important, since many studies have suggested that mold conidia only trigger immune responses in the early stages of germination [36, 73-75]. Hence the ability to germinate in-vivo becomes a key factor.

However, by selecting for molds that grow at 37°C, the method introduces a sampling bias. It is possible that allergenic fungi could be excluded from sampling. *A. versicolor* that was used for this study did not grow at 37°C and had to be cultivated at room temperature. Hence it would not have been possible to detect *A. versicolor* with the chosen experimental setup. Cultivating air samples at lower temperatures as well as at 37°C, and is something that should be considered for future studies.

A possible improvement of the impactor method could be to sample the air directly onto a water-based polymer agar [76]. This would enable the collection medium to be dissolved after sampling, and fungal particles could be collected on a filter for further analysis. The suspension would contain not only conidia, but also fungal fragments and other airborne particles which could be used in in-vitro exposure experiments. Using such a mixture could possibly provide insights on the effects of a wider range of fungal antigens on respiratory health. Such a suspension could also be used for identification purposes and population studies. It is possible to extract genetic information from both conidia and mold fragments, so it could be possible to identify several molds in the suspended sample in a single run, for example by using multiplex PCR or microarray chips.[77, 78].

5.1.2 PCR identification of molds

In order to extract DNA from the conidia and hyphae samples, the bead-beating procedure was optimized. This approach gave DNA from 24 out of 25 mold samples. For this study we followed the recommendations from Zhou et al. [26] for using a bead-beater for extraction of DNA from conidia. The same group also suggests that French-press and freeze-press methods are efficient methods for releasing DNA from conidia with about 80% conidia breaking efficiency. However, silica bead-beating was most efficient, reaching almost 100% conidia breakage. After observing conidia under a light microscope after bead-beating, the approach was found to be highly efficient at fragmenting conidia and hyphae. We thus decided to use the procedure also to

fragment conidia and hyphae to be in-vitro exposure experiments as well. This is discussed below.

The use of PCR/sequencing is a very efficient approach to the identification of molds. However, it was evident that the single step PCR sequencing method that was used in this study did not provide sufficient information to identify the sampled molds to the species level. The eukaryote specific primer set (EUK1209 / UNI1392) yielded PCR products for 23 out of 24 molds, whereas the indoor mold specific primers set (FF2 / FR1) yielded PCR products for 9 out of 24 molds, with 6 of these being mold samples from damp or water damaged rooms. Combining the two primer sets enabled the identification of 6 out of 24 molds at the species level. Of the 6 PCR products that were sampled in damp rooms and sequenced by both primer sets, 5 were identified as *Aspergillus sp.* and one as *Emericella nidulans.* If single set PCR is to be used for identification, there is a need to develop primers that will amplify a more variable region of fungal DNA. This will yield PCR products that will vary in their DNA sequence according to species of mold, and such sequences would be better to use for identification [23, 26]. More exact results and a longer were recently obtained using a third primer set (EUK1A/EUK516). This enabled a more specific identification of the two environmental isolates were chosen for cell culture studies.

There are other molecular identification methods that could be considered as a replacement for single set PCR identification. Rather than using only one or two primer sets for identification, other molecular methods are capable of identifying many different species of molds. With some of these methods it is be possible to identify several molds in one sample. By sampling both airborne conidia and fungal fragments on a waterbased polymer with an impactor air sampler, a suspension containing both conidia and fungal fragments from many molds can be obtained. This sample could be analyzed with a multiplex PCR. Here, several primers specific for different molds are loaded in the same PCR reaction. All the primers are designed to yield PCR products of different lengths. If several molds of interest are present in the same sample, a multiplex PCR will yield PCR products for each of these. The PCR products can then be separated by size, thus revealing the identity of the molds in the sample [77]. However, multiplex PCR will only be capable of identifying molds that are covered by the primers used. Molds that do not react with any of the primers will go undetected through a multiplex PCR.

A better candidate for identifying specific indoor molds in air samples are microarray based methods. In this system, several DNA probes are fixed on a solid surface such as a glass slide, which together make up a microarray chip. Each probe can be specifically designed, and each glass slide can accommodate thousands of DNA probes. In theory, each probe could represent a

specific mold. Sampled DNA is then amplified with PCR and at the same time marked with fluorescence. Target DNA will then hybridize to the specific probes and be bound to the microarray chip, and the microarray can then be scored for fluorescence. Fluorescence on the microarray will then identify the presence of the molds specific to the fluorescent probe spots. [78]

5.1.3 Mycotoxins

There are several methods available for detecting mycotoxigenic molds. The most common methods are analytical methods like commercially available ELISA kits for mycotoxins, high-performance layer chromatography (HPLC), and thin layer chromatography (TLC)[16]. Some mycotoxins, like aflatoxin, are possible to detect using culture methods as well. As the sampled molds were partly identified by PCR sequencing, most of the molds were identified as *Aspergillus* genus. Since aflatoxins only are produced by some species of *Aspergillus* and *Penicilliums*, it was decided to test if any of the isolates were aflatoxigenic strains. No other known mycotoxins are associated with *Aspergillus* [35].

Aflatoxin emits blue fluorescence under ultra-violet (UV) light. A previous study has shown that the fluorescence from aflatoxin can be greatly enhanced by supplementing the growth agar with β-cyclodextrin, thus adding to the sensitivity of this method of detection [16]. This culture method for detecting aflatoxin provided a simple, low-cost, and fast way to detect aflatoxigenic molds. Analytical methods like ELISA, HPLC and TLC all provide quantifiable measures for toxin production. However, such approaches are costly, and require time consuming extraction steps, which are not necessary with culture methods [16]. It is possible to test for other mycotoxins as well, but unless other species, like trichothecene toxin producing *Stachybotrys chartarum* is observed in the samples there is little reason to test for such toxins. Trichothecene toxins have been reported to be found in low concentrations in air samples in previously flooded houses. However, dust or surface samples have been shown higher concentrations [18].

Aflatoxins are considered to be carcinogenic, whereas trichothecenes induce vomiting, diarrhea and headaches. These toxins are also considered to be immunosuppressant [15]. However, the dose of mycotoxins that is acquired through inhalation has rarely been found to be sufficient to induce any adverse health effects. Generally only industry workers that handle large quantities of crops or foods are exposed to enough respirable mycotoxins to develop adverse health effects [35].

All of the sampled molds in this study tested negative for production of aflatoxin. Thus the damp and water damaged rooms do not seem to promote the growth of aflatoxin producing strains. This may indicate that adverse health effects in response to these molds would not be due to

mycotoxins, and that these molds should rather be studied for their effect on developing adverse cellular immune responses.

5.2 The Effect of Mold Conidia on Release of Inflammatory Mediators in In-vitro Exposure Experiments

5.2.1 Mold Conidia

Cell mono-cultures and co-cultures were exposed to conidia from several different species of molds. Of the molds used to expose cells, two of them, *Emircella nidulans* and one unknown *Aspergillus sp.* Isolated from air samples from damp rooms. Three other molds, *Aspergillus versicolor, A. niger,* and *A. fumigatus,* were selected based on their known prevalence in damp indoor environments and associations with health effects.

