Damp building moulds: Assessment of sensitization in patients and studies into mechanisms of airway inflammation using experimental models

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ACADEMIC DISSERTATION

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Contents

1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, referred to in the text by their Roman numerals. Some previously unpublished results are also presented.

I. Reijula K, Leino M, Mussalo-Rauhamaa H, Nikulin M, Alenius H, Mikkola J, Elg P, Kari O, Mäkinen-Kiljunen S, Haahtela T. IgE-mediated allergy to fungal allergens in Finland with special reference to *Alternaria alternata* and *Cladosporium herbarum.* Ann Allergy Asthma Immunol 2003 Sep;91(3):280-7.

II. Leino M , Reijula K, Mäkinen-Kiljunen S, Haahtela T, Mäkelä MJ, Alenius H. *Cladosporium herbarum* and *Pityrosporum ovale* allergen extracts share cross-reacting glycoproteins. Int Arch Allergy Immunol 2006;140(1):30-5.

III. Leino M, Mäkelä M, Reijula K, Haahtela T, Mussalo-Rauhamaa H, Tuomi T, Hintikka EL, Alenius H. Intranasal exposure to a damp building mould, *Stachybotrys chartarum*, induces lung inflammation in mice by satratoxin-independent mechanisms. Clin Exp Allergy 2003 Nov;33(11):1603-10.

IV. Leino MS, Alenius HT, Fyhrquist-Vanni N, Wolff HJ, Reijula KE, Hintikka EL, Salkinoja-Salonen MS, Haahtela T, Mäkelä MJ. Intranasal exposure to *Stachybotrys chartarum* enhances airway inflammation in allergic mice. Am J Respir Crit Care Med 2006 Mar 1;173(5):512-8.

2. ABBREVIATIONS

AHR	airway hyperresponsiveness
APC	antigen presenting cell
BAL	bronchoalveolar lavage
B-lymph.	bone marrow derived lymphocytes
CAP-FEIA	Pharmacia CAP-fluorescence enzyme immunoassay
CCL	C-C family chemokine ligand
CCR	C-C family chemokine receptor
CD	cluster of differentiation
CTL	cytotoxic T lymphocytes
CXCL	C-X-C family chemokine ligand
CXCR	C-X-C family chemokine receptor
cDNA	complementary deoxyribonucleic acid
Con A	Concanavalin A
ELISA	enzyme-linked immunosorbent assay
FAM	fluorescent reporter dye (6-carboxy-fluorescein)
GM-CSF	granulocyte-macrophage colony-stimulating factor
HP	hypersensitivity pneumonitis
HPLC	high performance liquid chromatography
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kDa	kilo Dalton
LIX	lipopolysaccharide induced CXC chemokine
MCh	methacholine
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MCP	monocyte chemoattractant protein
mRNA	messenger ribosomal nucleic acid
MW	molecular weight
OVA	ovalbumin
PAF	platelet activating factor
PAMP	pathogen associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PVDF	polyvinyl diflouride
RANTES	regulated upon activation normally T-cell expressed and secreted
SPT	skin prick test
TBS	tris buffered saline
TGF	transforming growth factor
T_H	T helper
T-lymph.	thymus derived lymphocytes
TNF	tumor necrosis factor
TLR	Toll-like receptor

3. ABSTRACT

Exposure to water-damaged buildings and the associated health problems have evoked concern and created confusion during the past 20 years. Individuals exposed to moisture problem buildings report adverse health effects such as non-specific respiratory symptoms. Microbes, especially fungi, growing on the damp material have been considered as potential sources of the health problems encountered in these buildings. Fungi and their airborne fungal spores contain allergens and secondary metabolites which may trigger allergic as well as inflammatory types of responses in the eyes and airways. Although epidemiological studies have revealed an association between damp buildings and health problems, no direct cause-and-effect relationship has been established. Further knowledge is needed about the epidemiology and the mechanisms leading to the symptoms associated with exposure to fungi.

Two different approaches have been used in this thesis in order to investigate the diverse health effects associated with exposure to moulds. In the first part, sensitization to moulds was evaluated and potential cross-reactivity studied in patients attending a hospital for suspected allergy. In the second part, one typical mould known to be found in water-damaged buildings and to produce toxic secondary metabolites was used to study the airway responses in an experimental model. Exposure studies were performed on both naive and allergen sensitized mice.

The first part of the study showed that mould allergy is rare and highly dependent on the atopic status of the examined individual. The prevalence of sensitization was 2.7% to *Cladosporium herbarum* and 2.8% to *Alternaria alternata* in patients, the majority of whom were atopic subjects. Some of the patients sensitized to mould suffered from atopic eczema. Frequently the patients were observed to possess specific serum IgE antibodies to a yeast present in the normal skin flora, *Pityrosporum ovale*. In some of these patients, the IgE binding was partly found to be due to binding to shared glycoproteins in the mould and yeast allergen extracts. The second part of the study revealed that exposure to *Stachybotrys chartarum* spores induced an airway inflammation in the lungs of mice. The inflammation was characterized by an influx of inflammatory cells, mainly neutrophils and lymphocytes, into the lungs but with almost no differences in airway responses seen between the satratoxin producing and non-satratoxin producing strain. On the other hand,

when mice were exposed to *S. chartarum* and sensitized/challenged with ovalbumin the extent of the inflammation was markedly enhanced. A synergistic increase in the numbers of inflammatory cells was seen in BAL and severe inflammation was observed in the histological lung sections.

In conclusion, the results in this thesis imply that exposure to moulds in water damaged buildings may trigger health effects in susceptible individuals. The symptoms can rarely be explained by IgE mediated allergy to moulds. Other non-allergic mechanisms seem to be involved. *Stachybotrys chartarum* is one of the moulds potentially responsible for health problems. In this thesis, new reaction models for the airway inflammation induced by *S. chartarum* have been found using experimental approaches. The immunological status played an important role in the airway inflammation, enhancing the effects of mould exposure. The results imply that sensitized individuals may be more susceptible to exposure to moulds than non-sensitized individuals.

4. INTRODUCTION

Individuals are being continually exposed to fungi, since these micro-organisms are an important part of our natural environment. Airborne fungal spores are inhaled into the airways or individuals may come into contact with them directly via the skin. Indoor fungal spore levels normally reflect the outdoor levels and thus vary according to the season (Burge 2002). The highest spore levels are observed in late summer and autumn in Finland. However, under certain circumstances such as in water damaged buildings, the growth of fungi on wet building material can be favoured and increased growth can occur indoors (Li and Yang 2004). Later fungal species normally not found indoors, start to grow on the wet material and this may give rise to health problems of inhabitants of these buildings, varying from allergic types of symptoms in the upper and lower airways to more diverse respiratory symptoms (Bush *et al.* 2006).

Fungi and fungal spores contain allergenic proteins and glycoproteins that can evoke sensitization in susceptible individuals (Kurup *et al.* 2000). Sensitization to moulds has been linked with an increased risk of developing allergic rhinitis and allergic asthma. Fungi are allergenic to varying degrees depending on the species. Two of the most common allergenic fungi tested in the clinic today are *Alternaria alternata* and *Cladosporium herbarum*. *A. alternata* is a worldwide sensitizer whereas *C. herbarum* is more common in cooler climates. These fungi are commonly present in the outdoor air but may occasionally also be found indoors.

Exposure to fungi may induce non-allergic, inflammatory airway responses. The fungal cell wall consists of components known to trigger innate immune responses. In addition, some fungal species can produce potentially harmful secondary metabolites (Assoulin-Daya *et al.* 2002). For example, *Stachybotrys chartarum* is one of the fungal species that has attracted attention due to its ability to produce highly toxic mycotoxins (Hossain *et al.* 2004, Kuhn and Ghannoum 2003). *Stachybotrys* grows well on materials containing wet cellulose and this micro-organism can occasionally be found in large numbers in water damaged buildings. *Stachybotrys* has been linked to serious cases of health problems in buildings where water damage has occurred.

Health problems associated with exposure to damp buildings has been responsible for a greatdeal of alarm and concern. The users of these buildings experience a variety of

symptoms but linking the symptoms to the use of a damp building has proved to be a controversial task (Hardin *et al.* 2003). Intensive investigations have been performed during the last 20 years in order to identify the agents responsible for the problems as well as clarifying the health effects or symptoms associated with the exposure (Terr 2004). However, there is little hard evidence for direct cause-and-effect relationships between exposure to damp buildings, the moulds and the health problems of the building inhabitants (IOM 2004, Nevalainen and Seuri 2005). Few, if any, markers can be used to assess exposure in the subjects residing in moisture problem buildings.

In an attempt to investigate the health effects associated with exposure to fungi or moulds present in water-damaged buildings, we utilized two different approaches. The prevalence for sensitization to moulds was assessed in patients attending a hospital for suspected allergy. The prevalence for sensitization and IgE reactivity to two common allergenic fungi (*Alternaria alternata* and *Cladosporium herbarum*) were evaluated. The potential cross-reactivity for the IgE binding between mould and yeast allergen extracts was further characterized. Direct airway effects of exposure to a common fungus often detected in water-damaged buildings, namely *Stachybotrys chartarum*, were studied using experimental models. Furthermore exposure to *S. chartarum* was studied together with experimental asthma in order to determine whether the immunological status has any effect on the response.

5. REVIEW OF THE LITERATURE

5.1. The immune response

The immune response against a foreign agent is commonly divided into innate immunity and adaptive immunity (Janeway *et al.* 2005). Innate immunity is characterized by an immediate attempt to eliminate the foreign agent by the host's defence system. The agent is engulfed into specialized cells called phagocytes which then ingest and destroy the agent. The adaptive immune response on the other hand is triggered when the innate immune response fails to eliminate the pathogen. The adaptive immune response can more specifically recognize virtually any foreign agent and in addition it leaves a pool of memory cells which triggers a rapid release of inflammatory cells and mediators on a second encounter with the antigen. Therefore, the adaptive immune response has been considered as the more sophisticated system. In recent years however, it has been recognized that a highly complex and specific system also underpins innate immunity.

5.1.1. Innate immunity

In innate immunity, the phagocytic cells hold in the key positions. The cells that play a role are mainly dendritic cells, macrophages and granulocytic leucocytes such as neutrophils. They provide the first line of defence against foreign agents such as microbes invading the host, some of which might be pathogenic (Bals and Hiemstra 2004). Pathogenic microbes are recognized by receptors on the phagocytosing cells, and then the micro-organisms are engulfed by the cell and destroyed. The receptors on the cell surface recognize and bind to conserved patterns or features common to pathogens. These receptors include mannose, glucan and scavenger receptors on macrophages known to bind to bacterial carbohydrates. Toll-like receptors (TLR), of which at least 10 functional members have been identified, bind specifically to conserved bacterial, viral and fungal components called pathogen associated molecular patterns (PAMPs) (Janssens and Beyaert 2003). Binding leads to engulfment and digestion of the pathogen inside the cell which triggers the release of inflammatory mediators such as cytokines and chemokines and the activation of adhesion molecules. Chemokines recruit monocytes that rapidly

differentiate into macrophages as well as attracting neutrophils to the site of inflammation. Subsequently also lymphocytes and eosinophils may be activated to participate in the immune response.

5.1.1.1. Inflammatory mediators

Cytokines are critical molecules involved in the signalling between the cells of the immune system. Cytokines are small glycoproteins, including interleukins, interferons and growth factors, produced by a variety of cells after activation. In a local inflammatory response, the primary cytokines involved are the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 (Janeway *et al.* 2005). Activated macrophages are the primary cells secreting TNF-α which is intended to provide local control of the inflammation. TNF-α along with IL-1β and IL-6 are the main mediators of the acute phase of the inflammation (Dinarello 2000, Netea *et al.* 2002). Later in the response, other cytokines such as IL-12 may be induced, leading to activation of yet more inflammatory cells. IL-12 favours the activation of T cell differentiation into T helper 1 type, thus enlisting adaptive immunity into the response. Early interferons such as IFN-α and -β are commonly activated in viral infections while the later-appearing IFN, called IFN-γ is involved in the acute immune response to intracellular pathogens.

Chemokines are chemotactic molecules or ligands (L) attracting cells with appropriate receptors (R) to migrate towards them (Luster 1998). Chemokines are divided into two main families based on the position and order of the conserved cysteine (C) amino acid residues in the molecule (Nickel *et al.* 1999). Chemokines involved in the recruitment of monocytes belong to the so called C-C family of which CCL3/MIP-1α, CCL4/MIP-1β and CCL2/MCP-1 are commonly known. Neutrophils are mainly attracted by chemokines from the C-X-C family such as by CXCL8/IL-8 in humans and CXCL5/LIX and MIP-2 in mice (Takaoka *et al.* 2001). CCL11/eotaxin-1 specifically recruits eosinophils while lymphocytes are attracted by several chemokines which can also recruit monocytes to the site of inflammation. The main chemokine receptors involved in innate immune responses include CCR1 and CCR5 which are expressed on T cells, monocytes and dendritic cells. Neutrophils commonly express receptors for CXC chemokines of which CXCR2 is the most common. On the other hand, CCR3 is typically found on eosinophils.

5.1.2. Adaptive immunity

The innate immune system and its receptors cannot recognize all pathogens and the adaptive immune system has developed to provide more specific recognition of a particular pathogen or antigen (Clark and Kupper 2005). In adaptive immunity, lymphocytes play a major role. Lymphocytes are unique in their ability to build up an immune response which can recognize and respond specifically to virtually any foreign antigen (Janeway *et al.* 2005). When a lymphocyte recognizes its specific antigen, it is activated and starts to differentiate. T-lymphocytes (thymus derived) differentiate into effector cells and these cells take on many different functions after activation. B-lymphocytes (bone marrow derived) on the other hand detect antigens outside the cells and start to produce antibodies against the antigen when activated. In adaptive immunity, the main features are the specificity of antigen recognition and memory. A subset of the activated specific lymphocytes develops into memory cells that can readily respond to the same antigen if it is encountered again (McHeyzer-Williams and McHeyzer-Williams 2005).

5.1.2.1. Antigen recognition

The immune response is initiated when an immature dendritic cell, which is the main phagocytosing cell in adaptive immunity, locates a pathogen or antigen in infected tissue and starts to ingest it. The dendritic cell becomes activated and matures into an antigen presenting cell (APC). The APC carries the degraded antigen to the nearest lymph node and effectively presents it to T-lymphocytes (von Bubnoff *et al.* 2001). The antigen is presented via molecules on the surface of APCs called the major histocompatibility complex (MHC). Peptides found in the cytosol of the APC such as viral antigens are collected and displayed by MHC-I class while MHC-II gathers and presents peptides from intracellular vesicles. Should a T-lymphocyte recognize its specific antigen in the lymph node, then the cell becomes activated. T cells differentiate into killer or helper cells, depending on the interaction between the antigen, APC and the T cell. Some of the activated T cells help B-cells to become activated when they have encountered their specific antigen. B-cells recognize their specific antigens through direct binding to the B

cell receptor. The activated lymphocytes leave the lymph node as effector cells that induce the adaptive immune response.

