Activation of lung epithelial cells by group 2 mite allergens

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Till mamma och pappa
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Abstract

Throughout many parts of the world house dust mites (HDM) are considered as a major source of indoor aeroallergens and they are powerful inducers of allergic diseases. Proteolytic HDM allergens are recognised as being able to directly activate respiratory epithelial cells and thereby actively participate in innate immune responses. Although several major HDM allergens lack proteolytic activity, their possible ability to similarly interact with epithelial cells is not known.

The overall aim of this thesis was therefore to elucidate if and how major non-proteolytic group 2 allergens from different mite species interact with respiratory epithelial cells. The effects of the structurally related Der p 2, Der f 2 and Eur m 2 from different HDM species as well as the storage mite allergen Lep d 2 were studied in vitro using human respiratory epithelial cells. Also the non-proteolytic, but structurally dissimilar, Fel d 1 from cat, Can f 2 from dog, Bet v 1 from birch and Phl p 5a from timothy were studied.

In this thesis evidence that major group 2 mite allergens activate bronchial epithelial cells is presented. Following allergen exposure the secreted amount of the inflammatory mediators G-CSF, GM-CSF, IL-6, IL-8, MCP-1, MIP-3α and sICAM-1 was increased. Surface expression of ICAM-1 was also increased following allergen exposure. Moreover, Fel d 1 and Can f 2 induced secretion of the same mediators from bronchial epithelial cells, representing two additional protein structures being able to directly induce cell activation. In experiments using specific inhibitors and siRNA transfection, it was shown that the mite allergens engage TLR4 and activation through MyD88, MAPK and NF-κB signal transduction pathways.

In conclusion, the novel findings in this thesis provide knowledge on how major aeroallergens, in addition to their ability to provoke specific adaptive immune responses, may aggravate a respiratory airway disease by adjuvant-like activation of inflammatory responses in bronchial epithelial cells. This differs from previously reported allergen-induction of epithelial cells by the clear independency of proteolytic activation.
**SELECTED ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Bet v 1</td>
<td>Betula verrucosa allergen 1</td>
</tr>
<tr>
<td>Can f 2</td>
<td>Canis familiaris allergen 2</td>
</tr>
<tr>
<td>Der p 2</td>
<td>Dermatophagoides pteronyssinus allergen 2</td>
</tr>
<tr>
<td>Der f 2</td>
<td>Dermatophagoides farinae allergen 2</td>
</tr>
<tr>
<td>Eur m 2</td>
<td>Euroglyphus maynei allergen 2</td>
</tr>
<tr>
<td>Fel d 1</td>
<td>Felis domesticus allergen 1</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HDM</td>
<td>House dust mite</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Lep d 2</td>
<td>Lepidoglyphus destructor allergen 2</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte-chemotactic protein</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NHBE</td>
<td>Normal human bronchial epithelial</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>Phl p 5a</td>
<td>Phleum pretense allergen 5a</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>T&lt;sub&gt;C&lt;/sub&gt;</td>
<td>T cytotoxic</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;</td>
<td>T helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
</tbody>
</table>
PAPERS IN THIS THESIS

This thesis is based on the papers listed below, which will be referred to in the text by their corresponding roman numerals.

I. Österlund C, Grönlund H, Polovic N, Sundström S, Gafvelin G and Bucht A.
   The non-proteolytic house dust mite allergen Der p 2 induce NF-κB and MAPK dependent activation of bronchial epithelial cells.

II. Österlund C, Grönlund H, Gafvelin G and Bucht A.
   Non-proteolytic aeroallergens from mites, cat and dog exert adjuvant-like activation of bronchial epithelial cells.

III. Österlund C and Bucht A.
   Activation of bronchial epithelial cells by the house dust mite allergens Der p 2 and Der f 2 is mediated through TLR4 signalling while activation by the cat allergen Fel d 1 is mediated through TLR2.
   *Submitted*

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INTRODUCTION

The normal response to innocuous inhaled agents originating from e.g. mites, animal dander, pollen, cockroaches or mould is tolerance. For patients who suffer from allergic diseases, exposure can however be far from harmless. Allergy is a rather wide term, covering adverse immune reactions to a wide range of substances that can be administered through numerous exposure routes. In its strictest meaning, type I allergy is defined as an immunoglobulin (Ig E) antibody mediated hypersensitivity disease of the immune system, which misinterprets *per se* harmless proteins (allergens) as disease agents. It requires both sensitisation and a later encounter of the same allergen. Common IgE mediated allergic reactions are allergic rhinoconjunctivitis, allergic asthma, gastrointestinal allergy and anaphylaxis.