The literature shows that a variety of methods are used to inactivate conidia, so that they will not germinate during the course of the experiments. One study used 5 kilogrey (unit of absorbed radiation dose) gamma radiation to inactivate conidia [79], whereas some studies have used chemical inactivation such as formaldehyde [75] or exposed cells to conidia with a fungistatic agent [73]. Many studies have used heat inactivated conidia, achieved either by autoclaving (120^{o}C for 20 minutes) or by heating the conidia in 100^{o}C water bath for 60 minutes as method for inactivation [75, 80, 81]. Untreated conidia has also been used in several studies, but they have been shown to germinate as early as 8 hrs. post-exposure [75]. This was also confirmed in our study, as we saw conidial germination within 8 hrs. of exposure for several species of mold. Using untreated native conidia can then become a limiting experimental factor since the formation of hyphae can severely affect the cell culture conditions, and in consequence it will become difficult to run exposure experiments longer than 8 hrs. We found that when monocytes were exposed to conidia that formed hyphae, the cells would no longer be in suspension but be bound by the hyphae at the bottom of the well. A study by Murphy et al. [82] found that when macrophages and monocytes were exposed to particles that were longer than the cells were capable of phagocytosis, they induced a greater pro-inflammatory response by the cells than if the particles were short. Cells would also attain macrophage properties, and latch on to the particles in an effort to encapsulate them. This could suggest that fungal growth induces macrophage maturation from monocytes, and could potentially explain why monocytes were no longer floating suspended in the culture medium.

In preparing the in-vitro exposure experiments, we considered adapting several of these methods for inactivation of conidia to use in cell exposure models. We tested heat inactivation,

chemical inactivation with methanol, and repeated shock-freezing of the conidia and hyphae in liquid nitrogen followed by thawing them in a 40°C water bath and finally bead-beating them to create conidia fragments. Early in the study we excluded the methanol treated approach, as it showed no advantages when compared to heat-treated conidia, both when considering the practicality of the inactivation procedure and the inflammatory response in cell-cultures (data not shown).

The protocol for heat-inactivating of conidia was also modified during the study. Initially conidia were inactivated by autoclaving suspended conidia at 120°C for 20 minutes. This was modified to heat-inactivating the suspended conidia to 60°C for 60 minutes. The temperature was lower since we suspected that a higher temperature could possibly destroy or alter epitope structures on the surface of the conidia that are involved in inflammatory signaling pathways. This suspicion has also been shared by other studies [75]. We found that for several species of mold 60°C is a sufficient temperature to achieve inactivation of the conidia [83, 84]. This was confirmed in our study, as exposure of conidia treated with 60°C showed no germination after 8 hrs. and treated spores streaked onto malt extract agar did not show any sign of growth after 48 hrs. of incubation.

It was also necessary to adjust the protocol for freeze-thawing fragmenting of mold conidia after the first in-vitro exposure experiment with fragmented conidia. After 8 hrs. of exposure conidia germination was detected in several wells with fragmented conidia. Initially the conidia were only subjected to 15 cycles of freeze-thawing, but when this proved to be insufficient to inhibit germination, it was increased to 30 cycles of freeze-thawing. This was sufficient to inhibit germination as it was confirmed by streaking the conidia out on malt extract agar.

The maturation stage of the conidia used for exposure is probably also important to consider, as studies have shown that *Aspergillus fumigatus* has a stage-specific display of β-glucans [73, 75]. The studies suggest that β-glucans, which acts as triggers for immune responses, are displayed on the surface of the conidia just prior to germination. The conidia should be inactivated at the proper stage, just prior to germination when the conidia are swollen, to induce proper inflammatory responses [73-75]. The maturation stage of the conidia was not accounted for in this study, but future work should take this into account.

All conidia that were used in this study came from colonies that were cultivated on MEA. It is a question how molds cultivated on agar plates compare to molds that grow in damp environments. A mold may cause varying immune responses depending on what material the mold grows on, since they can produce various metabolites and display different properties depending on the growth surface. Molds cultured in the core of plasterboards are for example

more potent inducers of immune responses than molds cultured on the surface of plasterboards [85, 86]. This could suggest that using mold conidia and fragments from molds cultivated in a laboratory on agar might not give a complete representation of actual conditions in damp building environments.

5.3 Immune Responses by Mold Exposure to Cell Cultures

5.3.1 Conidia induced IL-8 release from monocyte mono-cultures

IL-8 is an important inducer of inflammation. When released at sites of inflammation it will recruit neutrophils and monocytes to the site of inflammation. When mono-cultures of monocytes were exposed to conidia from *A. niger, A. versicolor, E. nidulans,* and one unknown *Aspergillus sp.* we found that all native mold conidia except *A. niger* caused significant increases in the release of IL-8. We also saw that heat inactivated *A. versicolor* conidia induced an IL-8 response similar to the response induced by native conidia. Perhaps surprisingly, the results showed that freeze-thaw fragmented conidia only induced low IL-8 response compared to that of native conidia. We had suspected that fragmentation would increase exposure of inflammatory β-glucans, but that did not reflect in the induction of IL-8 release from monocytes. Several studies have implied that conidia have to be swollen to induce release of inflammatory mediators, since conidia only expose β-glucans at this stage. Most of these studies have been done on *Aspergillus fumigatus*, but it is very likely that this would apply to other species of mold as well [73-75, 80, 87].

The low levels of IL-8 release seen in the present study with most of the inactivated conidia might be related to the fact germination is necessary for the conidia to present its surface antigen structures. No procedures were used to determine whether conidia used in this study swollen or not. This could provide an explanation as to why the induced release of mediators was not consistent with results from other studies. It is only just prior to germination that β-glucans are exposed on the conidia surface [36], and when the conidia have been inactivated it is possible that they never fully display the surface β-glucans. Furthermore, fragments have been hypothesized to be potent inducers of immune responses. This is because conidial fragments have surface proteins exposed constantly. A study by Pei et al. (2001) has shown that THP-1 monocytes when exposed to fragments of *A. versicolor* show a significant increase in the release of IL-8 [88]. In that study, mold fragments were obtained by sampling fragments directly from cultivated colonies. However, in the present study, only the high concentration of fragmented *A. versicolor* conidia induced a significant increase in the release of IL-8. All other molds failed to induce or induced poorly the release of IL-8, when exposed to monocytes. This could indicate

that freeze-thaw fragmenting causes some alteration in the fragments so they fail to induce any response.