5.1.2.1.1. T-effector cells

When a T-cell is activated, it starts to proliferate and differentiate into the effector cells needed to destroy the antigen (Janeway *et al.* 2005). The effector cells may be grouped into $CD8^+$ cytotoxic T cells (CTL) also called killer cells or into $CD4^+$ helper cells (Figure 1). The killer cells strive to achieve immediate clearance of pathogens to prevent them from replicating inside the cell. T killer cells recognize the antigen on the APC bound to MHC class I and kill the micro-organism through direct contact (Fig.1a).

T helper cells ($CD4^+$) recognize antigens bound to MHC class II on APCs. T helper cells with different functions develop depending on the antigen as well as on the immunologic predisposition of the responder (Lanzavecchia and Sallusto 2000). The T helper cells are commonly grouped, in order to distinguish between the responses, into subtypes called Th1 and Th2. Generally infectious bacteria induce a Th1 type of response while allergens induce a Th2 type of response in susceptible individuals. The differentiation into Th1 or Th2 type cells is a crucial step for effective immunity. The mechanisms that control the differentiation are not fully understood but cytokines present at the initial stage seem to play a major role but the process is also controlled by the regulatory cells (O'Garra 1998). The differentiation steps are strictly controlled and the activation of one type of responses inhibits the other type.

Th1 cells are activated in order to help macrophages prevent infectious microbes from starting to grow and replicating inside the host cell (Fig. 1b). Th1 cells activate macrophages and secret cytokines and chemokines that recruit more macrophages to the site of infection (Murphy *et al.* 2000). IFN-γ is the main trigger for Th1 cell differentiation, and this cytokine inhibits the activation of Th2 responses and the production of IgE. The production of Th1 type cytokines, and especially IFN-γ, is stimulated by two early Th1 type cytokines, IL-12 and IL-18 (Dinarello 1999). IL-12 and IL-18 are primarily secreted by the macrophages involved in the induction of pro-inflammatory cytokines attracting inflammatory cells to the site of inflammation (Kumano

et al. 1999). Th1 cells, macrophages and the cytokines secreted are the main participants in cell mediated inflammatory reactions of Th1 type.

Th2 cells help the antigen to activate B-lymphocytes to produce antibodies and therefore they play a crucial role in the development of humoral immunity. The allergen is presented to the T- cell through the MHC-II on the APC and IL-4 production is induced (Fig. 1c). IL-4 stimulates T helper cells to differentiate into type 2 cells (Th2) and these cells in turn induce the production of subsequent activators of the response such as the cytokines IL-5 and IL-13. IL-4 and IL-13 are involved in the activation of B-lymphocytes allowing these cells to produce IgE antibodies. The recruitment of eosinophils is tightly connected with the allergic Th2 type of response. IL-5 increases the formation, prolongs the survival and induces the degranulation of eosinophils at the site of inflammation (Hamelmann and Gelfand 2001). Activation of Th2 cells therefore triggers a response, such that the interaction of Th2 cells and cytokines will favour humoral immunity i.e., the production of IgE and an allergic type of response.

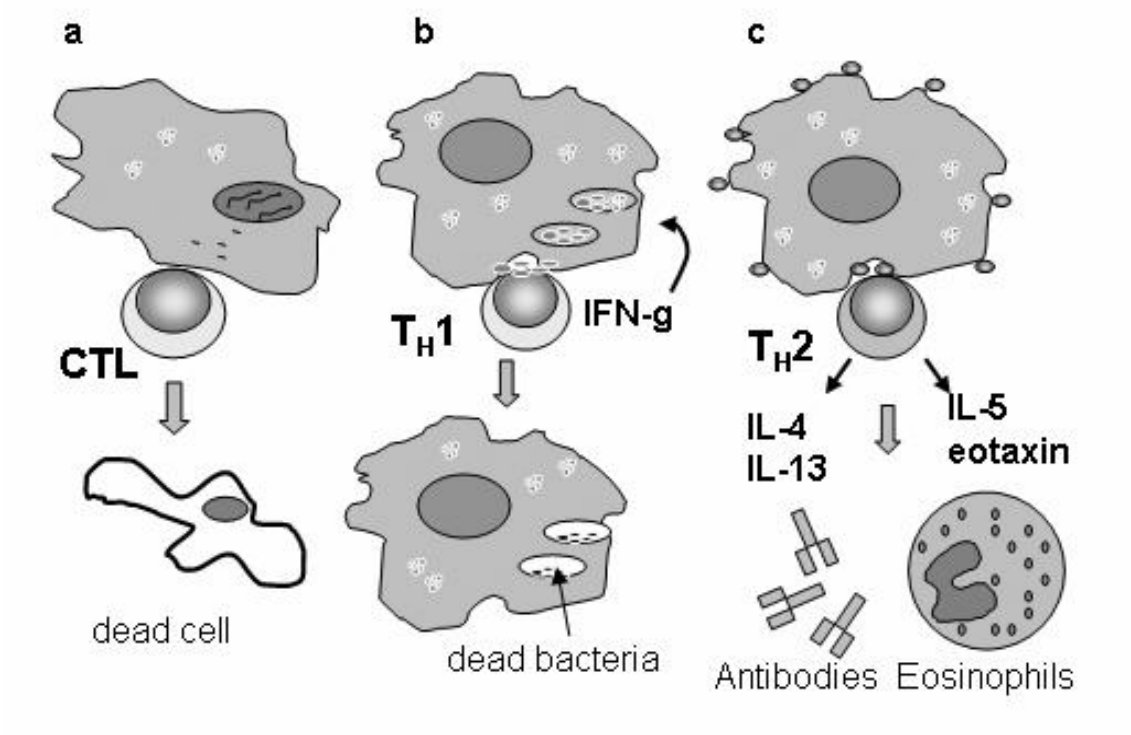

Figure 1 *Different types of effector T cells specialized to deal with different antigens. Modified from Janeway et al. 2005.*

5.1.2.2. Activation of B-lymphocytes

The main function of B cells is to produce antibodies against a foreign agent and to maintain a pool of memory cells (Gold 2002). When a B lymphocyte has recognized its antigen, with the help of T helper cells, it becomes arrested and activated. The cell enlarges, transforming itself into a lymphoblast which then starts to divide. The dividing lymphoblasts produce cells with identical specificity (a clone) and these then differentiate into antibody secreting plasma cells (Ollila and Vihinen 2005). The antibodies are secreted into the bloodstream and other extracellular fluids where they exert their action by binding to the antigen or the pathogen. Binding then designates the antigen for elimination by other cells. After the antigen has been eliminated, some of the differentiated plasma cells remain, building up the so called memory. These memory lymphocytes react more rapidly and effectively to a second infection.

5.1.2.2.1. Immunoglobulin classes

There are five main antibodies or immunoglobulin (Ig) isotypes IgM, IgD, IgG, IgA and IgE which all have different effector functions and localizations in the body (Janeway *et al.* 2005, Jelinek 2000). The initial antibodies secreted by B cells are of the M isotype. IgM is found mainly in the blood where it is involved in the activation of the complement system. IgG is the main isotype found in blood and extracellular fluids where it binds to pathogens in order to facilitate their opsonization by phagocytic cells. Although IgE is present only at low levels in blood, it plays an important biological role in immune responses (Vercelli 2001). IgE binds very effectively to receptors on mast cells upon antigen recognition and this triggers the release of the mediators responsible for the (allergic) symptoms seen in IgE mediated allergy. Finally, IgA is found mainly in secretions where it acts as a neutralizing antibody.

5.1.3. Immunomodulation

As mentioned above, the antigen on the APC determines the kind of effector T cells which will be activated. Activation of a T helper type 1 response via secretion of IFN-γ inhibits the cell's differentiation to the type 2 response while cytokines such as IL-4 on the other hand inhibit differentiation to type 1. The kind of cytokine milieu induced plays an

important role in determining the direction of the response. In allergic diseases, it is the Th2 response which predominates. In attempts to treat or modulate allergy, Th1 type responses are activated in order to skew the response away from Th2. Bacteria or bacterial components are strong Th1 immunostimulants and they have been successfully used to modulate allergic responses in experimental models (Jain *et al.* 2003, Walker *et al.* 2003). In that way, the allergic type of inflammation with eosinophilic infiltrates and the production of IgE antibodies can be downregulated by appearance of Th1 type cytokines.

Regulatory T cells have lately been recognized to play an important role in the early development and activation of an immune response. They have been shown to be important in the maintenance of self-tolerance and they probably control the development of autoimmune diseases and transplant rejections. They might therefore also be involved in the control of allergic diseases (Hawrylowicz and O'Garra 2005, Robinson *et al.* 2004). $CD4^+CD25^+$ regulatory T cells (Treg) have been found to be able to suppress T cell proliferation. The interaction occurs through cell contact and the secretion of immunosuppressive cytokines such as IL-10 and TGF-β. The development of $CD4^+CD25^+$ regulatory T cells is tightly regulated by a transcription factor, Foxp3. There are two other groups of regulatory T cells that are $CD25^-$, these cells have been named T_H3 and T_R1 (Umetsu *et al.* 2003). T_H3 are regulatory helper 3 type cells that produce cytokines such as IL-4, IL-10 and TGF-β while the regulatory cell T_R1 secretes IL-10 and TGF-β.

5.1.4. Hypersensitivity reactions

When the immune response is evoked against non-infectious antigens, hypersensitivity reactions or allergy can develop and these can cause tissue damage. Coombs and Gell classified the hypersensitivity reactions into four types called type I, II, III and IV (Coombs and Gell 1963). The type I reaction is IgE mediated while type II and III are IgG mediated. Type IV hypersensitivity reactions are T cell mediated and can be divided into three subgroups based on the reactions they activate. In the following section only the hypersensitivity reactions involved in the immune responses studied in this thesis will be explained in more detail.

5.1.4.1. IgE mediated allergy (type I)

IgE mediated allergy is characterized by the production of specific antibodies to an allergen driven by Th2 effector cells. On a second encounter with the allergen, an allergic immune response is induced. The immune response is characterized by an early and a late response. In the early response, pre-formed inflammatory mediators, such as histamine, are secreted when the antibodies produced against the allergen bind to the high affinity IgE receptors present on mast cells (Broide 2001). The release of histamine and other inflammatory mediators such as leukotrienes and prostaglandins causes a rapid increase in vascular permeability and smooth muscle contraction. In the late response, cytokines and chemokines participate in the activation and recruitment of more inflammatory cells such as eosinophils and basophils. IL-5 activates and increases the formation of eosinophils, and together with CCR3 binding chemokines such as CCL11/eotaxin-1 and CCL5/RANTES, attracts these cells to the site of inflammation. If this delayed-phase inflammation continues for a long time it can lead to a situation of chronic inflammation with eosinophils and effector Th2 cells constantly being present. The reaction can cause severe symptoms such as impaired airflow in the airways and sustained edema at the site of inflammation.

The allergic reaction produces different clinical symptoms depending on the route of allergen entry (Janeway *et al.* 2005). Inhalation of allergen is the most common route of entry and is associated with symptoms in the nose (allergic rhinitis) or in the lower airways (allergic asthma).

5.1.4.1.1. Allergic asthma

Allergic asthma is characterized by both an early (immediate) response, that can be classified as type I hypersensitivity, and a late (delayed) response of type IV with Th2 effector cells orchestrating the inflammation (Figure 2). The allergic asthma reaction starts when the allergen is inhaled and becomes bound to its specific antibodies situated on submucosal mast cells in the lower airways. Mast cells are activated and trigger the release of histamine, leukotrienes, prostaglandins and platelet activating factor (PAF) (Williams and Galli 2000). The immediate asthmatic reaction is characterized by smooth muscle contraction in the bronchial airways. The delayed response is initiated with the secretion of IL-4 by mast cells to activate T-lymphocytes. Activated Th2 cells and basophils secrete

IL-4 and IL-13, activating B-cells to produce more IgE antibodies. Th2 cells further secrete IL-3, IL-5 and GM-CSF in order to recruit eosinophils (Kay 2001). Then, the eosinophils release inflammatory mediators such as cationic proteins, leukotrienes and PAF to the site of inflammation (Busse and Lemanske 2001). The late response of the asthmatic reaction is characterized by swelling of the airway mucus membrane, increased mucus secretion and increased (bronchial) airway hyperresponsiveness (AHR). If the inflammation is allowed to proceed, Th2 lymphocytes and eosinophils will secrete more IL-5 and GM-CSF, stimulating the bone marrow to produce more eosinophils. Epithelial lesions start to develop due to the devastating effects of the cationic proteins constantly being released by the eosinophils. The airflow is impaired and the inflammation may develop into chronic asthma (Cohn *et al.* 2004).

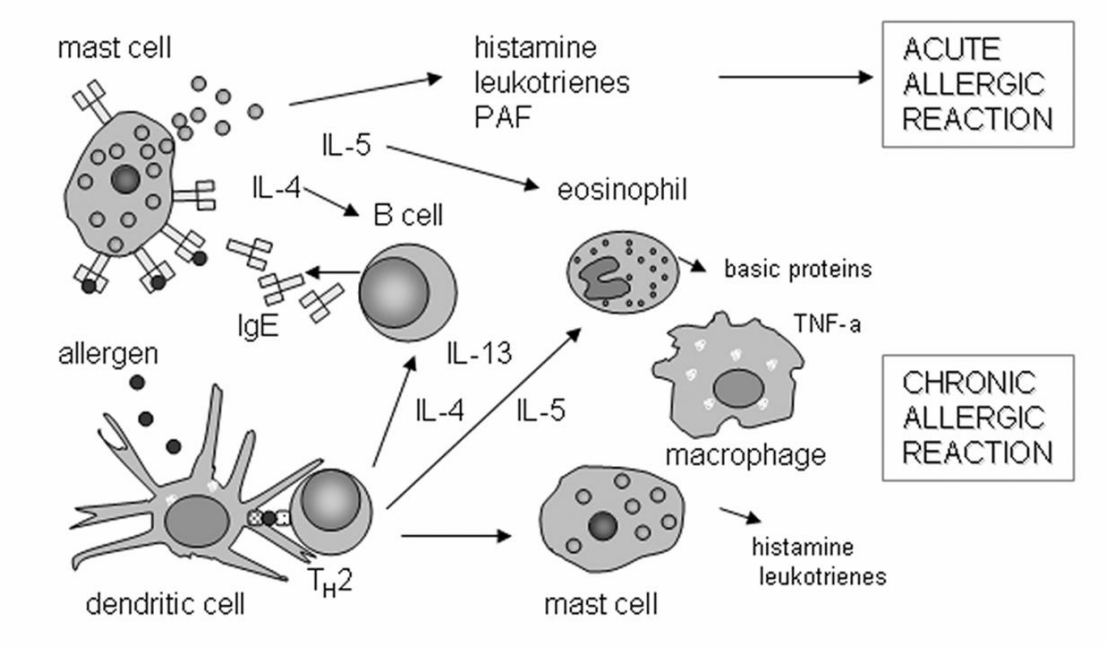

Figure 2 *Cells and mediators involved in the acute and chronic phase of allergic asthma. Modified from (Kay 2001).*

5.1.4.2. Type III hypersensitivity

Type III hypersensitivity reactions result from the inadequate removal of the immune complexes formed in response to antigens. Repeated inhalation of antigenic material induces the production of IgG antibodies which form immune complexes in the lung intended to facilitate phagocytosis. The persistent presence of immune complexes

activates mediators that induce local lung inflammation. Sustained exposure to high levels of antigen may lead to alveolar cell wall membrane destruction. The high levels of mouldy hay dust occasionally found in farming environments can induce a disease condition called farmer's lung (Reboux *et al.* 2001, Terho 1982). Farmer's lung disease exhibits the characteristics of type III hypersensitivity reactions such as IgG antibodies to the offending antigen and the formation of immune complexes in the lung. IgG antibodies to actinobacteria and fungi such as *Aspergillus fumigatus* are commonly found in the sera from these patients (Erkinjuntti-Pekkanen *et al*. 1999). The lung histology reveals granulomatous reactions and chest X-ray findings reflect the presence of immune complexes in the lung (Sutinen *et al.* 1983).