The prevalence of allergic diseases has been shown to increase over several decades, especially in industrialised countries [1]. Although genetic predisposition plays a big part in the development of allergic diseases, it cannot alone be responsible for the rising trend. Several other factors that might contribute have been proposed, for instance obesity [2], increased indoor allergen exposure [3-6], decreased number of siblings [7-9] and lack of severe or repeated infections [10, 11]. The hygiene hypothesis first proposed in 1989 [8], which suggests that as our living environment becomes cleaner the reduced exposure to microbes and parasites in early childhood has resulted in disordered regulation of the immune system and in increased prevalence of inflammatory disorders, is however heavily disputed.
**Immune responses in the lung**

The host response against inhaled substances is complex, involving an integrated response with both innate and adaptive reactions. The innate immune system is non-specific and comprises the basic immune mechanisms that an individual is born with. The airway epithelium is the first site of contact with the inhaled agent and constitutes both a physical and chemical barrier. Many cell types may aid in the clearance of the invader and in the recruitment of immune cells to the site of infection. Essential in the activation of immune cells are the so called pattern recognition receptors (PRRs), which recognise a wide range of pattern-associated molecular patterns (PAMP), such as lipopolysaccharide (LPS) and β-glucans on the invading pathogens [12, 13]. The human toll-like receptors (TLRs) discovered in 1997 [14], is the most well characterised group of PRRs. PAMP recognition triggers downstream signalling pathways, including nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, which induce production and secretion of proinflammatory cytokines and chemokines that may either propagate or suppress the inflammatory response.

The phagocytic macrophages and dendritic cells are the first inflammatory cells to recognise pathogens. Macrophages are a source of a wide range of inflammatory mediators and they promote the recruitment of neutrophils, which are considered the hallmark of acute inflammation, to the lung. They can destroy the invading pathogen by phagocytosis as well as initiate an oxidative burst.

PRRs are not only expressed by professional immune cells, but also by nonprofessional immune cells, including epithelial cells lining the respiratory tract [12]. It is well established that these cells not only
constitute a barrier against inhaled substances but also an active partner that may participate in innate immune responses upon activation by inhaled agents.

If the innate immune reactions fail to rapidly eliminate an invading pathogen, the more slowly developing adaptive or specific immune system is activated. In contrast to the innate immune system, which has no immunological memory and does not provide long-lasting immunity, the adaptive immune system evolves throughout life and is rapidly reactivated upon later encounter of the same antigen. Dendritic cells provide the link between innate and adaptive immunity. After antigen uptake the activated dendritic cell migrate to regional lymph nodes to present processed antigens on a major histocompatibility complex (MHC) class II molecule to naïve T helper (T\textsubscript{H}) cells, initiating either antigen-specific immune responses or tolerance. The presence or absence of a co-stimulatory signal during antigen presentation and various other environmental factors, like the surrounding cytokine milieu, will decide in which direction the T\textsubscript{H} cell will polarise. Interferon gamma (IFN-\gamma) and interleukin (IL)-12 favours polarisation into T\textsubscript{H}1 cells that subsequently activate cytotoxic T cells and thus aid in cell mediated responses. IL-4 on the other hand favours polarisation into T\textsubscript{H}2 cells that in turn activate B cells to switch from IgM to other Ig isotypes and thus favour the development of a humoral response. Cytokines secreted by T\textsubscript{H}1 cells suppress the T\textsubscript{H}2 subset and vice versa. In addition, polarisation to other subsets, such as regulatory T cells or T\textsubscript{H}17 cells, also occur. It takes the activated dendritic cells 6-24 h to travel to the lymph nodes and 3-4 days to induce the T cell response.
**Airway allergies**

The development of allergic airway diseases in an atopic individual is initiated by sensitisation. The presentation of allergen epitopes by an antigen presenting cell, usually a dendritic cell as described in brief previously, induces polarisation of the $T_{H1}$ cell to the $T_{H2}$ subtype. The $T_{H2}$ cells subsequently activate allergen-specific B cells to undergo isotype switching to IgE. The IgE secreted by the plasma B cell sensitises mast cells and basophils by binding to high-affinity FcεRI receptors on their cell surface (*Figure 1*). The production of IgE distinguishes allergic reactions from other humoral responses.