Several studies have shown that monocytes exposed to *Aspergillus versicolor* or *Aspergillus fumigatus* have a marked increase in the release of pro-inflammatory cytokines like IL-8 and TNF-α [42, 88, 89]. Our study confirms these results since they showed a significantly increased release of IL-8 when exposed to native conidia of all molds that were used, not only *A. versicolor.* Most of the in-vitro experiments where molds have been studied have used *A. fumigatus,* and some have used *A. versicolor* or *Stachybotrys chartarum.* Other molds need to be explored using these models also.

5.3.2 Conidia induced IL-8 release from co-cultures

Two variations of co-cultures were exposed to heat inactivated conidia of *A. niger and A. versicolor.* These were non-contact co-cultures of pneumocytes and immature DCs (P/IMDC), and contact co-cultures with monocytes and pneumocytes in non-contact with immature DCs (M+P/IMDC). Only exposure to *A. versicolor* induced a significantly increased release of IL-8, from the contact co-culture. In the non-contact co-culture there was only a slight non-significant increase in release of IL-8. This correlated partly with what was observed when the respective cells of the co-cultures were exposed to conidia as mono-cultures, since only the monocytes showed a significant increase in the release of IL-8, whereas immature dendritic cells showed only a slight, non-significant increase. *A. niger* did not induce any significant response measure in IL-8 response in co-cultures and in mono-cultures.

The increased release of IL-8 in both monocyte and immature dendritic cell mono-cultures did not sum up to the release measured in the contact co-culture. This would suggest that there is some interaction between the cells, which causes a greater release of IL-8 than what is seen in mono-cultures. There have been studies which indicate that cellular interaction could play an important role in enhancing the inflammatory response in co-cultures [62]. A study has shown that when monocytes and pneumocytes are exposed to particles as mono-cultures they release low levels of pro-inflammatory cytokines. However, when these cells are cultured as non-contact (M/P) or contact (M+P) co-cultures they release much higher levels of pro-inflammatory cytokines, with the non-contact co-culture releasing the highest levels [62, 90]. This could suggest that monocytes possibly induce a release of pro-inflammatory mediators, such as IL-8, from pneumocytes. This exemplifies not only the value of using co-culture in-vitro models, but also that the design of such co-cultures should be carefully considered and optimized to mimic in-vivo situations as closely as possible [33].

Other studies using molds and conidia to expose co-cultures could not be found. These methods have been used in a number of studies where other particles, like road dust or nanoparticles have been used for exposure [63, 91, 92]. Adapting co-culture models for use to assess the effect of mold particles could be of great value. It would give more information than what is available now only from mono-culture models. To our knowledge, this is the first study that has adapted lung co-cultures to learn more about the immune effects of mold conidia and fragments.

5.3.3 Conidia induced IL-4 release from mature dendritic cells mono-cultures

Dendritic cells initiate the adaptive immune responses to invading pathogens. They present the processed pathogens to T helper cells which in turn activate the adaptive immune response. If the activation of T helper cells by dendritic cells is marked by high levels of IL-4 in-vivo it is often thought to favor the activation of Th2 cells which is often seen as a marker for allergy and asthma pathogenesis [28].

Only mature dendritic cells exposed to heat inactivated *A. versicolor* showed a significant increase in the release of IL-4 in our study, whereas exposure to all other species or conidia treatments did not induce any significant IL-4 response. Heat inactivated *A. versicolor* did also induce a significant release of IL-8 from monocytes. This finding was interesting as it possibly suggests that *A. versicolor* might be a potent immune system stimulating mold. No studies could be found that had exposed DCs in monocultures to mold particles. Studies with DCs was mostly from animal models and some co-cultures. To our knowledge, no studies have exposed mono-cultures of DCs to mold conidia or fragments.

One possible explanation for why there was so little response from the mature dendritic cells in the release of IL-4 could be that mature dendritic cells are less predisposed for searching and engulfing pathogens. In vivo, most dendritic cells in tissues where infections occur exist as immature dendritic cells, and they are much more active when it comes to engulfing pathogens and initiating immune responses [64]. The THP-1 derived immature DCs that were used in this study have been confirmed be Berges et al. (2005) to attain these properties [93]. Dendritic cells also play an important role in mediating the intricate interplay between cells that are active during inflammation. Most exposure studies with dendritic cells are conducted in contact with other cells, such as in animal models or co-culture experiments [43, 64]. Thus a possible reason for the lack of response from DCs could be due to lack of cellular interaction or lack of contact with other cells.

5.3.4 Conidia induced release of allergy mediators from co-cultures

Neither *A. niger* nor *A. versicolor* induced any significant response measured in IL-4 release from co-cultures. IL-4 along with TSLP and IL-13 are markers of adaptive immunity activation and

Th2 responses [94]. TSLP and IL-13 were not detected in co-cultures, even when exposed to mold conidia. It is not unexpected that there was little response of IL-4 and no detection of IL-13, as these cytokines are reported to mainly be produced by T-cells [61, 94]. However, IL-13 has been found to be released from DCs [59].In the present study, IL-4 did increase significantly in mono-cultures of mature DCs that were exposed to heat inactivated *A. versicolor*, suggesting that they have the potential of releasing IL-4. One study has also seen that the release of IL-4 from DCs could possibly be induced by exposure to mold conidia and fragments [57] . TSLP is mainly released from epithelial cells, so it could be expected that co-cultures with pneumocytes would have released TSLP [61]. However, for this study, no TSLP was detected. In-vivo, it takes between 12 and 24 hrs. to complete maturation of DCs and migration to peripheral lymphoid organs. This possibly suggests that the 8 hrs. of exposure used in this study might not be sufficient to allow for allergy mediators like IL-4, IL-13 and TSLP to respond. One study detected increased release of IL-13 from DCs after 48 hrs. of exposure [59].

5.3.5 Particle-binding of cytokines

Cytokines and chemokines can bind to particles [68, 69]. This can lead to misinterpretations in in-vitro cell experiments when studying the immune responses by exposure to mold particles. In our particle binding test, native, heat inactivated and freeze-thaw fragmented conidia from both *A. niger* and *A. versicolor* was demonstrated to bind between 20-30% of IL-4 and IL-8. Native conidia from *A. niger* bound almost 40% of available IL-4 and IL-8. These results confirm that quantitative measurements of IL-4 or IL-8 in conidia exposed cell-cultures might be biased due to binding by mold conidia. Several studies have examined the binding of cytokines to particles, and have found that this often occurs [69]. The amount of cytokine that binds varies from particle to particle, as some do not bind cytokines at all, whereas other particles bind up to 85% of available cytokine [68]. It is essential to test the binding of more cytokines and several molds, as the relative binding might vary from mold to mold. If the relative binding by a mold is particularly high, it should be considered to adjust results measured from in-vitro experiments to account for relative binding. For the present study the results from particle-binding tests was not used to correct results from in-vitro exposure experiments. These tests have to be quality controlled, and estimated for more concentrations of cytokine. When particle-binding results are consistent and reliable they can be used to estimate a correction factor that results should be corrected by.