5.1.4.3. Cell mediated immunity (type IV)

Type IV hypersensitivity reactions are delayed T cell mediated immune responses which are divided into three types based on the reactions they induce (Roitt *et al.* 2001). In cell mediated immunity, antigen specific T effector cells are activated upon recurrent encounters with the antigen. The tissue damage is due to the activation of Th1 cells recruiting macrophages or Th2 cells that recruit mainly eosinophils. Direct tissue damage is caused by cytotoxic T cells.

5.1.5. Allergens

An antigen able to activate the immune system to produce specific IgE antibodies and to trigger an allergic reaction in susceptible individuals is called an allergen (Stewart and Robinson 2003). Allergens enter the body via the airways, skin, gastrointestinal tract or parenteral route and are usually presented to the host at very low doses which is thought to favor the induction of the allergic Th2 type response (Janeway *et al.* 2005). Allergens are mostly proteins or glycoproteins ranging in molecular weight from 3-80 kDa. They are usually highly soluble and are resistant to heat, proteases and denaturing agents. Allergens have several antigenic determinants on their surface called epitopes and it is to these regions that the antibodies bind. The antigenic determinants are commonly composed of discontinuous amino acids brought close together in the three dimensional structure of the

protein. The epitope recognized in its 3D form is called a conformational epitope. Antibodies may also recognize small continuous fragments on the polypeptide chain of the protein called linear epitopes. The conformation is however most crucial for antibody binding to the epitopes (Aalberse 2000).

5.1.5.1. IgE cross-reactivity

The IgE produced in response to an allergen might also bind to similar structures on other antigens leading to cross-reactivity. In particular, taxonomically related antigens often share antibody binding epitopes that cross-react for IgE binding (Aalberse *et al.* 2001). The high degree of homology in the primary structure of related proteins may produce homologous 3D structures potentially leading to cross-reactivity. More than 70% sequence identity between the proteins seems generally to be required for cross-reactivity to occur (Aalberse 2000). Also non-related preserved proteins (pan-allergens) might possess cross-reacting parts. Postsynthetic modifications such as glycosylation of proteins are commonly involved in cross-reactivity between unrelated proteins due to cross-reactive carbohydrate determinants (Ferreira *et al.* 2004). Cross-reacting antibodies may therefore induce positive results to allergens to which the individual has not primarily been sensitized, due to the similarities in the structure between the allergens.

5.2. Health effects linked to fungal exposure

We are constantly inhaling low levels of airborne fungal spores. Fungi are an integral part of our natural environment and normally do not have harmful effects to human health. The airborne fungal spores can originate from outdoor or indoor sources (Burge 2002, Flannigan *et al.* 1991). Usually the levels of outdoor spores are slightly higher compared to indoor levels (Shelton *et al.* 2002). The outdoor spore levels vary greatly depending on climate and season. In subarctic climates, outdoor spore levels are extremely low during the winter season (Reponen *et al.* 1992). In Finland, fungal spore levels reach their maximum during late summer and autumn. During these periods, susceptible individuals might experience sensitization to allergenic fungi originating mainly from outdoor environments. A water damaged building with moist materials however, is a suitable

growth area for fungi not normally found indoors (McGrath *et al.* 1999). Under these circumstances, fungal growth and consequently fungal spore levels in the air may be higher indoors than outdoors and health problems may arise. The health problems commonly linked with exposure to microbes in damp buildings are allergic diseases and nonspecific airway inflammation (IOM 2004, Portnoy *et al.* 2005).

5.2.1. Fungi

Fungi comprise a large kingdom of eukaryotic organisms divided into several taxonomical groups and species. In the following section, only a brief introduction to the fungal groups studied in this thesis will be described. Fungi are typically filamentous where the hyphae grow at the tip and form branches called mycelium. Fungi may reproduce both sexually or asexually by forming spores or conidia (Carlile *et al.* 2001, Deacon 1997). Fungi with asexual morphology have artificially been grouped together into a group called Mitosporic fungi, previously known as Deuteromycetes or Fungi Imperfecti. Mitosporic fungi are further divided into classes and the fungi studied in this thesis belong to the class hyphomycetes including asexual yeasts (Hawkswort *et al.* 1995). The hyphomycetes class include many common soil and plant fungal species such as *Cladosporium* and *Alternaria*. Food or grain spoilage fungi, including *Penicillium* and *Aspergillus* species which are potential mycotoxin producers also belong to the hyphomycetes class. There are several potential mycotoxin producers, e.g. *Stachybotrys* species commonly contaminate wet wood and hay. The budding yeasts in this class are sometimes separated into a subclass called blastomycetes of which *Candida* and *Pityrosporum* species (spp.) are typical examples. Generally however, when the name fungus is used here it includes both yeasts and fungi of the hyphomycetes class (Figure 3). Filamentous (micro)fungi of the hyphomycetes class that reproduce asexually by forming conidia or spores, are also commonly called moulds (Gravesen *et al.* 1994).

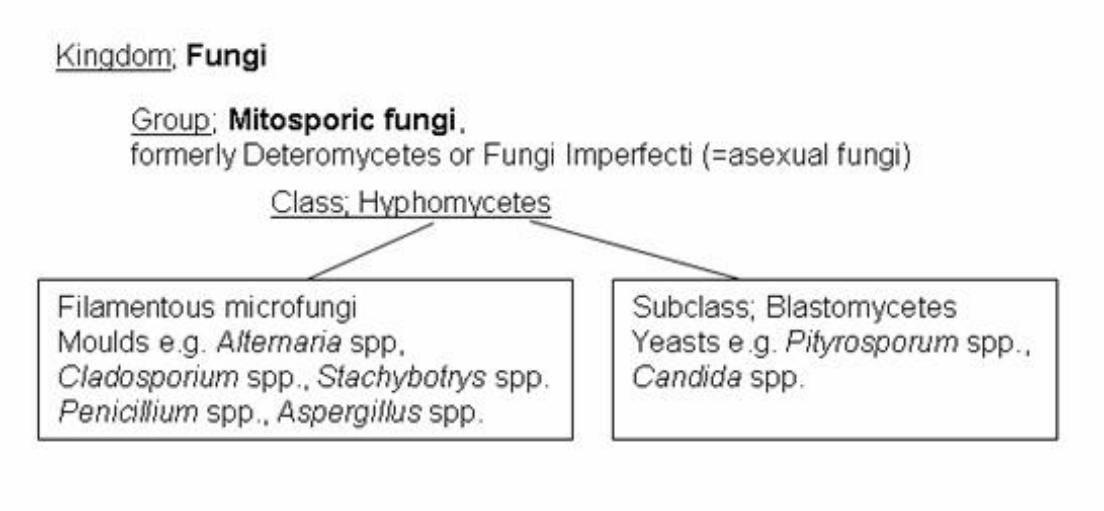

Figure 3 *Moulds and yeasts belong to the hyphomycetes class.*

5.2.2. Fungal growth in water damaged buildings

Water damage in buildings can lead to mould problems if the structures are not dried properly. The prevalence of significant moisture damages in buildings was evaluated in Finland in the 1990s and estimated to be as high as 50 % depending on how the damages were defined (Koskinen *et al.* 1999, Nevalainen *et al.* 1998). The proportion is estimated to be approximately the same in other countries with similar climates e.g. the northern European countries and North America (Brunekreef *et al.* 1989, Platt *et al.* 1989). Buildings are still often imperfectly designed and poorly constructed which may lead to potential water leakage from outdoor or indoor sources (Reijula 2004). Modern buildings are often tightly constructed in order to save energy and to keep them warm but this makes the drying process difficult should water damage occur. Fungal growth may develop on damp building materials and this can range from a few mouldy spots to large areas of contamination.

5.2.2.1. Microbes indicating moisture problem indoors

A damp or water damaged building favours the growth of certain microbes that can be considered as indicators of moisture problem (Gravesen *et al.* 1999). The typical microbes found in material from water damaged buildings in Finland (Reijula 2004) are shown in Table 1. Most of the microbes are moulds but also yeasts and bacteria (eg. *Actinomycetes*)

are found (Andersson *et al.* 1997). Among the first microbes to colonize wet buildings are *Penicillium* and *Aspergillus* species, while *Cladosporium* species are usually considered as secondary invaders. Typical tertiary colonizers are *Phoma herbarum* and *Stachybotrys chartarum* (Gravesen *et al.* 1994).

Table 1. *Typical microbes found in material samples from water damaged buildings in Finland (modified from Reijula 2004).*

Penicillium spp.
Phoma spp.
Aspergillus versicolor
Acremonium
Aspergillus sydowii
Cladosporium spp.
Fusarium spp.
Trichoderma viride
Aureobasidium spp.
Stachybotrys chartarum
Chaetomium globosum
Phialophora spp.
Yeasts
Actinomycetes

Damp building moulds, and some of the yeast species, commonly belong to the group of fungi that reproduce asexually. Moulds reproduce by forming spores whereas asexual yeasts replicate by budding off new cells. Mould spores can become airborne and inhaled into the airways (Burge 2002). It is not known at which spore level a contaminated building becomes a risk to an individual's health. Exposure limits have been difficult to quantify due to the fact that some microbes seem to be more harmful than others. National guidelines on exposure limits have been published in relation to the microbes found in moisture damaged buildings (STM 2003). Generally however, exposure to an average of more than 1000 colony forming units (cfu)/m^3 air has been considered as a potential health risk (Flannigan *et al.* 1991). Exposure to fungi is commonly estimated by taking air samples, collecting settled dust or by examining the building material.

Exposure to inhaled fungi and fungal spore levels in the air can be assessed by air sampling and microscopic identification of the species. Different air sampling techniques have been used, but basically a certain volume of air is sampled and the spores are collected by size or sampled directly onto culture plates in order to count and identify the spores and species (Portnoy *et al.* 2004). Semi-quantitative cultures may be used in order to identify species and to determine the viable colony forming units (cfu) not only from air samples but also from settled dust samples. Culturing techniques may however, favour the growth of the fast growing species while the slow growing and non-viable specimens are left undetected. New molecular biology techniques are being developed in order to identify and quantify moulds in indoor air or dust samples using species specific primers and probes for detection (Meklin *et al.* 2004, Zeng *et al.* 2004). The secondary metabolites produced by the fungi are commonly detected from air or material samples using HPLC or mass-spectrometry techniques (Engelhart *et al.* 2002, Tuomi *et al.* 1998).

5.2.2.2. Immunomodulatory fungal components

Fungal elements that might induce activation of the immune system are the fungal spore, secondary metabolites, hyphae or part of the fungal cell wall. Part of the fungal proteins and glycoproteins may also be allergenic and trigger the activation of the adaptive immune system in susceptible individuals. However, commonly the first line of defence is directed against those common parts of the fungi recognized as foreign to the host.

Cell wall components

The fungal cell wall contains a variety of molecules including proteins, polysaccharides linked to proteins, lipids and carbohydrates. The major components of the fungal cell walls are the polysaccharides (Carlile *et al.* 2001). Depending on the class of fungi, different polysaccharides in varying proportions are embedded into the wall. Mould cell walls contain mainly chitin and glucan while in yeast cell walls, glucans and mannans predominate. The immune system responds to fungal cell wall components through innate immune receptors such as Toll-like receptors (Netea *et al.* 2004). In particular, (1→3) β-D-glucan and mannan have been shown to be potential fungal PAMPs recognized by the receptors triggering the innate immune system (Roeder *et al.* 2004). Also the more

common cell wall constituent of moulds, chitin, has been shown to have immunomodulatory properties in airway inflammation (Strong *et al.* 2002).

Secondary metabolites

Many of the fungal species found in damp buildings can produce secondary metabolites under certain optimal growth conditions such as high humidity, a suitable medium and temperature (Nielsen 2003). The secondary metabolites are produced by the fungi in an attempt to fight for living space and have been shown to be highly species specific. Many fungal species found in moisture damaged buildings are potential toxin producers and their toxins, mycotoxins, have been isolated and identified from the building materials (Tuomi *et al.* 2000). Mycotoxins are capable of inhibiting protein synthesis and may exert immunosuppressive, hemolytic, cytotoxic or neurotoxic effects depending on the toxin (Sudakin 2003). Fungi can also produce other secondary metabolites e.g., enzymes with protease or proteolysing activities, which might also be potent immunostimulators. Fungal proteases, especially those produced by pathogenic fungi, have been observed to trigger the production of pro-inflammatory cytokines *in vitro* (Kauffman *et al.* 2000).

5.2.2.3. Health effects

Exposure to damp buildings has commonly been linked to many different health effects often described as coldlike symptoms. Epidemiological clinical studies have revealed that people exposed to damp buildings suffer more often from upper and lower respiratory tract infections and allergic diseases compared to people living in healthy buildings (Andrae *et al.* 1988, Andriessen *et al.* 1998, Bornehag *et al.* 2001, Norback *et al.* 1999, Verhoeff and Burge 1997). Typical allergic diseases which may be associated with exposure are rhinitis and asthma. Eye irritation, cough, headache, dizziness, nausea and joint pain are some of the more nonspecific symptoms reported. These symptoms are thought partly to be caused by exposure to fungi and the secondary metabolites produced by the fungi growing on damp building materials (Garrett *et al.* 1998, Peat *et al.* 1998, Sudakin 1998). In rare cases, fungi may be infectious. With respect to the typical damp building moulds, *Penicillium* and *Aspergillus* are allergenic while certain species such as

Aspergillus versicolor and *Stachybotrys chartarum* are potential toxin producers and therefore considered as a health risk (Bush and Portnoy 2001).