*Figure 1.*  A simplified overview of the type I hypersensitivity reaction. Allergen exposure and $T_{H2}$ cells activate allergen-specific B cells to become IgE secreting plasma cells. This is aided by IL-4 and IL-13 secreted by $T_{H2}$ cells. Secreted IgE sensitises mast cells and basophils, which upon re-exposure degranulate due to receptor cross-linking.
Upon re-exposure to the same allergen, the allergens bind to FceRI-bound IgE. Binding to multiple epitopes on the allergen molecule results in cross-linking of receptors and subsequent degranulation of the cell, e.g. histamine, leukotriens and prostaglandines are released (Figure 1). These mediators cause the hallmarks of the acute or immediate response; bronchoconstriction, vasodilation, vascular permeability and mucus secretion, within minutes.

A late phase response develops hours after exposure and leads to long-lasting inflammation in the lung. This is caused by infiltration of inflammatory cells in the lung, mainly eosinophils and neutrophils, as a result of mediators released during degranulation and by T_{H2} cells. Mediators released by eosinophils may cause e.g. extensive tissue damage in the lung.

### Allergens

An allergen is an environmental agent capable of initiating a type I hypersensitivity reaction in atopic individuals, i.e. persons who have a hereditary predisposition to produce IgE antibodies against common environmental allergens. Depending on their ability to bind IgE, allergens are designated as major, intermediate or minor. Major allergens bind IgE in more than 50% of sera from patients allergic to the allergen source in question, whereas minor allergens bind IgE in less than 10% and those in between are defined as intermediate allergens [15]. An allergen source usually encloses all three allergen types. However, other criteria need to be fulfilled, for the allergen to make a difference, e.g. that the allergen accounts for a substantial amount of the specific IgE produced (>10%) in a patient and that the
allergen is present in significant quantities in the source material [16, 17].

Allergens targeted by the immune system represent a very small fraction of the proteins humans are exposed to but worldwide the same molecules behave as allergens. The question why these act as allergens in susceptible individuals is still unanswered. A majority of allergens are proteins or glycoproteins. Proteolytic activity has for a long time been proposed as an adjuvant for allergenicity, but lipid binding is a far more common characteristic and found for more than 50% of the major allergens [18].

**Allergen nomenclature**

Many allergens have homologues in related species and the classification of allergens is therefore based on sequence homology or function. Since 1986 allergens are named using the systematic nomenclature established by the Allergen Nomenclature Sub-Committee of the World Health Organization (WHO) and International Union of Immunological Societies (IUIS). The name of an allergen consists of the first three letters of the genus, space, the first letter of the species, space and a Arabic numeral indicating the chronologic order of allergen purification [19]. The same number is used for homologous allergens of related species. Consequently, the first characterised allergen from the house dust mite (HDM) species *Dermatophagoides pteronyssinus* is named Der p 1 and the corresponding allergen from *D. farinae* is named Der f 1, for example.
Aeroallergens

Exposure to aeroallergens poses a major risk for sensitisation and development of allergic diseases. Aeroallergens may have either perennial or seasonal distributions. Indoor allergens, derived from e.g. mites and animal dander, are perennial allergens present throughout the year and sensitisation to these is strongly associated with the development of asthma [3, 4, 20-23]. Seasonal outdoor allergens typically include airborne pollen and fungi. Sensitisation to these is instead associated with seasonal rhinitis, rather than asthma [4, 23]. Bronchial provocation of asthmatic patients with HDM or pollen extract results in similar early asthmatic reactions, whereas the late asthmatic response was associated with HDM provocation only [24].

The sensitisation profile reflects the environment and there are geographical variations as to which allergen sources dominate sensitisation. Mites are regarded as the most important source of allergens nearly worldwide and in regions where mites thrive they are the main cause of allergic sensitisation with prevalence reaching over 90% among allergic individuals [25]. In regions with cold or dry climate sensitisation to mites is less common [26, 27]. For instance, in Scandinavia and at high altitudes, sensitisation to cat and dog allergens dominate instead [28-32].

Dust mite allergens are not easily airborne in undisturbed air but rather found locally for example in beds, while cat allergens are easily airborne for extended periods of time and therefore found everywhere in the home [33]. This explains why persons allergic to cats develop symptoms rapidly when visiting a house with a cat. Cat as well as dog allergens are also spread by their owners to other places in substantial
quantities and may be a significant source of exposure for sensitised individuals [34, 35]. Exposure to cat allergen in early childhood seems to have a protective effect on subsequent sensitisation and asthma development [36-39]. In contrast, exposure to increasing quantities of dust mite allergens is associated with an increasing risk of sensitisation and asthma development [5, 20, 37, 40, 41]. Individuals may, however, vary widely in their susceptibility and therefore no absolute threshold levels that would determine the risk of sensitisation have been identified.