5.3.6 Evaluations if in-vitro exposure experiments

In-vitro exposure provides reproducible models in which it is possible to study immune responses to fungal conidia and fragments. Compared to animal models the in-vitro exposure approach allows a more broad-based way to investigate immune effects of molds. The costs are

lower and the laboratory preparations are less demanding than animal models. Therefore a larger range of molds can be investigated than in animal models. Mono-culture experiments provide a good indication of what molds could potentially be of interest with respect to respiratory health. In-vitro studies simplify the studies of specific responses and signaling pathways. However, in-vitro studies are limited, and findings will eventually have to be confirmed in animal studies.

We found that in-vitro experiments it made more sense to use immature DCs than mature DCs. As indicated above, they are more reactive towards pathogens, and will actively seek out pathogens to engulf them and process their antigen structures and mediate the recruitment of other immune cells to the site of infection [43]. Mature DCs have a significantly decreased ability to phagocytose pathogens, and are in a state where they present MHC 2 proteins on their surface [95]. In order to make co-culture exposure experiments as realistic as possible, we used immature DCs rather than mature DCs.

Co-culture models are useful for assessing immune responses. In contrast to mono-cultures, a co-culture model will account better for the cellular interplay that occurs in vivo when mediating immune responses[63, 91] . In co-culture models in this study we saw that the release of IL-8 was far greater in contact co-cultures with M+P/IMDC exposed to *A. versicolor* than in monocultures of the same cells. Upon exposure it is likely that monocytes induce a release of IL-8 from pneumocytes, whereas pneumocytes in mono-cultures did not respond to *A. versicolor [90, 91]*.

Only two models of co-cultures were tested in this study. However there are many variants of co-culture models that have been used in other studies [29, 30, 63, 96]. These vary in cell types or in construction of the co-culture. Other types of co-cultures in addition to those that have been tested in this study should be considered in future work. Other co-cultures might present a more accurate representation of actual in vivo responses.

When deciding how to expose in-vitro cultures to conidia and fragments, we considered several methods. Initially the concern was to find a method for inactivating germination of the conidia, as we considered that hypha growth would decrease the reproducibility of experiments, and also minimize the risk of contamination in the laboratory. As mentioned earlier, not all of species of native conidia germinated within 8 hrs of incubation, but some did, hence the germination had to be inactivated. For this study, conidia germination was inactivated by heat inactivation at 60^{o}C and by freeze-thaw fragmentation. The idea was to inactivate germination in the gentlest way possible, so that cellular response to inactivated conidia would be as similar as possible to

the response induced by native conidia. 60°C was sufficient to inactivate all conidia [97], and we concluded that this was the preferred method for conidial inactivation.

The freeze-thaw fragmentation method was good for inactivating growth and fragmenting conidia. However we are not certain as to whether this is the best approach for future studies. When transferring the fragmented conidia from the bead-beating tube with silica beads at the end of freeze-thaw fragmentation was complicated, the silica had to be rinsed in order to extract most of the fragments. However, fragments were still lost to the silica beads, which adds creates difficulty in estimating how many fragments were exposed to the cells. It might be possible that freeze-thawing alone could cause some fragmentation of the conidia [26], but this would have to be confirmed before it could have been used in in-vitro exposure models.

The next logical step for improving in-vitro experiments would be to increase the time of exposure. So far, cells have only been exposed to conidia for 8 hrs. This might not be sufficient to induce the adaptive immune responses [95]. By increasing the time of exposure to 24 and 48 hrs, more time would allow the development of adaptive immune responses. It is likely that pro-inflammatory cytokines like IL-8 have a quicker response time, and allergy inducing cytokines like IL-4 could possibly respond with a longer exposure [50].

For this study we measured the immune response to molds as the release of mediators, such as IL-8 and IL-4. These have different functions and will mediate immune responses differently. IL-8 is a pro-inflammatory cytokine and will mediate an inflammatory response to pathogens, by recruiting inflammatory cells to the site of infection. Increased levels of IL-8 are closely linked to inflammation. IL-4 on the other hand may be anti-inflammatory, and functions to stimulate adaptive immunity, in particular Th2 cell stimulation which can lead to allergy. Increased IL-4 is often observed in allergies and asthma [28].

6 Future Considerations

This study has focused on developing methods that that can be applied in a systemized approach to the characterization and identification of molds in damp indoor environments in Norway. The isolated molds and their stimulatory effects on immune responses in mono cell-cultures and lung co-cultures were investigated.

Since this study focuses on molds in relation to respiratory health, it made sense to characterize damp indoor environments by collecting airborne conidia. By sampling the air with an impactor, it was possible to collect mainly airborne conidia. The air samples were cultivated at 37°C in an attempt to focus on species capable of germinating in vivo. Thus we did not included molds that are incapable of germinating at this temperature. This includes a bias in the sampling procedure, as other molds could potentially also induce immune responses. Future studies should include incubation of air samples at room temperature. The importance of sampling at room temperature was exemplified in our study. We observed a strong inflammatory response towards *A. versicolor*, which does not grow at 37°C, in the lung co-culture models, whereas *A. niger* cultivated at 37°C did not induce any response.

Fungal fragments are thought to play an important role in initiating immune responses [31, 32], and by collecting air samples on a water base polymer rather than on agar, it could be possible to also account for hyphal fragments and not only conidia. The polymer can be resuspended in water after air sampling and DNA use for identification could potentially be extracted from both conidia and fragments. This would give a more complete picture of molds in the damp environment and enable population studies. We are currently developing the polymer method using samples collected from a water-damaged school.

The process of identifying molds could be improved. The PCR primer sets used in this study were for the most part only capable of identifying molds from damp rooms to the genus level. Thus molds had to be cultivated and identified with other methods to determine the species name. Studies have developed DNA probes that are specific for a variety of mold species [78], and selected DNA probes could be mounted on a microarray chips. The chip could then be used to analyze for the presences of many different molds in one sample, like water based polymer air samples. Toxin genes could also be included. As studies develop, it could potentially be possible to bypass the cultivation step when identifying molds. If molecular identification methods become specific enough it could be possible to identify species of molds using only suspended conidia and hyphal fragments collected from air samples.

In this study, the only mycotoxin that was tested for was aflatoxin. This was because mainly *Aspergillus* species were observed in damp indoor environments. If future studies detect other

molds, like *Stachybotrys* species, it could be of interest to test for other mycotoxins, like trichothecenes.

In vitro mono-cultures are helpful for initial evaluation of molds. They can be used to determine which molds should be studied in co-culture experiments. As they only give a rudimentary presentation of how molds affect immune system cells, molds should also be evaluated in co-culture experiments. We have not found other studies that have exposed mono-cultures of DCs to mold particles, and this could be something to consider for future studies.

To our knowledge, this is the first study that has used lung co-cultures with DCs to learn about the immune effects of mold conidia and particles. This is something that future studies should build on, as co-cultures provides a better in-vitro model for understanding immune responses as they would occur in-vivo. Other co-culture models could be considered, as they might provide more relevant models for lung immune responses, but this is something that would have to be evaluated.