5.2.3. Allergy to fungi

Individuals, especially atopic individuals with an inherited tendency to produce IgE antibodies, can become sensitized to allergenic moulds (Kurup *et al.* 2000). Sensitization is characterized by IgE antibodies and positive skin test reactions to the allergen. Sensitization can occur through inhalation in the airways, mucous membranes or in the skin. Sensitized individuals may develop allergic symptoms upon subsequent exposure, manifesting itself as allergic rhinitis or asthma if they have been sensitized and challenged via the airways. Sensitization to moulds through the skin may occur in situations where mouldy materials are handled; in this case the moulds can induce allergic symptoms such as eczematous skin reactions.

5.2.3.1. Allergenic fungi

Fungi contain many allergenic components but the spores have been found to contain the most allergenic (Horner *et al.* 1995). Most of the allergenic fungi belong to the taxonomical group Deuteromycetes which replicate asexually (imperfect fungi) by forming spores or conidia. Fungal spores have been shown to be allergenic to varying degrees depending on the species. Species that are considered highly allergenic are *Alternaria alternata, Cladosporium herbarum, Aspergillus fumigatus* as well as *Penicillium* species (Bush and Portnoy 2001).

Cladosporium herbarum* and *Alternaria alternata

Cladosporium herbarum and *Alternaria alternata* are two of the most common allergenic fungi used in allergy tests when allergy to fungi is suspected. *A. alternata* is a worldwide sensitizer and despite its rather large spore size (20-80 μm) has been shown, together with Aspergillus species, to be one of the most potent sensitizers in terms of spore numbers (Beaumont *et al.* 1985, Gautrin *et al.* 1994). *C. herbarum* is found in high spore numbers especially in cooler climates but, in comparison with its high spore counts, it is not as

potent a sensitizer as *Alternaria*. *C. herbarum* and *A. alternata* are typical fungi found in the outdoor air. They are common saphrophytes found on decaying plants, foodstuffs, in the soil and on decaying wood (Gravesen *et al.* 1994). Should there be water damage indoors they may also start to colonize the wet building material.

5.2.3.2. Cross-reactivity between fungal allergens

There is known to be considerable cross-reactivity between fungi (Kurup and Banerjee 2000). Commonly cross-reactivity is encountered between species of the same genus but can also be seen between different genera and classes of fungi. Fungi, in general, contain conserved allergens such as enolases (Breitenbach *et al.* 1997). These components are found in many species and plants which are not related to each other. In addition, moulds contain cross-reacting conserved allergens such as aldehyde dehydrogenase, aldolase and acidic ribosomal proteins (Kurup and Banerjee 2000, Shen *et al.* 1998, Weichel *et al.* 2003). Phylogenetically related moulds such as *Aspergillus* and *Penicillium* species seem to share several cross-reacting allergens (Schmechel *et al.* 2005). Yeasts share cross-reacting protein allergens such as enolase and peroxisomal membrane proteins between species but cross-reactivity to *Aspergillus fumigatus* mould allergens has also been observed. Typically yeasts seem to share similar cell wall carbohydrate components such as mannans between different genera and species (Doekes *et al.* 1993, Lintu *et al.* 1999).

5.2.3.3. Prevalence of sensitization

Sensitization to fungi may occur outdoors as well as indoors and is therefore not always linked to exposure to indoor moulds. Sensitization to airborne fungal spores occurs mainly via the airways. The prevalence of respiratory allergy to fungi has been estimated as being about 6% in the general population (Horner *et al.* 1995, Kurup *et al.* 2000) with only minor variations between countries (Bush and Portnoy 2001). The sensitizing fungal species vary slightly depending on the living conditions and the climate. Different fungal allergen extracts and diagnostic techniques also account for some of the variation in prevalence seen in epidemiological studies. In addition, the selection criteria for the study population strongly affect the prevalence. In an atopic population, the prevalence for

sensitization has been shown to be higher (20-30%) due to their inherited susceptibility to develop IgE mediated diseases. The age of the study group also affects the prevalence, showing higher values in children (Kauffman *et al.* 1995, Koivikko *et al.* 1991). The prevalence of sensitization to *C. herbarum* and *A. alternata* was shown to be highest at the age of eight years, declining with increasing age (Nolles *et al.* 2001).

5.2.3.4. Association with mould induced asthma

Prolonged exposure to increased levels of allergenic fungal spores is able to induce allergic asthma or to exacerbate asthma symptoms in susceptible individuals (IOM 2004). Exposure to some of the allergenic fungal species, such as *Alternaria alternata* and *Aspergillus fumigatus*, has been linked with an increased risk to develop allergic asthma (Downs *et al.* 2001). Epidemiological studies have shown that sensitization, especially sensitization to *Alternaria*, is common among subjects with asthma (D'Amato *et al.* 1997). The association was shown to be more pronounced in children and to decrease with age in accordance with sensitization (Nolles *et al.* 2001).

5.2.4. Non-IgE mediated airway responses linked to exposure

Exposure to fungi can also induce irritation and inflammation of the mucus membranes in the eyes and in the respiratory tract. The airway response may resemble allergic responses but are not IgE mediated. Other antibodies such as IgG may play a part in the initiation of these airway responses (Gravesen *et al.* 1994). The inflammatory airway responses may result in irritant asthma or other asthma like symptoms (IOM 2004).

5.2.4.1. Innate airway response

Inhalation of airborne spores activates the defence system of the host to attempt to eliminate the foreign agent. Initially, when fungal spores enter the upper airways, the largest spores are filtered and washed away by the nasal mucosa. The smaller spores, less than 10 μm in diameters, may be inhaled into the lungs (Geiser 2000). Only fungal spores less than 3 μm in diameter are small enough to reach the terminal bronchioles and alveoli but once there alveolar macrophages recognize them as foreign and start to eliminate the

spores (Knox and Homer 2003). The inflammatory immune response is activated with the release of pro-inflammatory cytokines. This immune response is in most cases sufficient to eliminate the antigen. If however, the antigen is still presented on the inflamed mucosa after a period of a few days or weeks, then the adaptive response may be activated to eliminate the antigen.

5.2.4.2. Hypersensitivity syndrome due to fungi

Prolonged exposure to extremely high concentrations of fungal spores may lead to a disease condition which exhibits the characteristics of both type III and IV hypersensitivity reactions. IgG to moulds is found in sera of exposed persons but the role of IgG in this hypersensitivity reaction is not clear (type III) (Marinkovich 2004). The cell mediated immune response of Th1 type with activated macrophages (Type IV) seems to play the main role in these reactions. If the macrophages are unable to eliminate the fungal spores, then they fuse into epitheloid cells and giant cells leading to the granuloma formation typical of extrinsic allergic alveolitis or hypersensitivity pneumonitis (HP). The high fungal spore concentrations that might induce this condition can occasionally be found in barns or workplaces where mouldy hay (*A. fumigatus*) or wood pulp (*A. alternata*) is being processed (Gravesen *et al.* 1994).

5.2.4.3. Infection

In rare cases, such as in immunocompromised individuals, fungi may be infectious. *Aspergillus fumigatus* is one of the moulds that may colonize the lungs of immunosuppressed individuals and induce a disease condition called aspergillosis. Fungal colonization of lungs places a heavy burden on the immune system and frequently, the individual has to be treated with antifungal drugs in order to eliminate the fungi (Wark *et al.* 2003). Also opportunistic yeasts, such as *Candida albicans*, which normally colonizes mucosal surfaces, may be converted into a pathogen in immunosuppressed individuals and induce a systemic infection (Nucci and Marr 2005).

5.2.5. Diagnosis of health effects associated with fungal exposure

Many investigations have been undertaken to examine the health problems experienced by individuals occupying damp buildings but still controversy exists concerning the cause and effect of exposure to moulds. Sensitization to moulds can be demonstrated with allergy tests but there are few tests or markers which can be used for the non-specific symptoms associated with exposure. Commonly the first step taken in a damp building case with linked health problems is a microbial evaluation of the building. The health problems may be evaluated by using questionnaires or clinical examinations of the patients. Allergy *in vivo* tests such as skin prick tests (SPT) and *in vitro* antibody measurements from sera are widely used when diagnosing sensitization to fungi (Trout *et al.* 2004). In the context of diagnosing occupational diseases, potential asthma associated with exposure is examined with lung function tests. Allergen provocation test are often needed to confirm the association between the sensitizing agent and the disease.

The non-allergic health effects linked to exposure to damp building moulds have however been difficult to demonstrate experimentally or in patient samples. The occupants of a damp building "feel sick" in many different ways but the symptoms are diverse and difficult to relate directly with their exposure to moulds at the level of the individual. Epidemiological and cross-sectional studies have clearly shown increased incidences of upper and lower airway symptoms in occupants of damp buildings (Brunekreef 1992, Dales *et al.* 1991, Koskinen *et al.* 1999, Savilahti *et al.* 2000, Taskinen *et al.* 1999). Many attempts have been made to identify inflammatory markers in the respiratory organs such as the nose or the lungs. Changes in cell composition or in cytokine levels in broncho-alveolar lavage samples have been used to study lung diseases in general but there have been few studies conducted on patients exposed to fungi. Nasal lavage and induced sputum are also used in the diagnosis of airway disease and some of the studies have focused on exposure to damp buildings (Walinder *et al.* 2001). Their results suggest that higher levels of inflammatory mediators are present in nasal fluid from patients in a moisture damaged building compared to controls (Hirvonen *et al.* 1999).

5.2.6. *Stachybotrys chartarum*

In 1986 Croft et al. reported adverse health effects linked to a damp building where *Stachybotrys chartarum* growth was found in large areas of the building (Croft *et al.* 1986). Since then, many other cases of health problems have been associated to exposure to *S. chartarum* (Dill *et al.* 1997, Hodgson *et al.* 1998, Johanning *et al.* 1996). The commonest health complaints have been headache, sore throat, fatigue, dermatitis, general coldlike symptoms and malaise. Special attention was drawn to a cluster of cases in Cleveland Ohio in the 1990's where several infants became seriously ill suffering from idiopathic pulmonary hemosiderosis (Dearborn *et al.* 1999). *S. chartarum* was found in large numbers in the children's bedrooms. However it has never been confirmed whether *S. chartarum* was actually responsible for the children's symptoms (CDC 2000). One of the reasons for blaming *S. chartarum* for health effects is that under suitable circumstances it possesses the ability to produce highly toxic macrocyclic trichothecenes (Hintikka 1978, Hintikka 2004).

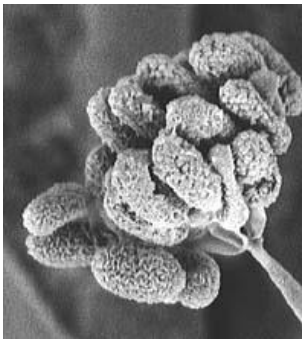

Figure 4 *Stachybotrys spores (conidia). Spore size range from 5-12 µm. Scanning electron micrograph with permission from an article by Berlin D. Nelson in November 2001 APSnet, courtesy of the American Phytopathological Society.*

5.2.6.1 Secondary metabolites produced by S. chartarum

Stachybotrys may produce different secondary metabolites with many different potential health effects depending on the strain (Nielsen 2003). The *Stachybotrys* strains found in Cleveland have been characterized in detail and the toxins analyzed (Jarvis 2002). Many of the strains produced not only the macrocyclic trichothechenes, satratoxin G and H, roridins, stachybotrylactones and lactams, but also proteases, atranones and hemolysin. Trichothechenes have been shown to inhibit protein synthesis and to be cytotoxic to

eukaryotic cells while the lactones and lactames are immunosuppressive. *Stachybotrys* derived proteases have been reported to cause tissue damage whereas the atranones have only been recently identified and their function is still unknown (Kordula *et al.* 2002, Yike *et al.* 2002). The hemolysin (stachylysin) has been shown to cause hemolysis of red blood cells (Vesper *et al.* 2001).

5.2.7. Experimental studies

Experimental models are frequently used to study exposure to moulds. The experimental models can be used to study physiological, immunological and histological changes in the lungs after exposure to mould allergens and mould spores, also toxic spores, which would not be possible in human studies. In order to mimic the natural exposure route for inhalants, the airways are commonly exposed directly. This can be achieved by nebulizing the antigen into a closed chamber where experimental animals such as mice inhale the aerosolized antigen (Korpi *et al.* 2002). The antigen can also be installed directly into the airways using intratracheal (i.t.) instillation (Jussila *et al.* 2002). One method representing the situation in between inhalation and i.t. can be achieved by intranasal instillation (Cooley *et al.* 2000). In intranasal instillation, a drop of the antigen suspension is installed on the nose of an anaesthetised animal which then inhales it into the lungs. In allergen sensitization studies, in order to induce allergic asthma, the allergen is commonly injected intraperitoneally at first to induce sensitization and later directed to the airways by inhalation.

Exposure to *Stachybotrys*

Stachybotrys chartarum has often been the microbe of choice when exposure to damp building moulds has been investigated in experimental models (Yike and Dearborn 2004). Toxin producing and non-producing strains have been used as well as pure toxins. *S. chartarum* spores and toxins have mostly been installed intratracheally into mice followed by evaluation of histological and morphometric changes in lung tissue (Rand *et al.* 2003). Rats have also been used and commonly changes in the cell population in the BAL fluid have been studied after intratracheal installations (Rao *et al.* 2000). An attempt to mimic a real life exposure situation has also been made where mice inhaled spores of *S. chartarum*

directly from agar plates (Wilkins *et al.* 1998). The technique of installing *S. chartarum* spores intranasally repeatedly was introduced by our group in order to mimic long time exposure (Nikulin *et al.* 1996, Nikulin *et al.* 1997). The main effects of exposure were studied from the histological lung sections.

Allergic fungal asthma

Experimental models studying sensitization and the development of allergic fungal asthma have commonly used typical allergenic moulds such as *Penicillium* or *Aspergillus* species. In fact, *Apergillus fumigatus* has been the most widely evaluated fungus. Exposure to *A. fumigatus* has been linked to allergic asthma and sensitization and exposure to *A. fumigatus* allergenic extracts has been extensively studied (Haczku *et al.* 2001, Kurup *et al.* 2001). These models of acute and chronic asthma induced by *A. fumigatus* have added important data concerning the role of chemokines and chemokine receptors in the pathogenesis of the disease (Blease *et al.* 2001, Schuh *et al.* 2002). *Penicillium* species are commonly found in damp buildings and allergic inflammation was recently induced in a murine model using a purified protease allergen from *Penicillium chrysogenum* (Schwab *et al.* 2004).

Allergic asthma model

The acute allergic asthma model has proved to be useful for studying the pathogenesis and possible treatment of allergic asthma (Epstein 2004, Gelfand 2002). The mouse model of allergic asthma exhibits the main characteristics associated with human asthma. The allergized mouse present with airway hyperreactivity (AHR), an eosinophilic lung infiltrate, specific IgE antibodies to the sensitizing allergen and a Th2 type inflammatory response in the lungs (Tomkinson *et al.* 2001). The protocol involves a sensitization period (allergen injected intraperitoneally) after which the reaction is directed to lungs by challenging the airways with the allergen. The most commonly used allergen in this model is ovalbumin (OVA).