**Mite allergens**

There are thousands of mite species but only some of them are associated with allergic sensitisation. Hypersensitivity reactions to mites, including asthma, perennial rhinitis and atopic dermatitis, are estimated to affect 10-20% of the population and 50-85% of asthmatics are typically allergic to mites [42]. The most important allergy-causing mites are those found in large quantities in human dwellings. These can broadly be divided into the pyroglyphid mites, referred to as HDMs and the non-pyroglyphid, referred to as storage mites (Figure 2).

![Figure 2. HDMs and storage mites associated with allergic diseases.](Image)
HDMs thrive in modern homes with high temperature, high humidity and sparse ventilation. Since they feed on human and pet dander, beds constitute their prime habitat but they can also be found in carpets, curtains and furniture (Figure 3). Because storage mites are typically found in grain, flour and hay, they have been considered as inducers of mainly occupational allergy among e.g. bakers and farmers [43]. Many storage mites are, however, also commonly found in house dust and cause allergic sensitisation from domestic exposure, especially in damp houses [44-49].

Figure 3. Habitats of HDMs. Beds are the main habitat of HDMs, but they are also found in e.g. carpets and curtains.

There are great geographical differences in the distribution of mites. The HDMs *D. pteronyssinus* and *D. farinae* are the two most common species causing allergic diseases in Europe and North America [17, 50]. Another HDM species of importance for sensitisation is *Euroglyphus maynei* [51]. *D. pteronyssinus* is also found in tropical parts of the world, where it co-exists with *Blomia tropicalis*, the most abundant storage mite in these regions. *Lepidoglyphus destructor*, on the other hand, is the dominating
storage mite in non-tropical regions [52]. Other storage mite species associated with allergic sensitisation are those belonging to *Glycyphagus, Acarus* and *Tyrophagus*.

So far over 20 mite allergen groups have been characterised, some of which are species specific while others are found in several species, but with varying homology and cross-reactivity. The characterised allergens from the main allergic HDM and storage mite species are summarised in *Table 1*.

**Table 1. Overview of characterised HDM and storage mite allergens**

<table>
<thead>
<tr>
<th>Group</th>
<th>Der p 1</th>
<th>Der f 1</th>
<th>Eur m 1</th>
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<tbody>
<tr>
<td>Group 2</td>
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<td>Der f 2</td>
<td>Eur m 2</td>
<td>Blo t 2</td>
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<td>Eur m 3</td>
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<td>Der f 4</td>
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<td>Blo t 4</td>
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<td>Blo t 7</td>
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<td>Blo t 8</td>
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<td>Gly d 13</td>
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<td>Eur m 14</td>
<td>Blo t 14</td>
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<td>Group 24</td>
<td>Blo t 24</td>
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</table>
The group 1, 3, 6 and 9 allergens possess proteolytic activity, a property which has been proposed as an adjuvant for allergenicity [18]. Based upon the frequency of patients sensitised, the group 1, 2, 3, 9, 11, 14 and 15 allergens are major IgE binders [53]. However, the group 1 and 2 proteins are considered the only major mite allergens since specific IgE account for the majority of the total IgE induced in patients and the content of these allergens in mite extracts is high [17, 53-56]. Both Der p 1 and Der p 2 have both been extensively characterised, but studies on the direct effect on respiratory epithelial cells has mainly focused on the non-proteolytic mite allergens Der p 1, Der p 3 and Der p 9 [57-63].

**Group 2 mite allergens**

The group 2 allergens are, as previously mentioned, major allergens of dust mites, to which up to 80-90% of mite-sensitive patients have IgE antibodies [17, 53, 54, 64-66]. In contrast to the major group 1 allergens, the group 2 allergens lack proteolytic properties.

The molecular weight of these proteins is about 14 kDa. The predominating pyroglyphid Der p 2 and Der f 2 are highly homologous, sharing approximately 88% amino acid sequence identity [67]. Their protein structures are therefore quite similar and they are both made up of two anti-parallel β-sheets, with a hydrophobic cavity in between [68-73]. Three pairs of disulfide bonds are important for the correct conformation [74] and disruption of these by mutations results in reduced IgE antibody binding capacity [75-77].
Eur m 2 of the HDM E. maynei shares about 84% sequence identity with Der p 2 and Der f 2 [78] and the degree of cross-reactivity between these related allergens is high [51, 64, 79]. Similarly, the cross-reactivity among group 2 storage mite allergens is high [80-82]. In contrast, there is little cross-reactivity between the group 2 allergens from HDM and storage mite species [80, 83-85]. The major allergen of L. destructor Lep d 2 shares about 52% and 57% identity with Der p 2 and Der f 2, respectively [86]. The other non-pyrogllyphid group 2 allergens share even less identity with the corresponding pyrogllyphid allergens [84]. Their tertiary structures are, nonetheless, almost identical, since differences are located mainly to the surface [80]. Similar protein folds can be obtained with as little as 25% sequence identity [87]. In contrast, cross-reactivity is rarely observed if allergens share less than 50% identity, but usually requires more than 70% amino acid sequence identity.