The adaptive immune response along with Th2 activation is a crucial component in the development of asthma. In this study, little activation of the adaptive immune system was observed, both in mono-cultures and co-cultures. Cell cultures were only exposed for 8 hrs. which might not be sufficient time to allow for adaptive immune responses to occur. Future studies should also include exposure times of 24 and 48 hrs. to see if adaptive immune responses activate at a later time.

For inactivation of conidia, treatment at 60^{o}C was sufficient. Future studies should continue with this method for inactivating, as it is the gentlest method for activation we have observed. Other methods might be more prone to alter antigen structures on the conidia, thus altering their ability to initiate immune responses in in-vitro studies. For future studies, the maturation stage of conidia to be used for exposure experiments should also be considered. Studies have shown that mold conidia only expose antigen structures on the surface when conidia are swollen, just prior to germination [73, 74]. If conidia are not swollen they are less potent at inducing immune responses. Methods have been developed to inactivate conidia when they are swollen and should possibly be considered for future studies.

It should be considered to measure other factors along with those factors measured in this study. Other cytokines, like IL-1 or TNF-α could provide additional information about immune responses. Measuring cell death should also be considered, as conidia might induce cell death in-vitro, and this could potentially alter the measured releases of mediators.

If species of molds are found to be of particular interest for causing immune responses in in-vitro models, eventually they should be evaluated in animal models. They provide the best models for evaluating immune responses, as in-vitro studies will not be fully able to replicate actual in-vivo responses. However, by using in-vitro models to determine what molds are of interest for animals models, it is possible to ensure higher quality animal studies, as only known immune stimulating molds will be tested.

7 Conclusion

Techniques that have been tested in this study could, when combined, make it possible to schematically evaluate and identify molds in damp indoor environments. This study found that of molds isolated from damp and water damaged rooms, a majority was *Aspergillus* species. Molecular identification methods still need to be improved, possibly by using other methods, such as microarray chip identification.

Molds isolated from damp environments and characterized standard molds were studied in in-vitro models. This study found that the immune responses caused varied greatly depending on the specie of the mold. In contact co-culture experiments, *Aspergillus niger* induced no response whereas *Aspergillus versicolor* induce a strong release of IL-8. The importance of identifying mold to the species level is exemplified by these results. Since two molds of the same genera induce very different immune response, it shows it is necessary to identify molds by their specie when assessing if a damp indoor environment is unhealthy.

With use of methods tested and developed in this study, it is possible to study actual isolated molds from damp environments. When combining methods for identification of molds in damp environments with in-vitro methods to study the molds immunological affects the understanding of damp/moldy indoor environments in relation to respiratory health problems can be improved.

8 References

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9 Appendix

9.1 Appendix 1. Air sampling protocol

Protocol for Air Sampling

At each site, a site information form should be filled out (**Informasjonsskjema for takning av luftprøver i fuktige rom**), and samples are to be taken at three locations.

- Damp room
- Healthy room
- Outdoors (control)

For each location, two or three samples are taken according to sampling procedure; one Malt Extract Agar (MEA), one Rose Bengal Agar (RBA) and possibly one Dichloran Glycerol Agar (DG18). Before the samples are taken, one should record the temperature, humidity and CO^2 levels using the Q-Trak and measure the size of the room in cubic meters.

Sampling Procedure

1. Place SAS Super Iso (SAS) in center of the room where the sample is taken in upright position at about 1.5 m height.
2. Unscrew the SAS drilled cover plate, and clean both the cover plate and the air sampler before the contact agar plate is inserted.
3. When apparatus is cleaned, insert contact agar plate (MEA or DG18) with lid on. Then remove the contact agar lid, and replace SAS cover plate
4. Set SAS to 500 liters and start the sampling.
5. When sampling is completed, unscrew the cover plate and place the lid on the contact agar.
6. Contact agar is then to be removed and should be sealed with tape. The plate should then be placed in incubation as soon as possible at 37^{o} C for 48 hours.
7. Procedure is then repeated with one more contact agar plate so that each room has one MEA sample, one RBA sample and one DG18 sample.
8. Each plate is labeled with ID number.
    a. Example, site 3 sample 1 (damp room) on DG18 agar
        i. S3P1 DG18

Protocol is modified from:

Morris, G., M. H. Kokki, et al. (2000). "Sampling of Aspergillus spores in air." Journal of Hospital Infection **44**(2): 81-92.

9.2 Appendix 2. PCR sequences for sequenced molds

Table 1: (1) Rhizomucor pusillus

GenBank. HQ845298.1

TCAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGATGAAGGCAACGAGTTTA
TAACCTTGGCCGGAAGGTCTGGGTAAACTTTTGAAACTTCATCGTGCTGGGGATAGAGCATTGCAATT
ATTGCTCTTCAACGAGGAATTCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCCCTGCCCT
TAGATGTTCTGGGCCGCACGCGCGCTACACTGATGAAGGCAACGAGTTTATAACCTTGGCCGGAAGGT
CTGGGTAAACTTTTGAAACTTCATCGTGCTGGGGATAGAGCATTGCAATTATTGCTCTTCAACGAGGA
ATTCCTAGTAAGCGCAAG

Table 1: (2) Aspergillus sp.

TCAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGATGAAGGCAACGAGTTTA
TAACCTTGGCCGGAAGGTCTGGGTAAACTTTTGAAACTTCATCGTGCTGGGGATAGAGCATTGCAATT
ATTGCTCTTCAACGAGGAATTCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCCCTGCCCT
TAGATGTTCTGGGCCGCACGCGCGCTACACTGATGAAGGCAACGAGTTTATAACCTTGGCCGGAAGGT
CTGGGTAAACTTTTGAAACTTCATCGTGCTGGGGATAGAGCATTGCAATTATTGCTCTTCAACGAGGA
ATTCCTAGTAAGCGCAAG

Table 1: (3) Aspergillus sp.

Primer set 1:
TTGGTTTCTAGGACCGCCGTAATGATTAATAGGGATAGTCGGGGGCGTCAGTATTCAGCTGTCAGAGG
TGAAATTCTTGGATTTGCTGAAGACTAACTACTGCGAAAGCATTCGCCAAGGATGTTTTCATTAATCA
GGGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCGAC
TAGGGATCGGGCGGTGTTTCTATGATGACCCGCTCGGCACCTTACGAGAAATCAAAGTTTTTGGGTTC
TGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACAAGGCGTGGAGC
CTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACAAAATAAGGATT

Aspergillus sp
Aphanoascus fulvescens
JN941603.1

Primer set 2:
CTACTAGGCATTCCTCGTTGAGAGCAATAATTGCAATGCTCTATCCCCAGCACGACAGGGTTTAACAA
GATTACCCAGACCTCTCGGCCAAGGTGATGTACTCGCTGGCCCTGTCAGTGTAGCGCGCGTGCGGCCC
AGAACATCTAAGGGC

Table 1: (4) Rhizopus microsporus

GenBank. FN182240.1

CCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGATGAAGTCAGCGAGTTTATAACCTTGGCCGGA
AGGTCTGGGTAAACTTTTGAAACTTCATCGTGCTGGGGATAGAGCATTGTAATTATTGCTCTTCAACG
AGGAATTCCTAGTAAGCGCGAGTCATCAGCTCGCGTTGATTACGTCCCTGCCCTTT

Table 1: (5) Aspergillus sp.