6. AIMS OF THE STUDY

Exposure to allergenic or toxic fungal spores is considered to be one of the major inducers of the health problems linked to water damaged buildings. Sensitization to moulds may explain part of the symptoms but other components such as the toxins in mould spores may also play an important role. In addition, the immunological status of the exposed individual may modulate the extent of the response.

The specific aims were:

1. To evaluate the prevalence of sensitization to two common moulds in patients attending a hospital for suspected allergy.

2. To characterize the IgE binding to fungal allergen extracts in sera from mould sensitized patients in order to study the specificity of the binding to moulds.

3. To study the airway responses to a toxin producing damp building mould, *Stachybotrys chartarum*, in an experimental model.

4. To study the interaction between experimental allergy and exposure to *Stachybotrys chartarum* in the airways in order to determine whether the immunological status affects the response.

7. SUMMARY OF METHODS

A more detailed description of the material and methods used in this study can be found in the original publications (I-IV).

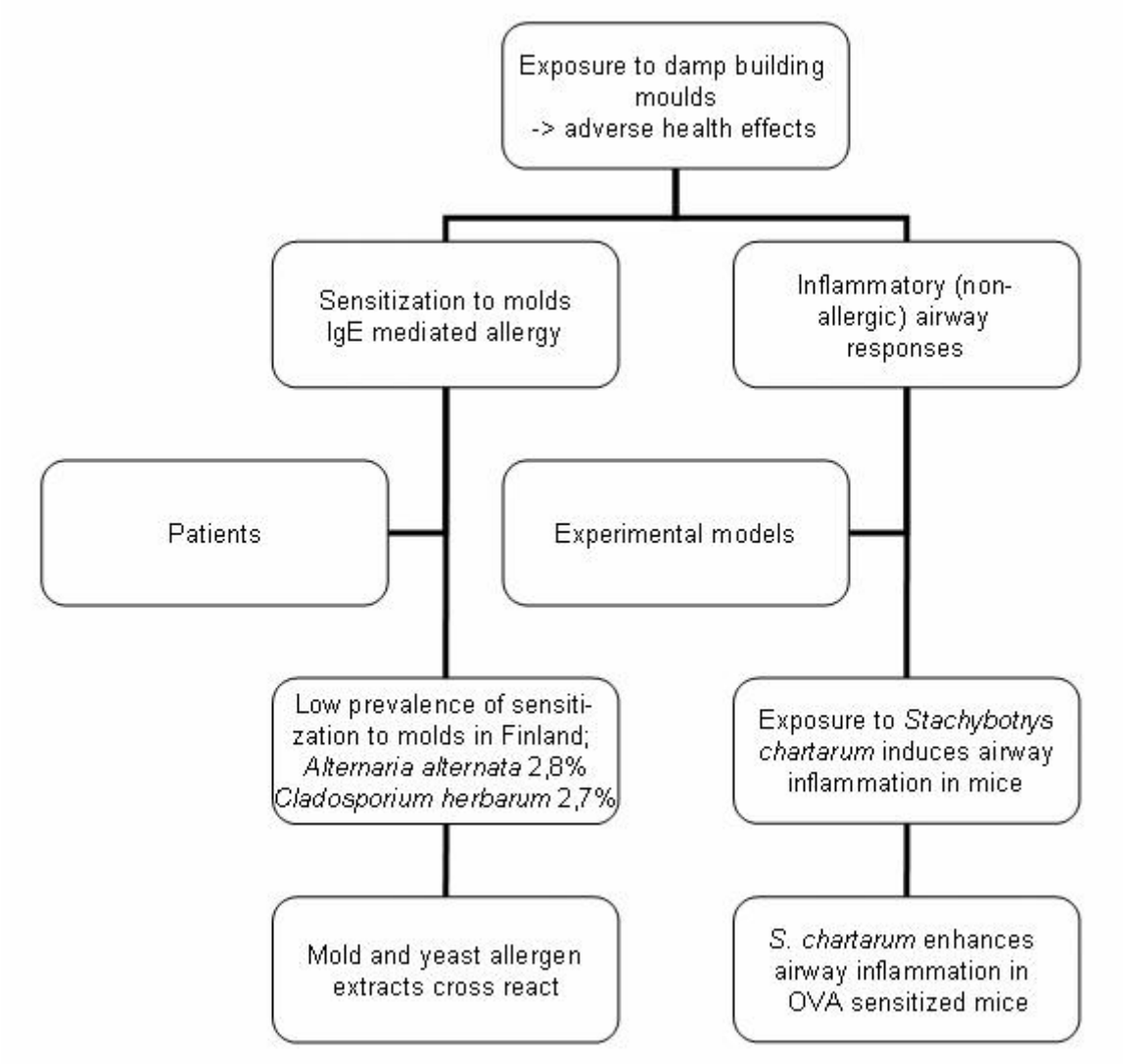

Figure 5 *Summary of study design.*

7.1. Sensitization to moulds, PATIENT STUDIES

7.1.1. Study subjects

Patient screening (I)

A group of 6376 patients examined at the Skin and Allergy Hospital in Helsinki during January 1996 to May 1997 was skin prick tested for 11 common inhalant allergens including *Cladosporium herbarum*. The patients had attended this hospital due to suspected allergy. Of these, 1504 patients with a history of indoor air problems were further tested with a special fungal allergen panel.

Mould sensitized patients (I, II)

Serum samples were obtained from 40 patients sensitized to moulds. All patients demonstrated a SPT wheel diameter ≥ 4 mm to *C. herbarum* and part of them also to *A. alternata*, 23 were females and 17 males (mean age 34 years, range 14 – 60).

Control patients (I, II)

Serum samples from 20 non-atopic (16 females and 4 males, aged 25-57) and 20 atopic (14 females and 6 males, aged 16-57, with no mould positive SPT results) persons were used as controls. All patients gave their informed consent to participate in the study. The study was approved by the ethical committee of the Skin and Allergy Hospital, Helsinki University Central Hospital.

7.1.2. Allergen extracts

Commercial allergen extracts (I, II)

Skin prick tests (SPT) and water soluble fungal extracts were from ALK, Abello (Denmark/Spain). The 11 common inhalant allergens were cat, dog, horse and cow epithelium; birch, timothy, mugwort and meadow grass pollen; *D. pteronyssinus*; natural rubber latex (SQ 10 HEP, ALK) including *C. herbarum* (1/20 w/v). The allergen extracts in the fungal panel were *A. alternata, Aspergillus fumigatus, Aureobasidium pullulans, Fusarium roseum, Mucor racemosus, Penicillium notatum, Phoma herbarum, Rhizobus niger, Rhodotorula rubra* (1/20 w/v) and *Acremonium kiliense (5 mg/ml)*.

Preparation of in-house fungal extracts (I)

Alternaria alternata (strain LS14/2) and *Cladosporium herbarum* (strain HRA MI) isolated from water-damaged buildings (referred as FIOH antigen in article I) were grown in mycological peptone liquid medium for three weeks at room temperature. The fungal cultures were homogenized (Ultra-Turrax, IKA, Labortechnik, Staufen, Germany) and centrifuged for 30 min at 4^{o}C 3600 x g (Heraeus Minifuge RF, Hanau, Germany). The supernatants were preparatively purified using HPLC (Source RPC15, Pharmacia, Uppsala, Sweden) with a linear acetonitrile gradient (15% to 100%). The eluted protein fractions were pooled, concentrated by Speed-Vac evaporation and the protein concentration of the extract was determined.

Enzymatic deglycosylation of fungal extracts (II)

The SPT extracts were enzymatically deglycosylated (Enzymatic Protein Deglycosylation Kit, Sigma-Aldrich Inc., St. Louis, MO, USA). The enzymes included in the kit can cleave and remove all N-linked and simple as well as complex O-linked carbohydrates from glycoproteins. The extent of deglycosylation of the extract was assessed on SDS-PAGE gels and the protein concentration measured.

Separation of glycoproteins from the extract with ConA Sepharose affinity chromatography (II)

The glycoproteins were separated from the allergen extract by running the extract through a mannan binding ConA Sepharose affinity chromatography system. Briefly the extract was diluted into the running buffer (0.01 M Tris HCl, 0.5 M NaCl, 0.01% NaN_3, pH 7.4) according to the manufacturer's instruction and applied to a column packed with ConA Sepharose (Pharmacia Biotech). The non-binding portion was collected as the extract free of glycoproteins. The column was washed and the bound part was eluted from the column with borate solution. The fractions were desalted (Sephadex G25), concentrated by Speed-Vac evaporation and the protein concentration was measured.

7.1.3. Clinical *in vivo* allergy tests (I)

SPT was performed on the volar forearm according to standard procedures at the Skin and Allergy Hospital in Helsinki. SPT was repeated in the 40 patients showing positive SPT reactivity to *A. alternata* or *C. herbarum* using both commercial extracts (ALK) and the specially prepared in-house *A. alternata* extract. The in-house SPT extract of *A. alternata* was diluted to a concentration of 100 mg/ml in phosphate-buffered saline, pH 7.4.

Conjunctival challenge was performed by an ophthalmologist according to standard procedures at the Skin and Allergy Hospital in Helsinki. Water-soluble extracts of *A. alternata* and *C. herbarum* (1/100 w/v, ALK) were used.

7.1.4. Immunochemical *in vitro* allergy tests

IgE-antibodies to *A. alternata* and *C. herbarum* and total IgE concentrations were measured by the Pharmacia CAP FEIA method (Pharmacia AB, Uppsala, Sweden) at the Skin and Allergy Hospital in Helsinki. In addition, IgE antibodies were measured by an ELISA method.

ELISA and inhibition ELISA (I and II)

IgE reactivity to fungal extracts was assessed by ELISA. Briefly microtiter plates (Immunoplate, Nunc, Roskilde Denmark) were coated with fungal extracts at a concentration of 15 μg/ml in sodium carbonate buffer (pH 9.6) overnight at 4°C. The antigen on the plate was pre-blocked with a residual coat (human serum albumin in sodium carbonate buffer), washed with PBS-Tween and incubated with the serum (diluted 1:10). The IgE was coupled to biotinylated anti-human IgE (Vector Laboratories) after which alkaline phosphatase (AP)-Streptavidin (Zymed, San Francisco) was added. The AP substrate was added and the absorbance was measured at 405 nm using an ELISA plate absorbancereader (Multiskan MS, Labsystems, Turku, Finland).

In inhibition-ELISA, the serum samples were pre-incubated with the inhibitor for one hour before they were added to the plates. All the other steps were as for ELISA.

Immunoblot (II)

IgE binding to the allergens in the fungal extracts were visualized by immuno-blotting. Briefly, the proteins in the mould extract were run on a SDS-PAGE gel (MiniProtean system, Bio-Rad) and then transferred to a polyvinyldifluoride (PVDF, Millipore) membrane in a Western Blot system (MiniBlot, Bio-Rad). The membrane was pre-blocked with 5 % milk powder in Tris buffered saline (TBS) and the serums (diluted 1:5 in TBS-milk) were added onto the membrane and incubated overnight at 4°C using a mini-blotter apparatus (Intermedica). The blot was washed with TBS-Tween and incubated with biotinylated anti-human IgE, AP-Streptavidin and finally with the AP-substrate (Color Development kit, Bio-Rad).

In inhibition-immunoblot studies, the serum samples were pre-incubated with the inhibitor for one hour before being added onto the blot. All the other steps were as for immunoblotting.

7.2. EXPERIMENTAL STUDIES

7.2.1. Animals

BALB/c female mice, 6-8 weeks of age, were used in experimental works III and IV. In addition, transgenic mice carrying the ovalbumin $(OVA)_{323-339}$-specific DO11.10 T cell receptor (OVA-TCR) were used in work IV. The mice were housed in specific pathogen free conditions and maintained on an ovalbumin free diet. All the experiments were approved by the Social and Health Care Department at the State Provincial Office of Southern Finland.

7.2.2. Microbe preparations

Stachybotrys chartarum was chosen as a model organism in order to study the airway responses to a mycotoxin producing damp building mould. The *S. chartarum* strains s. 29 and s. 72 (NRRL 6084) were grown on rice flour agar for two weeks at room temperature and for an additional two weeks at 4˚C to induce toxin production. The spores were suspended from the plates into phosphate buffered saline (PBS, pH 7.2) and counted.

An antigen suspension was prepared from the spore suspension. The spores were broken on ice using an ultrasonic processor (Branson Sonic Power Comp.). The unbroken spores were centrifuged down (3500 rpm, 4°C, Minifuge RF Heraeus) and the supernatant was filtered (0.45 μm pore size) and used as the antigen suspension. The protein concentration was determined from the suspension and used in the antibody analysis.

A yeast, *Saccharomyces cerevisiae,* was used as a control in order to be able to compare the responses of *S. chartarum* to a benign microbe. *S. cerevisiae,* also known as the bakers yeast is generally considered harmless (Anadon *et al.* 2006). The yeast (Rajamäki, Alko) was grown on malt extract agar for 48 hours in 37°C, the cells were suspended into PBS and counted. The spore- and cell-suspensions were γ-irradiated at intensity 10 kGy before use.

Preparation and analysis of mycotoxin samples (III)

Mycotoxins were extracted from the *S. chartarum* spore suspensions with aqueous 95% methanol, purified by a hexane wash and solid-phase extraction, separated by reverse-phase HPLC, identified by tandem mass spectrometry, and quantified using electrospray ionization on a quadrupole ion trap mass analyzer, as described previously (Tuomi *et al.* 1998).

Mass spectrometric analysis verified that the non-satratoxin producing strain (s. 29) did not produce any detectable amounts of either satratoxin G or H and only minor amounts of stachybotrylactam and stachybotrylactone (< 1ng/10^5 spores). On the contrary, the satratoxin producing strain (s. 72) produced satratoxin G and H, stachybotrylactone and stachybotrylactam in amounts of 4 ng, 10 ng, 8 μg and 2 μg respectively per 10^5 spores.

7.2.3. Experimental procedures

Intranasal administration of microbe preparations (III, IV)

The fungal spore and yeast cell suspensions were administered intranasally (i.n.) under light anaesthesia (using methoxyflurane in work III and isoflurane in work IV) two times a week for three weeks. The fungal spores (strain s. 29 and s. 72) and yeast cells were administered into the mouse nostrils at two different concentrations ($1x10^3$ and $1x10^5$ in

25 µl PBS in work III while $2x10^5$ spores in a volume of 50 µl were used in work IV), the control group received PBS only.

Exposure to *S. chartarum* spores in experimental asthma (IV)

Mice were sensitized by intraperitoneal (i.p) injection of 20 µg ovalbumin (Grade V; Sigma) or sham sensitized with PBS emulsified in alum on days 0 and 10. On day 2, the intranasal instillations of mould spores, yeast cells or PBS were started. The suspensions were administered intranasally (i.n.) under light anesthesia (IsoFlo, Schering-Plough Animal Health Corp., Union, New Jersey, USA) two times a week in parallel with the ovalbumin sensitization and the challenge protocol. The spore or cell concentration per instillation was $2x10^5$ in 50 µl PBS. Finally, on days 20-22, the mice were challenged with 1% aerosolized OVA for 20 minutes.