Primer Set 1:
ATTTGCTGAAGACTAACTACTGCGAAAGCATTCGCCAAGGATGTTTTCATTAATCAGGGAACGAAAGT
TAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCGGGC
GGTGTTTCTATGATGACCCGCTCGGCACCTTACGAGAAATCAAAGTTTTTGGGTTCTGGGGGGAGTAT
GGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACAAGGCGTGGAGCCTGCGGCTTAAT
TTGACTCAACACGGGGAAACTCACCAGGTCCAGACAAAATAAG

Rhizomucor pusillus

GenBank. HQ845298.1

Primer Set 2:
TTAGATGTTCTGGGCCGCACGCGCGCTACACTGATGAAGGCAACGAGTTTATAACCTTGGCCGGAAGG
TCTGGGTAAACTTTTGAAACTTCATCGTGCTGGGGATAGAGCATTGCAATTATTGCTCTTCAACGAGG
AATTCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCCCTGCCCTT

Table 1: (6) Aspergillus sp.

Primer Set 1:
TTGGTTTCTAGGACCGCCGTAATGATTAATAGGGATAGTCGGGGGCGTCAGTATTCAGCTGTCAGAGG
TGAAATTCTTGGATTTGCTGAAGACTAACTACTGCGAAAGCATTCGCCAAGGATGTTTTCATTAATCA
GGGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCGAC
TAGGGATCGGGCGGTGTTTCTATGATGACCCGCTCGGCACCTTACGAGAAATCAAAGTTTTTGGGTTC
TGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACAAGGCGTGGAGC
CTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACAAAATAAGG

Penicillium sp.
Aspergillus sp.

Primer set 2:
GCCTAGTAGGCACGAGTCATCAGCTCGTGCCGATTACGTCCCTGCCCTTT

Table 1: (7) Aspergillus terreus

GenBank. JN831364.1

Primer Set 1:
TTTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGATAGTCGGGGGCGTCAGTATTCAGCTGT
CAGAGGTGAAATTCTTGGATTTGCTGAAGACTAACTACTGCGAAAGCATTCGCCAAGGATGTTTTCAT
TAATCAGGGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTAT
GCCGACTAGGGATCGGGCGGTGTTTCTATGATGACCCGCTCGGCACCTTACGAGAAATCAAAGTTTTT
GGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACAAGGCG
TGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACAAAATAAGGATTG

Aspergillus sp.

Primer Set 2:
TTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGGGCCAGCGAGTACATCACCTTGGCCGAGAGG
TCTGGGTAATCTTGTTAAACCCTGTCGTGCTGGGGATAGAGCATTGCAATTATTGCTCTTCAACGAGG
AATGCCTAGTAGGCACGAGTCATCAGCTCGTGCCGATTACGTCCCTGCCCTTTGTA

Table 1: (8) Aspergillus sp.

TCAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGGGCCAGCGAGTACA
TCACCTTGGCCGAGAGGTCTGGGTAATCTTGTTAAACCCTGTCGTGCTGGGGATAGAGCATTGCAATT
ATTGCTCTTCAACGAGGAATGCCTAGTAGGCACGAGTCATCAGCTCGTGCCGATTACGTCCCTGCCCT
TTGTA

Table 1: (9) Aspergillus sp.

CAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGGGCCAGCGAGTACAT
CACCTTGGCCGAGAGGTCTGGGTAATCTTGTTAAACCCTGTCGTGCTGGGGATAGAGCATTGCAATTA
TTGCTCTTCAACGAGGAATGCCTAGTAGGCACGAGTCATCAGCTCGTGCCGATTACGTCCCTGCCCTT
TGTACAC

Table 1: (10) Paecilomyces variotii / Aspergillus sp.

GenBank.JF416647.1

Primer Set 1:

GTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGATAGTCGGGGGCGTCAGTATTCA
GCTGTCAGAGGTGAAATTCTTGGATTTGCTGAAGACTAACTACTGCGAAAGCATTCGCCAAGGATGTT
TTCATTAATCAGGGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAA
ACTATGCCGACTAGGGATCGGACGGTGTTTCTATTATGACCCGTTCGGCACCTTACGAGAAATCAAAG
TTTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACA
AGGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACAAAATAAGGA
TTGA

Paecilomyces variotii

GenBank.JF416647.1

Primer Set 2:

TGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGGGCCAGCGAGTACATCACCTT
GACCGAGAGGTCTGGGTAATCTTGTTAAACCCTGTCGTGCTGGGGATAGAGCATTGCAATTATTGCTC
TTCAACGAGGAATGCCTAGTAGGCACGAGTCATCAGCTCGTGCCGATTACGTCCCTGCCCTTTGTACA
CACCGCC

Table 1: (11) Aspergillus sp.

GTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGGGCCAGCGAGTACATCAC
CTTGGCCGAGAGGTCTGGGTAATCTTGTTAAACCCTGTCGTGCTGGGGATAGAGCATTGCAATTATTG
CTCTTCAACGAGGAATGCCTAGTATGCAATTATTGCTCTTCAACGAGGAATGCCTAGTA

Table 1: (12) Aspergillus sp.

TTGGTTTCTAGGACCGCCGTAATGATTAATAGGGATAGTCGGGGGCGTCAGTATTCAGCTGTCAGAGG
TGAAATTCTTGGATTTGCTGAAGACTAACTACTGCGAAAGCATTCGCCAAGGATGTTTTCATTAATCA
GGGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCGAC
TAGGGATCGGGCGGTGTTTCTATGATGACCCGCTCGGCACCTTACGAGAAATCAAAGTTTTTGGGTTC
TGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACAAGGCGTGGAGC
CTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACAAAATAAGGATT

Table 1: (13) Aspergillus sp.

CTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGGGCCAGCGAGTACATCACCTTGGCCGAGAG
GTCTGGGTAATCTTGTTAAACCCTGTCGTGCTGGGGATAGAGCATTGCAATTATTGCTCTTCAACGAG
GAATGCCTAGTAGGCACGAGTCATCAGCTCGTGCCGATTACGTCCCTGCCCTTTGT

Table 1: (14) Emericella nidulans

GenBank. AB008403.1

Primer Set 1:

TGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGATAGTCGGGGGCGTCAGTATTCAGCTGTCAGA
GGTGAAATTCTTGGATTTGCTGAAGACTAACTACTGCGAAAGCATTCGCCAAGGATGTTTTCATTAAT
CAGGGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCG
ACTAGGGATCGGGCGGCGTTTCTTTTATGACCCGCTCGGCACCTTACGAGAAATCAAAGTTTTTGGGT
TCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACAAGGCGTGGA
GCCTGCGGCTTAATTTGACTCAACACGG

Aspergillus sp.

Primer Set 2:
TCAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGGGCCAGCGAGTACA
TCACCTTGGCCGAGAGGCCCGGGTAATCTTGTTAAACCCTGTCGTGCTGGGGATAGAGCATTGCAATT
ATTGCTCTTCAACGAGGAATGCCTAGTAGGCACGAGTCATCAGCTCGTGCCGATTACGTCCCTGCCCT
TTGTACACACCGCCCGTA

Table 1: (15) Lichtheimia corymbifera

GenBank. JQ004931.1

GGAATTCCTCGTTGAAGAGCAATAATTGCAATGCTCTATCCCCAGCACGATACAGTTTCAGAAGTTTA
CCCAGACTTGTCAGCCAAGGAAAGATAACTCGCTGCCTGCATCAGTGTAGCGCGCGTGCGGCCCAGAA
CATCTAA

Table 1: (16) Absidia corymbifera / Lichtheimia corymbifera

GenBank. AF113407.1 / JQ004931.1

GGAATTCCTCGTTGAAGAGCAATAATTGCAATGCTCTATCCCCAGCACGATACAGTTTCAGAAGTTTA
CCCAGACTTGTCAGCCAAGGAAAGATAACTCGCTGCCTGCATCAGTGTAGCGCGCGTGCGGCCCAGAA
CATCTAA

Table 1: (17) Engyodontium album / Halophytophthora vesicula / Artomyces pyxidatus

GenBank. JF797223.1 / HQ161089.1 / JQ086388.1

TAAACTCCGTCGTGCTGGGGATAGAGCATYGCAATTATYGCTCTTCAACGAGGAATCCCTAGTAAGCG
CAAGTCATCAGCTYGCGTTGATTACGTCCCTGCCCTT

Table 1: (18) Tritirachium oryzae / Paratritirachium cylindroconium / Hyphodermella corrugata

GenBank. JF797224.1 / JF797220.1 / JN940192.1

AATTATYGCTCTTCAACGAGGAATACCTAGTAAGCGTGAGTCATCAGCTCGCGTTGATTACSTCCCTG
CCCTT

Table 1: (19) Aspergillus sp.

Primer set 1:
GTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGATAGTCGGGGGCGTCAGTATTCAGCTGTCAGAG
GTGAAATTCTTGGATTTGCTGAAGACTAACTACTGCGAAAGCATTCGCCAAGGATGTTTTCATTAATC
AGGGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCGA
CTAGGGATCGGGCGGTGTTTCTATGATGACCCGCTCGGCACCTTACGAGAAATCAAAGTTTTTGGGTT
CTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACAAGGCGTGGAG
CCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACAAAATAAGGATTG

Penicillium sp.
Aspergillus sp.

Primer set 2:
TCTTGTTAAACCCTGTCGTGCT

Table 1: (20) Aspergillus sp.

AGGGCCAGCGAGTACATCACCTTGGCCGAGAGGTCTGGGTAATCTTGTTAAACCCTGTCGTGCTGGGG
ATAGAGCATTGCAATTATTGCTCTTCAACGAGGAATGCCTAGTAGGCACGAGTCATCAGCTCGTGCCG
ATTACGTCCCTGCCCTTTGTA

Table 1: (21) Hyphodermella corrugata / Hyphodermella rosae

GenBank. JN940192.1 / JN940191.1

CCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGAGCCAGCGAGTTTTTTTCCTTGGCCGGA
AGGTCTGGGTAATCTTGTGAAACTCTGTCGTGCTGGGGATAGAGCATTGCAATTATTGCTCTTCAACG
AGGAATACCTAGTAAGCGTGAGTCATCAGCTCGCGTTGATTACGTCCCTGCCCTTTGT

Table 1: (22) Aspergillus sp.

TCAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGGGCCAGCGAGTACA
TCACCTTGGCCGAGAGGTCTGGGTAATCTTGTTAAACCCTGTCGTGCTGGGGATAGAGCATTGCAATT
ATTGCTCTTCAACGAGGAATGCCTAGTAGGCACGAGTCATCAGCTCGTGCCGATTACGTCCCTGCCCT
TTGTACACACCGCCCGTA

Table 1: (23) Aspergillus terreus

GenBank. JN831364.1

Primer Set 1:
GTTCTATTTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGATAGTCGGGGGCGTCAGTATTC
AGCTGTCAGAGGTGAAATTCTTGGATTTGCTGAAGACTAACTACTGCGAAAGCATTCGCCAAGGATGT
TTTCATTAATCAGGGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATA
AACTATGCCGACTAGGGATCGGGCGGTGTTTCTATGATGACCCGCTCGGCACCTTACGAGAAATCAAA
GTTTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCAC
AAGGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACAAAATAAGG
ATTGA

Aspergillus sp.

Primer Set 2:

TCAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGGGCCAGCGAGTACA
TCACCTTGGCCGAGAGGTCTGGGTAATCTTGTTAAACCCTGTCGTGCTGGGGATAGAGCATTGCAATT
ATTGCTCTTCAACGAGGAATGCCTAGTAGGCACGAGTCATCAGCTCGTGCCGATTACGTCCCTGCCCT
TTGTACACACCGCCCGTA

Table 1: (24) Aspergillus sp.

GCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGGGCCAGCGAGTACATCACCTTGG*CCGA*
GAGGTCTGGGTAATCTTGTTAAACCCTGTCGTGCTGGGGATAGAGCATTGCAATTATTGCTCTTCAAC
GAGGAATGCCTAGTAGGCACGAGTCATCAGCTCGTGCCGATTACGTCCCTG

9.3 Appendix 3. Malt extract agar

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MALT EXTRACT (7341)

Intended Use
Malt Extract is a dehydrated extract of malt for use in preparing microbiological culture media.

Product Summary and Explanation
Malt Extract is a clarified water soluble extract of malted barley. Malt Extract is a useful ingredient of culture media designed for the propagation of yeasts and molds. This ingredient is suitable for yeasts and molds because it contains a high concentration of carbohydrates, particularly maltose. The approximate percentage of reducing sugars in Malt Extract is 60 – 63%. Malt Extract is generally employed in culture media at concentrations between 10 to 100 grams per liter.

Malt Agar, a medium recommended for the detection and isolation of yeast and molds from dairy products, food, and as a stock culture, contains Malt Extract. Wort Agar, used for the cultivation and enumeration of yeasts, has Malt Extract as one of the main ingredients in the formula. Several media containing Malt Extract are specified in standard methods.[1-3]

Principles of the Procedure
Malt Extract provides carbon, protein, and nutrients for the isolation and cultivation of yeasts and molds in microbiological culture media.