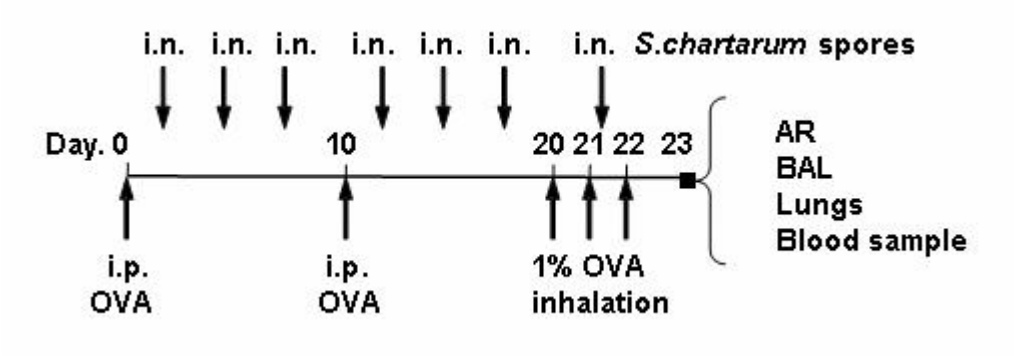

Figure 6 *Schematic illustration of the sensitization and exposure protocol.*

Sensitization of OVA transgenic mice and *in vitro* stimulation of splenocytes (IV)

Transgenic mice which express the OVA-specific TCR, were sensitized by i.p. injection of OVA emulsified in alum on days 0, 14 and 21. On day 22, single-cell suspensions of spleen cells from naive and sensitized mice were prepared in complete RPMI 1640 (Gibco, Invitrogen Corp. UK). Cells were cultured in the presence of OVA (50µg/ml) or *S. chartarum* spores (10^5) and collected after 6 hours.

7.2.4. Functional tests, sample preparation and measurements

Determination of airway responsiveness (III and IV)

Airway responsiveness was assessed using a single chamber whole body plethysmograph system obtained from Buxco (Troy, NY, USA). The airway responsiveness was measured 48 hours after the last i.n. microbe instillation (III) but 24 hours after the last OVA inhalation challenge (IV). Briefly, the unrestrained animal was placed into a chamber and exposed for 5 min to nebulized PBS and subsequently to increasing concentrations of methacholine (MCh) (Sigma) using an AeroSonic 5000 D ultrasonic nebulizer (DeVilbiss). After each nebulization, recordings were taken for 5 min. The enhanced pause (Penh) values were measured during each 5 min sequence and were expressed for each MCh concentration as the percentage of baseline Penh values following PBS exposure (% baseline Penh).

Sample collection (III and IV)

The mice were sacrificed by CO_2 asphyxiation. The blood was drained from the hepatic vein. The chest cavity was opened and the lungs were lavaged with PBS via the tracheal tube. The left lung was removed for RNA isolation. Part of the right lung was perfused with 10% formalin while the other part was embedded into Tissue-Tek O.C.T Compound and quick frozen.

BAL cytology and lung histology (III, IV)

The bronchoalveolar lavage (BAL) sample was cytocentrifuged (Cytospin, Shandon Ltd., UK) on a slide and the cells were stained with the MayGrünwald-Giemsa (MGG) stain and differentials counted under light microscopy. The lung specimens in formalin were embedded into paraffin, cut, affixed to microscope slides and deparaffinized. The slides were stained with hematoxylin and eosin (HE) stain and examined under light microscopy. The OCT lung samples were cut, stained immunohistochemically with monoclonal antibodies against CD3, CD4 and CD8 (BD Biosciences Pharmingen, San Jose, CA) and counted under light microscopy in study IV.

Real-time quantitative RT-PCR (III and IV)

Total RNA was extracted from the lungs and splenocytes using Trizol Reagent (Invitrogen, NY, USA) and was reverse transcribed into cDNA with MultiScribe Reverse Transcriptase (Applied Biosytems, Foster City, CA, USA) and random hexamers as primers according to the manufacturer's instructions. The real-time quantitative polymerase chain reaction (PCR) was performed with an AbiPrism 7700 Sequence Detector System (SDS) (Applied Biosystems), according to the manufacturer's instructions. PCR primers and probes were predeveloped assay reagents or generated by PrimerExpress version 1.5 software and ordered from Applied Biosystems. The PCR products were detected directly by monitoring the increase in fluorescence of the reporter dye, FAM or VIC™ labeled. The FAM signals of the target gene were standardized to the VIC signals of the endogenous reference gene, 18S rRNA, to control for sample loading and to allow normalization between samples. The final results were expressed as relative quantities as described in article III.

BAL cytokines and chemokines (III and IV)

The levels of cytokines (TNF-α and IL-13) and chemokines (CCL3 and CCL11) in the BAL fluid were measured with commercial kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Serum antibodies (III and IV)

Total IgE in serum was measured by ELISA. Briefly, a 96 well plate (Immunoplate, NUNC, Roskilde, Denmark) was coated with purified anti-IgE (Pharmingen, San Diego, CA, USA). The diluted serum samples were added to the plate and incubated overnight. Biotinylated anti mouse IgE and streptavidin horseradish peroxidase together with the substrate (ABTS Microwell Peroxidase Substrate System, KPL, Gaithersburg, Maryland, USA) was used to detect the bound antibody levels measured with an ELISA plate absorbancereader. Total IgE levels were calculated by comparing with known mouse IgE standards.

S. *chartarum* and OVA specific serum IgE, IgG1 and IgG2a were measured by ELISA. The plate was coated with either S. *chartarum* antigens (20 μg/ml) or OVA (2 μg/ml). The diluted serum samples were added to the plate and incubated overnight.

Biotinylated IgE, IgG1 or IgG2a (Pharmingen, San Diego, CA, USA) was added as the detecting antibody. The reaction was amplified with streptavidin horseradish peroxidase together with the ABTS substrate and detected with the ELISA plate absorbancereader. The OVA specific antibody levels were compared between the groups.

8. RESULTS

8.1. Sensitization to moulds in patients

8.1.1. Prevalence (I)

The prevalence of sensitization to moulds was evaluated in a material of 6376 patients visiting the Skin and Allergy Hospital in Helsinki for suspected allergy. Positive SPT results to *Cladosporium herbarum* were found in 2.7% of these patients. *C. herbarum* is one member of a panel of commonly used inhalant allergens. In cases of indoor air problems, a special series of fungal allergens is included. Indoor air problems were suspected in 1504 patients who therefore underwent skin prick testing also to other fungal allergens including *Alternaria alternata*. Positive SPT results to *A. alternata* were found in 2.8% of these patients. In addition, positive SPT results were found in 2.1% of the patients to *Fusarium roseum*, 1.7% to *Phoma herbarum*, 1.6% to *Mucor racemosus* and 0.9% to *Aspergillus fumigatus*.

8.1.2. Patients, clinical features (I, II)

Forty patients sensitized to either *C. herbarum* or *A. alternata* were invited for further testing and their clinical features were studied in more detail. Of these patients, 58% were found to have atopic eczema, 44% had asthma and 31% had rhinitis. In addition, the majority of these patients showed positive SPT reactions and IgE antibodies to several other fungal allergens and 75% to 80% had become sensitized to pet animal or pollen allergens. The patients were mainly high IgE responders. The mean total IgE was 1632 kU/L in the patients while the atopic controls showed a mean total IgE level of 196 kU/L with even lower values in the non-atopic controls, 61 kU/L.

The 40 mould sensitized patients were observed to also have IgE antibodies to a yeast belonging to the normal skin flora ie. *Pityrosporum ovale*. About 40% of the patients demonstrated specific IgE antibodies to the *P.ovale* SPT extract in ELISA. The non-atopic controls did not exhibit any IgE reactivity to *P. ovale*.

SPT was repeated on the 40 mould sensitized patients selected for further studies using commercial and an in-house (FIOH antigen) *A. alternata* allergen extract. The SPT results were compared and showed a good correlation between the extracts (r=0.63). The commercial and in-house fungal extracts exhibited also good correlation between the IgE reactivity results measured by ELISA for both *A. alternata* (r= 0.99) and *C. herbarum* (r=0.92). In addition, CAP-FEIA and ELISA results correlated well for both the commercial (ALK) and the in-house extracts. The correlation was r=0.91 for *A. alternata* and r=0.88 for *C. herbarum* when commercial extracts were used. When the in-house extracts were used, the correlation was 0.90 and 0.96 for *A. alternata* and *C. herbarum* respectively. The control patients, that were atopic but not SPT positive to moulds and the non-atopic individuals, had no IgE antibodies to either *A. alternata* or *C. herbarum*.

When the *in vivo* (SPT) and *in vitro* (specific IgE in sera) methods were compared no correlation or only a poor correlation was seen. *A. alternata* showed no correlation between SPT using the commercial extract and specific IgE to either the commercial (r=0.12) or the in-house extract (r=0.12). *C. herbarum* on the other hand, exhibited a poor correlation between commercial SPT and specific IgE to the commercial (r=0.47) and the in-house extract (r=0.63).

8.1.3. Characterization of cross-reacting components in mould and yeast allergen extracts (II)

In order to investigate potential cross reactivity between mould and yeast allergen extracts, ELISA and immunoblot inhibition assays were used. Sera showing IgE reactivity to all three fungi (*A. alternata, C. herbarum* and *P. ovale*) were chosen for further studies. At least two different binding patterns were seen in these patients. In some of the patients, the binding was weakly (9%) or not at all inhibited by the other fungal extracts. This was mostly seen in patients with high reactivity to one of the fungi studied but weak binding to the other fungi. On the other hand, in patients with strong or intense binding to all fungi, the binding was commonly inhibited by another fungal extract even as much as 100%. In some of these patient's sera, the IgE binding to a mould (*C. herbarum* or *A. alternata*) in the solid phase was dose dependently inhibited by addition of the yeast *P. ovale* to the

reaction and vice versa. The immunoblot revealed that the inhibited part consisted mainly of smeary bands indicating binding to carbohydrates.

The carbohydrate part was extracted from the yeast extract by ConA-Sepharose chromatography which binds the mannosyl and glycosyl residues present in mannans and glycoproteins in general. After the removal of the carbohydrate part from the yeast extract, inhibition of binding to the mould extracts was markedly reduced in the majority of those patients that previously had shown inhibition in ELISA and immunoblotting.

In addition, enzymatic deglycosylation was performed in order to specifically remove N and O-linked carbohydrates from the yeast extract. However, after enzymatic deglycosylation, cross-reactivity between the yeast and mould extracts was still seen. Part of the cross-reactivity between mould and yeast extracts was therefore due to the binding of IgE to the protein backbone in shared glycoprotein allergens.

8.2. Airway responses to mould spore exposure in experimental models

8.2.1. Characterization of airway responses to *S. chartarum* (III)

Experimental models were used to investigate the role of satratoxins in the potential health effects induced by *S. chartarum* spores. Two different strains, a satratoxin producing and a non-producing strain were used at two different concentrations (10^3 and 10^5 spores/instillation). *S. chartarum* spores, given twice a week for a total of three weeks, induced an airway inflammation characterized by a lymphocytic and neutrophilic cell infiltration in the lungs of the mice at the higher spore concentration (10^5 spores/instillation). The lower dose (10^3 spores/instillation) did not induce any inflammatory cell influx, the infiltrate was comparable to that seen in the control mice receiving PBS alone. However, there was no major difference in the airway responses evoked by the satratoxin producing compared to the non-producing strain.

S. chartarum (10^5 spores/instillation) triggered an inflammatory infiltrate seen both in the bronchoalveolar fluid as well as in the HE stained histological lung sections. Macrophages predominated in the cell infiltrate with an increase in their number, especially of neutrophils and lymphocytes, compared to the controls. Inflammatory cell clusters were seen in the lung sections of *S. chartarum* exposed mice, mostly in the lung parenchyma, but the inflammation was generally classified as mild.

The inflammatory infiltrate was accompanied by an increase in pro-inflammatory and inflammatory cytokine and chemokine gene expressions in the lungs. The pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α, were induced in the lungs with both strains when the higher spore concentration (10^5 spores per instillation) was used. The inflammatory chemokines, CCL2/MCP-1, CCL-3/MIP-1α and CCL-4/MIP-1β, which recruit inflammatory cells to site of inflammation, were also induced by both strains. However, one chemokine, CXCL5/LIX, that attracts neutrophils, was induced more strongly in mice that had received the satratoxin producing *S. chartarum* strain compared to the non-satratoxin producing strain although the difference was not statistically significant.

No signs of allergic sensitization to *S. chartarum* or induction of airway hyperresponsiveness were seen in this model. The airway responsiveness was measured with whole body plethysmography two days after the final intranasal instillation of *S. chartarum* spores. The response to methacholine, expressed as a change in the parameter Penh, was not different from Penh values seen in the control mice. Serum IgE levels were not significantly induced in *S. chartarum* exposed mice, neither were any mould specific IgE, IgG1 nor IgG2a antibodies detected in the serum.

8.2.1. Modulation of experimental asthma by *S. chartarum* exposure (IV)

In order to investigate the role of the immunological status in relation to the airway responses induced by exposure to *S. chartarum*, a well known allergic asthma model was used together with exposure to spores. The allergic asthma model on its own induces a Th2 type of allergic response with airway responsiveness, eosinophilia in the lung and IgE

production against the sensitizing allergen. The intranasal exposure to *S. chartarum* on the other hand as shown in work III induces a neutrophilic inflammation with upregulated levels of pro- and inflammatory cytokines and chemokines but no change in airway responsiveness. When these two models were combined the inflammation was markedly more severe although the airway responsiveness was decreased compared to the allergic asthma model. Intranasal exposure to *S. chartarum* spores in conjuction with sensitization and airway challenge to the allergen strongly enhanced the degree of airway inflammation (Figure 7).