Precaution
1. For Laboratory Use.

Quality Control Specifications
Dehydrated Appearance: Powder is homogeneous, free-flowing and light beige to tan.

Prepared Appearance (2% wt/vol): Prepared medium is clear to brilliant with or without a slight precipitate and light beige to tan.

Chemical Composition:

CAS #:	8002-48-0
Moisture:	< 6.0%
pH (2% Solution):	5.0 – 7.0

Microbiological Specifications: Standard Plate Count: < 5000 CFU / g

Growth Supporting Properties: Malt Agar: Satisfactory

Test Procedure
Refer to appropriate references for specific procedures using Malt Extract.

Results
Refer to appropriate references for test results.

Storage
Store sealed container of Malt Extract at 2 - 30˚C. Once opened and recapped, place container in a low humidity environment at the same storage temperature. Protect from moisture and light by keeping container tightly closed.

Expiration
Refer to expiration date stamped on container. Malt Extract should be discarded if not free flowing, or if the appearance has changed from the original color. Expiry applies to Malt Extract in its intact container when stored as directed.

PI 7341, Rev 02, April 2009

9.4 Appendix 4. Rose bengal agar

ROSE BENGAL CHLORAMPHENICOL AGAR (7664)

Intended Use

Rose Bengal Chloramphenicol Agar is used for the selective isolation and enumeration of yeasts and molds from foods.

Product Summary and Explanation

Rose Bengal Chloramphenicol Agar is a selective medium for the enumeration of fungi. In 1944, Smith and Dawson used Rose Bengal for the selective isolation of fungi from soil samples.[1] This formula was prepared with a neutral pH, deviating from the usual acidified medium. Several investigations have found a neutral pH with the addition of a selective agent have been successful in supporting fungal growth and restricting bacterial growth.[2,3] Rose Bengal Agar supplemented with Chloramphenicol is a modification of the Rose Bengal Chlortetracycline Agar formula of Jarvis.[4] Chloramphenicol is recommended as the selective agent in fungal medium with a neutral pH because of its heat stability and broad antibacterial spectrum.[5]

Rose Bengal Chloramphenicol Agar is recommended in standard methods for the enumeration of yeast and molds from foods and water.[5-7] Rose Bengal Chloramphenicol Agar is also referred to as Rose Bengal Agar and Rose Bengal-Malt Extract Agar.

Principles of the Procedure

Enzymatic Digest of Soybean Meal provides the nitrogen and vitamin sources required for organism growth in Rose Bengal Chloramphenicol Agar. The high concentration of Dextrose is included as an energy source. Monopotassium Phosphate is a buffering agent. Magnesium Sulfate provides trace elements. Rose Bengal is included as a selective agent to inhibit bacterial growth and restricts the growth of rapidly growing molds. Rose Bengal is incorporated in the cells of yeasts and molds, turning these colonies pink. Chloramphenicol is a broad-spectrum antibiotic inhibitory to a wide range of Gram-negative and Gram-positive bacteria. Agar, Bacteriological is the solidifying agent.

Formula / Liter

Enzymatic Digest of Soybean Meal	5 g
Dextrose	10 g
Monopotassium Phosphate	1 g
Magnesium Sulfate	0.5 g
Rose Bengal	0.05 g
Chloramphenicol	0.1 g
Agar, Bacteriological	15.5 g

Final pH: 7.2 ± 0.2 at 25°C
Formula may be adjusted and/or supplemented as required to meet performance specifications.

Precautions

1. For Laboratory Use.
2. TOXIC. Toxic by inhalation, if swallowed, or absorbed through the skin. Irritating to eyes, respiratory system, and skin. Possible risk of harm to unborn child. Possible carcinogen.

Directions

1. Dissolve 32.2 grams of the medium in one liter of purified water.
2. Heat with frequent agitation and boil for one minute to completely dissolve the medium.
3. Autoclave at 121°C for 15 minutes.

Quality Control Specifications

Dehydrated Appearance: Powder is homogeneous, free flowing, and pink.

Prepared Appearance: Prepared medium is trace to slightly hazy and bright pink.

PI 7664, Rev 2, January 2012

9.5 Appendix 5. Dichloran glycerol agar

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DICHLORAN GLYCEROL (DG-18) AGAR BASE (7592)

Intended Use

Dichloran Glycerol (DG-18) Agar Base is used for the selective isolation and enumeration of yeasts and molds from foods.

Product Summary and Explanation

Dichloran Glycerol Agar Base is based on the formulation by Hocking and Pitt.[1] This medium is recommended for enumeration and isolation of yeasts and molds from dried and semi-dried foods, including fruits, spices, cereals, nuts, meat, and fish products. The highly selective composition of this medium allows for enumeration of fungal growth. In a comparative study between DG-18 Agar Base and DRBC Agar, greater recovery of two molds commonly isolated in high numbers from dried foods grew poorly on DRBC Agar.[1]

A modification of DG-18 Agar Base, enhanced with Triton-X, is described as increasing inhibition of vigorously-spreading fungi.[2]

Principles of the Procedure

Peptone provides nitrogen and vitamins required for organism growth. Glucose is included as an energy source. Monopotassium Phosphate is a buffering agent. Magnesium Sulfate, Zinc Sulfate, and Copper Sulfate are inorganic salts used to stimulate fungal growth and sporulation. The antifungal agent, Dichloran, inhibits fungi from spreading and restricts colony size. Chloramphenicol inhibits growth of bacteria present in environmental and food samples. Chlortetracycline is a broad-spectrum antibiotic, inhibiting a wide range of Gram-positive and Gram-negative bacteria. Agar is the solidifying agent. Glycerol is added as a carbon source.

Formula / Liter

Peptone	5 g
Glucose	10 g
Monopotassium Phosphate	1 g
Magnesium Sulfate	0.5 g
Zinc Sulfate	0.01 g
Copper Sulfate	0.005 g
Dichloran	0.002 g
Chloramphenicol	0.05 g
Chlortetracycline	0.05 g
Agar	15 g

Supplement

Glycerol, 220 g

Final pH: 5.6 ± 0.2 at 25°C

Formula may be adjusted and/or supplemented as required to meet performance specifications.

Precautions

1. For Laboratory Use.
2. TOXIC. Toxic if swallowed, inhaled, or absorbed through the skin. Irritating to eyes, skin, and respiratory system. Possible risk of harm to unborn child. Possible carcinogen.

Directions

1. Suspend 31.6 g of the medium and 220 g of glycerol in one liter of purified water.
2. Heat with frequent agitation and boil for one minute to completely dissolve the medium.
3. Autoclave at 121°C for 15 minutes. DO NOT OVERHEAT.

Quality Control Specifications

Dehydrated Appearance: Powder is homogeneous, free flowing, and beige.

PI 7592, Rev 4, February 2011