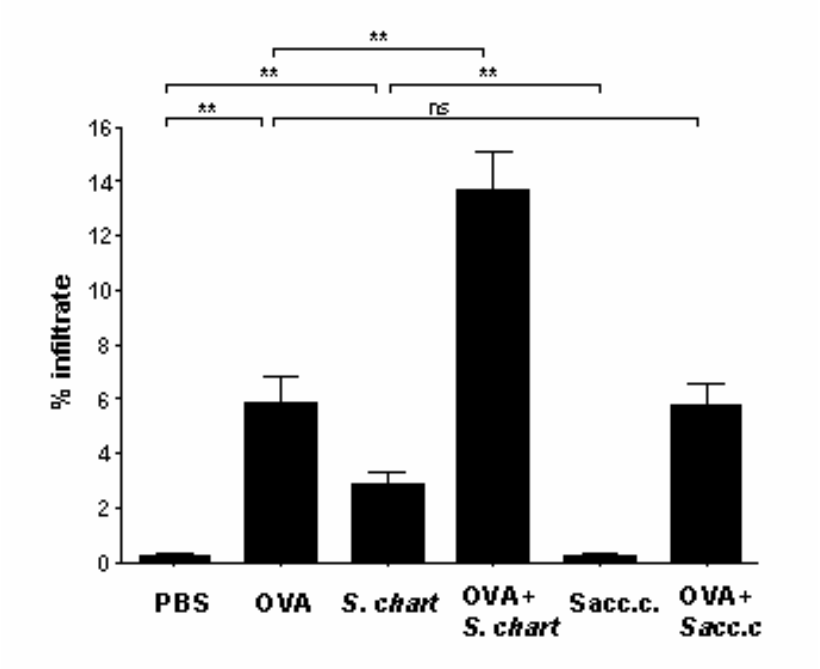

Figure 7 *Percent of inflammatory infiltrates in mice lungs. PBS=control; OVA=OVA sensitized/challenged; S. chart. = S. chartarum exposed; OVA+S.chart= OVA sensitized/challenged and S. chartarum exposed; Sacc.c= mice exposed to bakers yeast (Saccharomyces cerevisiae) and OVA+Sacc.c = OVA sensitized/challenged and Sacc.c exposed. Results are expressed as mean ± SEM for each group. Significant differences between the groups at **p<0.001, ns=not significant.*

Sensitization and airway challenge to OVA induced the features associated with allergic asthma. The OVA sensitized/challenged mice showed increased levels of Th2 type cytokines such as IL-4, IL-5 and IL-13 in the lungs. The airway responsiveness to inhaled methacholine was increased and IgE antibodies to OVA were found in sera. Histological lung sections revealed the presence of inflammation mostly in the peribronchial areas and the BAL showed that the eosinophils predominated in the infiltrate.

In ovalbumin sensitized/challenged mice which were also exposed to *S. chartarum* spores, the total number of inflammatory cells was markedly increased and the cell population had changed. The eosinophils still predominated as would be expected in this allergic asthma model but at the same time increased numbers of neutrophils and lymphocytes were seen in the inflammatory influx. The histological lung sections revealed an inflammation which was seen both peribronchially and perivascularly. In addition intense inflammation with granulomatous infiltrates and multinucleated giant cells was seen in all compartments of the lung.

The intense inflammation in the lungs of OVA sensitized/challenged mice exposed to *S. chartarum* was also seen as a strong induction in the levels of inflammatory and especially the proinflammatory cytokines and chemokines. TNF-α was markedly induced both at the mRNA level in the lungs and at the protein level in BAL. The pro-inflammatory cytokine, IL-1β, was also induced followed by increased levels of the inflammatory chemokines CCL2/MCP-1 and CCL3/MIP-1α and their receptors CCR5 and CCR3. MIP-2, which specifically recruits neutrophils to sites of inflammation in mice, was also induced as was the levels of one of its receptors CXCR2.

The typical Th1 and Th2 cytokine mRNA expressions revealed mixtured features in mice sensitized/challenged to OVA and exposed to *S. chartarum*. Exposure to *S. chartarum* in OVA sensitized/challenged mice showed a slight tendency to decrease the levels of Th2 cytokines compared to the OVA sensitized/challenged mice while it slightly increased the levels of IFN-γ and IL-12p40 compared to either model on its own.

The airway responsiveness to methacholine was increased in OVA sensitized/challenged mice but exposure to *S. chartarum* reduced the airway responsiveness to the same level seen in *S. chartarum* exposed alone. IgE antibody was produced to OVA in the OVA sensitized/challenged mice but the levels of the antibody were not affected by exposure to *S. chartarum*.

In order to clarify the role of the Th2 environment on the induction of the inflammation, splenocytes were isolated from OVA sensitized mice and stimulated *in vitro* with *S. chartarum* spores. *S. chartarum* markedly increased the production of TNF-α and IL-1β mRNA expressions in splenocytes from OVA sensitized mice. The increase was more than two times higher than the expression seen in splenocytes from naive, non-sensitized mice.

9. DISCUSSION

Exposure to increased levels of allergenic or toxic fungal spores has been considered to be one of the major sources of the health problems linked to water damaged buildings. In this thesis, different approaches to study the health effects associated with exposure to moulds have been made. The prevalence of sensitization to moulds was studied in patients attending the hospital for suspected allergy and was found to be low and highly dependent on the atopic status of the exposed. In addition, part of the IgE binding to the mould extract was shown to be due to cross-reactivity between moulds and yeasts. Sensitization to moulds could therefore only explain part of the symptoms experienced in damp buildings and therefore another approach using experimental models was taken. We studied the airway responses to a toxin-producing damp building mould, *Stachybotrys chartarum,* in an experimental model and found that it was able to induce airway inflammation. Further, since individual variation in the symptoms has been observed upon exposure, we hypothesized that the immunological status may play a role. We exposed mice to *S. chartarum* in parallel to sensitization to an allergen and found that the degree of airway inflammation was markedly enhanced.

9.1. Sensitization to moulds in patients

The prevalence of sensitization to moulds varies slightly, depending on the patient material, climate and extracts used. The prevalence of sensitization to two of the most commonly tested moulds in Finland was low (2.7% to *Cladosporium herbarum* and 2.8% to *Alternaria alternata*). In Europe, in general, the prevalence of sensitization to *C. herbarum* and/or *A. alternata* in atopic subjects has been found to be on average 9.4% but the prevalence does vary between countries with a rather wide range of frequencies e.g. 20% in Spain and 3% in Portugal (D'Amato *et al.* 1997). In the general population, the median prevalence of specific IgE to *C. herbarum* was 2.4% in a large European multicenter study (Burney *et al.* 1997) while positive skin test results to *A. alternata* in a large US study were found in 3.6% of the sample (Gergen *et al.* 1987).

The prevalence has been shown to be highly dependent on the population of patients examined with higher frequencies being detected in atopic populations (Nolles *et al.* 2001). Atopic individuals commonly have high total IgE serum levels with weak IgE reactivity to multiple allergens. Reactivity to several allergens in atopic individuals indicates that they have undergone multiple sensitization events due to their increased immunologic responsiveness (Aalberse *et al.* 2001). In our study, the majority of the patients were atopics, indicating that the frequency of sensitization to moulds may be even lower in the general population. In addition, it shoud be noted that the patients examined for positive SPT results to the whole fungal allergen panel comprised of selected individuals due to indoor air problems. Therefore, although this subpopulation was selected nonetheless the prevalence of positive SPT results to fungi remained low. The low prevalence of specific IgE to fungi in the subpopulation suggests that the prevalence might be low in the general population.

Sensitization to moulds seems to be rare but there are concerns that sensitized individuals may be at an increased risk of developing allergic asthma (Bush and Prochnau 2004, Zock *et al.* 2002). Epidemiological studies have pointed to an association between mould sensitization and asthma, particularly for sensitization to *Alternaria alternata* (Neukirch *et al.* 1999). In particular, the more severe form of asthma requiring emergency ward treatment has been associated with sensitization to fungi (Black *et al.* 2000, Denning *et al.* 2006). A European multicenter study showed that with increased severity of asthma, the frequency of sensitization to moulds was higher in all regions (Zureik *et al.* 2002). This was clearly shown for the fungal species *Cladosporium herbarum* and *Alternaria alternata* included in the test. A positive association was also seen between sensitization to house dust mite (*Dermatophagoides pteronyssinus*) and asthma severity but no association was seen for patients sensitized to pollen or cat allergens.

Reliable test methods and allergen extracts are needed to guarantee an accurate diagnosis of fungal allergy. The fungal allergen extracts used in the clinic today are still not well standardized (Esch 2004). The allergen composition may vary in extracts due to different strains, growing conditions and extraction methods used. Thus, the mould extracts show varying degrees of sensitivity and specificity. In our study, the correlation

was good between the in-house and commercial extracts when the same methods were used. On the contrary, the skin prick (*in vivo)* and ELISA (*in vitro)* allergy test results did not correlate, this being especially the case for the *A. alternata* extracts. Commonly when reliable and standardized extracts are used, there is an 85-95% correlation in the results between SPT and IgE measurements in sera (Demoly *et al.* 2003). SPT is the gold standard method, and considered to be more reliable and specific than IgE measurements (Dolen 2003). The poor correlation between the methods might be due to poor quality and unknown biological activity of the mould allergen extracts used. On one hand, positive skin prick tests results may be due to unspecific agents in the extract inducing histamine release which is not IgE mediated. On the other hand, the ELISA methods may fail to detect low levels of mould specific IgE in sera. The vast majority of the specific IgE has been speculated to be bound to mast cells and Langerhans cell in the skin leaving only trace amounts in sera.

9.2. Components in mould and yeast allergen extracts cross-reacting for IgE binding

In allergic patients, a clustering of IgE responses to fungal allergens has often been observed (Potter *et al.* 1991). It is more common to show reactivity to several fungal species than for an individual to be sensitized to only one specific fungus (Mari *et al.* 2003). Reactivity to multiple allergens may be due to a susceptibility or predisposition in the individual to produce IgE to many different inhalant allergens or due to cross-reactivity between the allergens (Aalberse *et al.* 2001).

One finding of special interest was that patients sensitized to moulds often also show reactivity to a yeast present in the normal skin flora *Pityrosporum ovale*, synonymous to *Malassezia* sp. (Nissen *et al.* 1998). It has been speculated that in atopic eczema where the skin barrier is reduced due to itching and scratching of the skin, growth of the saprophytic yeast *Malassezia* sp. might be favoured (Scheynius *et al.* 2002). Increased levels of *Malassezia* growth on the skin might induce the production of specific IgE antibodies to the yeast in these patients (Jensen-Jarolim *et al.* 1992). Of our mould sensitized patients, 58% suffered from atopic eczema and about 40% showed IgE reactivity to *P. ovale*.

Potential cross-reactivity between the fungal extracts was studied and it was demonstrated that mould and yeast extracts shared glycoproteins that cross-reacted for the IgE binding in the sera of mould sensitized patients.

It is well known that moulds share some common allergens and species can cross-react but also yeast species have shown cross-reactivity (Bisht *et al.* 2004, Kanbe *et al.* 1997). Cross-reacting glycoproteins between mould and yeast allergen extracts have not however previously been taken into account in evaluations of sera from atopic eczema patients. Cross-reactivity has been observed for certain allergens between part of the moulds and yeasts (Hemmann *et al.* 1997). *Aspergillus fumigatus* and *Candida albicans* share allergenic enzymes some of which seem to play a role as fungal virulence factors (Lopez-Ribot *et al.* 2004). The inhibited or cross-reacting component in our study was not however a specific band seen on the immunoblot. On the contrary, diffuse bands of size of 60-200 kDa indicated that shared high molecular weight glycoproteins may be present in both mould and yeast extracts. It is known that yeasts contain allergenic mannans in their cell wall but much less is known about the carbohydrates making up the mould cell wall. Moulds, especially their cell walls, do contain carbohydrates as well and some of the mould allergens have been shown to be glycoproteins. The major allergen from *Alternaria alternata* (Alt a1) and *C. herbarum* (Cla h2) are glycoproteins (Portnoy *et al.* 1998, Sward-Nordmo *et al.* 1988). The known glycoprotein allergens Alt a1 and Cla h2 are however in the molecular weight range of 20-50 kDa in size.

The cross reactivity seen between mould and yeast allergen extracts (II) implies that care should be taken when diagnosing allergy to moulds. Our results indicate that individuals suffering from atopic eczema with specific IgE antibodies to *P. ovale* may also show positive SPT results to moulds due to shared IgE binding glycoproteins in the allergen extracts. Therefore some of the patients diagnosed as being sensitized to moulds may indeed not be sensitized or allergic to moulds, but instead the primary sensitizer might be the yeast. In our study, the yeast tended to show stronger inhibition of IgE binding to moulds than vice versa indicating that *P. ovale* might be the primary sensitizer. The role of antibodies to the saprophytic yeast on the skin in association with the pathogenesis of atopic eczema is not clear (Faergemann 2002). Atopic eczema is a chronic

inflammatory skin disease where allergens are thought to be involved in the maintenance of the disease. High total IgE levels and antibodies to environmental allergens are associated with this disease (Broberg *et al.* 1992). The role of antibodies to *P. ovale* is hypothesized to be a contributing or triggering factor in the disease where there may be a reduced skin barrier. The cross-reactivity between moulds and yeasts for the IgE binding, might therefore exaggerate atopic eczema upon exposure to moulds. These factors need to be taken into account when diagnosing mould allergy, especially if the patient also suffers from atopic eczema.

Taken together these results suggest that mould allergy seems to be rare. The clinical significance of the positive results to fungal allergen extracts can further be questioned due to the cross-reactivity seen between extracts. Mould allergy appears to explain only part of the symptoms experienced by occupants of damp buildings, suggesting that other non-allergic factors and responses may be involved. We therefore chose one specific damp building mould that frequently has been implicated in the literature as a potential inducer of health problems, *Stachybotrys chartarum*, and studied the airway responses to this mould in experimental models. With the chosen mould, other non-IgE mediated mechanisms related to exposure to fungi were investigated.

9.3. Airway responses to exposure to *S. chartarum* spores in an experimental model

Stachybotrys chartarum is a damp building mould that is recognized for its ability to produce highly toxic secondary metabolites such as macrocyclic trichothecenes under suitable conditions (Jarvis *et al.* 1998). There are several macrocyclic trichothecenes, but satratoxins have been shown to be some of the most potent (Yang *et al.* 2000). A satratoxin producing and a non-producing strain of *S. chartarum* in two different concentrations were used in study III in order to study the effects of the microbe in the airways of mice.

Intranasal instillations of spores from both the satratoxin producing and the non-producing strain of *Stachybotrys chartarum* induced inflammation in the airways with

almost no difference between the strains. Although *Stachybotrys*-produced trichothecenes, satratoxins, have been shown by others to induce changes in the phospholipid composition of pulmonary surfactants (Mason *et al.* 1998, Rand *et al.* 2002) as well as the erythrocyte accumulate in the lungs (Rao *et al.* 2000, Yike *et al.* 2002), it is possible that other components of the secondary metabolites induced the airway inflammation seen here. Similar results were recently reported in a study using two different *Stachybotrys* strains, one of which was a trichothecene producer and the other an atranone producer (Flemming *et al.* 2004). Both strains triggered similar kinds of airway responses according to the cell population seen in BAL when low spore doses were used. Other biologically active components in addition to the trichothecenes might be involved, especially atranones and hemolysins have gained much attention lately (Miller *et al.* 2003). Atranones might be potential inducers of inflammation via activation of macrophages (Nielsen *et al.* 2002) while hemolysins on the other hand might play a role in causing haemolytic effects such as haemorrhage (Vesper and Vesper 2002).

In addition there are other, non-toxic components that might trigger or enhance the inflammatory effects in the airway. Fungal cell wall constituents such as β(1→3)-D-glucan and the recently recognized proteases are potential agents inducing inflammation (Rylander and Lin 2000). Fungal spores or conidia as well as hyphae from the pathogenic fungi *Aspergillus fumigatus* have been shown to bind to TLR2 and 4 and trigger the induction of pro-inflammatory cytokines in macrophage cell lines (Bellocchio *et al.* 2004). In the case of the pathogenic yeast *Candida albicans*, the component to which TLRs 2, 4 and 6 bind is a yeast cell wall mannan, phosholipomannan (Jouault *et al.* 2003). On the other hand, β-glucan has recently been found to be a potent inducer of the innate immune system also via other receptors such as Dectin-1 (lectin receptor for β-glucan) (Gantner *et al.* 2003). In addition it has been found that the proteases produced by the fungi may play an important perhaps adjuvant roles, e.g. proteases have recently been found to potentially favour the uptake of fungal spores into host cells (Reed and Kita 2004). A protease produced by *Stachybotrys*, called stachyrase A, is known to cleave structurally important proteins such as collagen in the lung environment (Kordula *et al.* 2002).

In order to mimic long term exposure the fungal spores were installed twice a week for a total three weeks. Two concentrations were used, 10^3 and 10^5 spores per instillation, these being chosen based on previous experience. By taking into account the lung volumes and breathing frequencies of mice in comparison to human lungs, estimations were made of potentially inhaled spore concentrations (Nikulin 1999). With intratracheal instillations, it is usual to assess the acute responses and concentrations ranging from $5x10^2$ to $5x10^5$ of *Stachybotrys* spores have been administered to mice (Flemming *et al.* 2004, McCrae *et al.* 2001). Instillations of spore concentrations above 10^5 are considered too high in relation to real-life exposure (Miller *et al.* 2003). However, with intranasal instillations, the exact amount of spores reaching the lungs cannot be estimated precisely. Part of the spores will stay in the nose and some are swallowed (Southam *et al.* 2002). It has been estimated that only 1/10 or perhaps as little as 1/50 of the spores ultimately reach the lungs after intranasal instillation (McCusker *et al.* 2002). With intranasal instillations performed repeatedly however, the variation between the mice and groups will be minimized and comparisons between the groups can be made.

There were no signs of allergy or sensitization to *S. chartarum* using this model. *Stachybotrys* spores or its allergenic extracts may also induce sensitization in experimental settings (Viana *et al.* 2002). Specific antibodies to *S. chartarum* have been found in patient studies and some of the allergens has been characterized (Barnes *et al.* 2002, Karkkainen *et al.* 2004). However, the airways in general or the intranasal instillations used here is not the most effective route to induce sensitization in experimental settings. Furthermore, the airway responsiveness did not increase after *S. chartarum* exposure in this model. More frequent instillations or alternatively sensitization (i.p. injections) and airway challenge using more allergenic spores might have been needed to induce allergic asthmatic reactions to mould spores and AHR.

9.4. Airway inflammation in OVA allergic mice exposed to *S. chartarum*

Exposure to damp buildings evokes a variety of poorly defined symptoms which are not easy to diagnose. Individuals seem to respond differently to the same exposure, implying

that the immunological status might play a role (Kauffman and Van Der Heide 2003). Exposure to a damp building mould might affect differently an individual with unbalanced immunity such as an atopic person. To study this phenomenom, a well known allergic asthma model was used together with exposure to *S. chartarum* (IV). When mice were both sensitized/challenged to an allergen (OVA) and exposed to *S. chartarum* spores, the extent of inflammation in the lungs was markedly increased, evidence of a clear interaction between the responses.

The enhanced inflammation seen in work IV suggests that the lungs were more susceptible to exposure to *S. chartarum* when the mouse had been sensitized to an allergen. As previously shown, without allergic asthma, *S. chartarum* was normally cleared from the lungs through the phagocytosing actions of macrophages and neutrophils, inducing only a mild inflammation (III). However, a severe inflammation was seen when allergen sensitization was employed together with exposure to *S. chartarum* (IV). The histological lung sections showed intense inflammatory infiltrates that were not seen in any of the individual models. In order to determine whether the Th2 environment had enhanced the inflammation to *S. chartarum*, OVA sensitized splenocytes were stimulated with *S. chartarum in vitro*. Th2 primed splenocytes did induce higher pro-inflammatory cytokine mRNA compared to the non-primed naive cells when stimulated with *S. chartarum*. The results imply that in an allergic Th2 type of environment, exposure to *S. chartarum* evokes a more severe inflammation.

The results also suggest that if the lungs are experiencing an ongoing Th2 type inflammation, then they may be more vulnerable and incapable of defending themselves against new invaders such as microbes. This can lead to the gathering of microbes into inflammatory clusters in macrophages which are typical of a disease condition called allergic alveolitis. The inflammation seen in the histological lung sections (IV) contained multinucleated giant cells and granuloma formation as is also seen in patients with allergic alveolitis. Extrinsic allergic alveolitis develops when intracellular particles or micro-organisms that the cell has not been able to destroy are gathered into activated macrophages which then become transformed into epitheloidal cells. The epitheloidal cells secrete increasing amounts of TNF-α in order to destroy the agent but this leads to

increased inflammation and the formation of giant cells and granulomatous inflammation in the lung (Roitt *et al.* 2001). Exposure to *S. chartarum* has been shown also to induce granuloma formation in mice lungs in a direct manner (Rand *et al.* 2003). However, in our model, sensitization/challenge to OVA was needed to induce this condition after exposure to *S. chartarum*.

Surprisingly, the airway hyperresponsiveness (AHR) was reduced in OVA sensitized/challenged mice also exposed to *S. chartarum*. Microbes or microbial products, especially bacteria, have been shown to modulate the airway inflammation and AHR in allergic mice (Hopfenspirger and Agrawal 2002). The decrease or increase in AHR has correlated with the degree of inflammation related to eosinophils and to the pulmonary levels of mediators such as IL-13 and IL-5 (Instanes *et al.* 2004). In our study, the inflammatory response in the lung was not reduced. We speculate that the effect on AHR might be due to the slight decrease in IL-13 and to some extent to the other Th2 cytokines (Wills-Karp *et al.* 1998). Another possibility is that the whole body plethysmography (Buxco system) used to measure airway responsiveness in conscious unrestrained mice, was not sensitive enough to recognize the airway changes occuring here. A parameter called enhanced pause (Penh) is calculated based on the pressure in the chamber in relation to breathing patterns of the animal (Hamelmann *et al.* 1997). However, it is still unclear whether this parameter actually reflects airway responsiveness and changes in lung function (Albertine *et al.* 2002, Bates and Irvin 2003, Lundblad *et al.* 2002). Therefore our results related to airway responsiveness have to be interpreted with caution. A more sensitive invasive method measuring airway resistance in anaesthetized animals might be needed in order to confirm if the decrease in Penh indeed is related to a change in airway function.

These results imply that sensitized individuals may be at a higher risk of developing health problems if they are exposed to moulds such as *Stachybotrys chartarum*, compared to non-sensitized individuals. The health effects associated with damp buildings might be highly dependent on the immunological status of the exposed. This is an interesting finding that may shed some light on why the symptoms associated with exposure to damp buildings seem to exhibit such inter-individual variation. Patient studies in damp buildings

have indeed revealed a higher risk of respiratory symptoms in atopic children (Verhoeff *et al.* 1995). The risk increased with maternal asthma pointing to a potential difference in susceptibility to exposure in atopic children (Belanger *et al.* 2003). It may further be hypothesised that individuals suffering from other diseases disturbing the normal immune response (allergy, rheumatic diseases, immunosuppression) might be more susceptible to the inflammatory effects of moulds. There are some indications that this might be the case i.e. a recent report described a clustering of rheumatic type of diseases in moisture damaged work-places (Luosujarvi *et al.* 2003, Myllykangas-Luosujarvi *et al.* 2002). Healthy individuals on the other hand, are probably able to handle exposure to at least low levels of mould spores without suffering any serious health problems and symptoms. However, if the exposure is long-lasting and the spore levels are high, then health problems may arise even in healthy individuals.

Although our results rely on experimental exposure studies only, it is realistic to believe that exposure to high levels of either allergenic or potentially toxic moulds might induce health problems in susceptible individuals. High levels of airborne fungal spores can occasionally be found in damp buildings and be responsible for health problems. In these cases, although allergy to moulds does seem to be rare, attention should be paid to sensitized individuals who may develop allergy and worsening of asthma symptoms. In addition, if moulds that are known to produce potentially harmful secondary metabolites are found in large amounts in the building, especially if high spore levels containing toxins are found in the air, then remedial actions need to be taken. Exposure to high levels of the damp building mould *Stachybotrys chartarum* should be avoided. Although the trichothecene, satratoxin, did not enhance the airway inflammation seen here in relation to the strain not producing satratoxin there is a body of evidence indicating that the mould is not innocuous. *Stachybotrys*, even without satratoxins, clearly induced a more severe pulmonary inflammation in mice compared to a benign microbe, *Saccharomyces cerevisiae*.

9.5. Future directions

In the future improved and standardized allergen extracts will be needed in order to diagnose allergy or sensitization to moulds accurately. The diagnosis of asthma and other hypersensitivity disorders depends on the availability of reliable allergy tests. Mould allergen extracts are a problem for the manufacturers of these products as well as for the clinician performing the allergy tests. Many attempts have been made in order to improve the quality of the extracts. Pure recombinant fungal allergens have shown promising results in allergy diagnosis, but much more work is needed to identify all the moulds and the allergens that might induce sensitization in susceptible individuals. Reliable allergy tests would, at least, help to identify those sensitized individuals.

As a model organism, to study the non-allergic airway responses to a damp building mould, *Stachybotrys chartarum* was used in the second part of the thesis. The results from the experimental model revealed that *S. chartarum,* may be considered a harmful mould if an individual is exposed to it at high concentrations. Many of the health problems linked to exposure to *S. chartarum* have been considered to be attributable to the produced trichothecenes. However, recent findings have recognized many other components in the cell wall that may be extremely important. More detailed studies into the mould wall components could reveal the most potent components involved in the induction of inflammation. One intriguing question concerns the interaction and potential co-operation between these components and metabolites i.e. can they facilitate each other's actions. It is known that microbes in general and pathogens in particular have developed sophisticated ways to avoid elimination by the host's defence system.

The mechanism behind the development of enhanced airway inflammation in allergic mice exposed to *S. chartarum* needs to be investigated in more detail. It was of interest to note that the airway inflammation in these mice resembled the disease condition extrinsic allergic alveolitis. Extrinsic allergic alveolitis has occasionally been seen in patients exposed to high levels of moulds. It is more commonly found in farmers handling mouldy hay but also some cases associated with damp buildings have been reported (Schwarz *et al.* 2000, Trout *et al.* 2001). Detailed studies are needed to identify the potential cells involved in the development of the inflammation. If the inflammation indeed resembles

extrinsic allergic alveolitis, then macrophages might play a major role. Stimulation of macrophages with *S. chartarum* in a Th2 environment might give initial clues to the process. In addition, since both a Th2 type of response and an innate response are induced, the interaction and the early phase of regulation of these responses might be the key inducers determining the final effect. A more detailed knowledge about the mechanism behind the inflammation could help to identify and potentially to treat some of the symptoms in order to dampen the overall effect. For example, if the allergic symptoms could be treated or dampened, the overall effect of the potential inflammation might be reduced.

In this thesis the airway responses to one damp building mould were studied but more attention should be paid to the other microbes, also non-toxic micro-organisms, present in the damp building and their possible interactions. A synergistic interaction between bacteria (*Streptomyces*) and *Stachybotrys* has been observed in macrophage cell lines inducing enhanced levels of inflammatory markers such as IL-6 (Huttunen *et al.* 2004, Penttinen *et al.* 2005). Many different microbes including bacteria and moulds with potential health effects may be living on damp material. Our present knowledge about microbes indicates that indeed a variety of health effects may occur, depending on the mould species and their numbers. Microbes may trigger allergic, inflammatory, immunostimulatory or immunosuppressive health effects. The final outcome of health problems experienced by occupants of damp buildings can therefore be a mixture of all of these responses. At the moment one microbe at a time has been tested for its potential health effects and this is the best way to start to assess their effects. In the future however, all the microbes found in the damp building will need to be taken into account when evaluating the potential health effects.

There are still many controversies in the field of damp building moulds and the health effects linked to these buildings. Fungi and their role as potential inducers of health problems in water damaged buildings have frequently been debated in the media during the last ten years. Different opinions exist, even among clinicians and therefore patients with health problems assumed to be related to a damp building are treated very differently. This is understandable especially since there are few, if any, markers to be used to

measure exposure in patients. Nonetheless, it is important to take into account the fact that there are likely individual differences in the health effects experienced in damp buildings. Therefore every damp building case should be treated as unique, depending on the microbes found and the patients involved.

10. CONCLUSIONS

The prevalence of sensitization to two commonly tested fungal species, *Cladosporium herbarum* and *Alternaria alternata* was found to be low in a Finnish population. Sensitization to fungi was mostly observed in patients suffering from atopic diseases such as atopic dermatitis, asthma and rhinitis. The patients were commonly sensitized to other inhalant allergens as well, such that specific sensitization to moulds seems to be of minor clinical importance.

Shared IgE binding glycoproteins in mould and yeast allergen extracts were detected in some of the mould sensitized patients sera. The majority of the patients sensitized to moulds suffered from atopic eczema. Atopic eczema patients, on the other hand, have often been observed to have antibodies in their sera to a yeast found on the skin *Pityrosporum ovale*. In this study, cross-reactivity between the mould and the yeast allergen extracts used in skin prick testing was observed. The cross-reactivity was found to be due to IgE binding to shared glycoproteins in the extracts. This should be taken into account if patients are considered to suffer an allergy to moulds, in order to avoid false positive results, especially in atopic dermatitis patients.

Stachybotrys chartarum is one of the most widely studied moulds associated with damp buildings due to its capability to produce a variety of highly toxic trichothecenes. Exposure to *Stachybotrys chartarum* induced an airway inflammation in mice. The airway inflammation was characterized by a neutrophilic and lymphocytic lung infiltrate. The trichothecenes, satratoxin G and H, did not appear however to be responsible for the inflammation induced in the airways measured here. Other components of the spores seem to also play a role. This study has added new useful information concerning the airway inflammation and the potential components involved in the airway inflammation induced by *Stachybotrys chartarum*.

The immunological status of the exposed individual seems to play an important role in determining the health effects and this has not been previously recognized. When mice were both sensitized/challenged to an allergen (OVA) and exposed to *Stachybotrys* spores,

then the inflammation detected in the lungs was markedly enhanced. The histological lung sections showed intense inflammatory infiltrates, evidence of a strong interaction between the responses. These results suggest that an atopic person might be at higher risk to develop airway inflammation or symptoms from exposure to a damp building compared to a non-atopic individual.

The results in this thesis indicate that exposure to certain moulds indoors might trigger health effects in susceptible individuals. One of the potent candidates to induce health problems in water damaged buildings is *Stachybotrys chartarum*. The symptoms experienced by some occupants of these buildings are most probably not attributable to IgE mediated allergy. In this thesis we have found new explanations, by using experimental models, for an inflammatory reaction in the airways induced by moulds.

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