The structure of group 2 mite allergens shows homology with MD-2 [88, 89], the LPS-binding protein of the TLR4 signalling complex (Figure 4) [90]. The similarities with MD-2 suggest that group 2 mite allergens are lipid-binding proteins [88, 89] and both Der p 2 and Der f 2 have been shown to bind LPS [91, 92].

The potential role of TLR4 signalling in HDM sensitivity has recently been studied both in animal models and in vitro. Mice lacking TLR4 or its main adaptor protein MyD88 in a model of HDM allergic asthma were shown to be protected from the cardinal features of asthma [93]. Furthermore, it has been demonstrated that the HDM allergic response is dependent on TLR4 expression on airway structural cells [94]. Der p 2 itself has been shown to exert adjuvant-
like activity by activation of TLR4 through functional mimicry of MD-2 [95].

**Figure 4.** A simplified figure illustrating cell activation through the TLR4/MyD88 pathway. LPS binds to TLR4 in association with CD14 and MD-2. This may induce signalling via the adaptor protein MyD88 and subsequent activation of MAPKs and the transcription factor NF-κB, which is released when the inhibitory IkB is phosphorylated.

**Pet allergens**

More than half of the households in Western countries are estimated to have at least one pet. Allergens derived from these are of great importance in the pathogenesis of asthma and allergic rhinitis, especially in regions free of mites [28-32].

The major allergens derived from dog are Can f 1 (*Canis familiaris*) and Can f 2, which elicits IgE responses in about 70% of dog allergic patients [96, 97]. The reported IgE prevalences do however vary
widely, ranging from as low as 25% to over 90% [98, 99]. Both Can f 1 and Can f 2 belong to the lipocalin family of proteins [100], together with the majority of other major mammalian allergens, from e.g. mouse, rat, horse and cow [101, 102]. The cat allergen Fel d 1 (*Felis domesticus*) is however an important exception, as it is an uteroglobin-like protein [103, 104]. Fel d 1 is the only major allergen identified from cat and cause IgE responses in over 90% of cat allergic individuals [96, 105]. Depletion of Fel d 1 from the cat allergen extract greatly reduces the allergenicity of it, in some cases by more than 90% [106].

**Pollen allergens**

Trees and grasses are major sources of seasonal pollen allergens, which are mainly responsible for symptoms in the upper airways and for causing allergic rhinitis [4, 23]. In Europe, birch and timothy are major producers of numerous allergens [107]. Bet v 1 (*Betula verrucosa*) is recognised as the major allergen of birch, to which up to 100% of birch allergic individuals may produce IgE antibodies [108]. The most important allergens in timothy grass pollen are Phl p 1 (*Phleum pretense*) and Phl p 5, to which a majority of grass pollen sensitised individuals have specific IgE antibodies [109].
**AIMS**

The overall aim of this thesis was to elucidate if and how major non-proteolytic group 2 mite aeroallergens may have a direct activating effect on respiratory epithelial cells. Other structurally dissimilar non-proteolytic aeroallergens from cat, dog, birch and timothy, representing different protein structures were studied as a comparison.

The specific aims of each paper were:

**Paper I** To examine if the major non-proteolytic HDM allergen Der p 2 from *D. pteronyssinus* may activate bronchial epithelial cells to produce inflammatory mediators. The role of MAPKs and NF-κB in activation was also studied.

**Paper II** To compare if different non-proteolytic allergens originating from HDM, storage mite, cat, dog, birch and timothy mount different responses in the bronchial epithelial cells.

**Paper III** To examine if the Der p 2 and Der f 2-induced activation of bronchial epithelial cells involves signalling through TLR2, TLR4 and MyD88. For comparison the structurally dissimilar cat allergen Fel d 1 was included.
MATERIALS AND METHODS

In this section the main materials and methods used are summarised in brief.

Cell culture

This thesis is based on in vitro experiments carried out using commercial respiratory epithelial cell lines, which are recognised as good model cells. These have also been used by others for examining the effects of proteolytic HDM allergens [58-63].

Bronchial cells

The human bronchial epithelial cell line BEAS-2B (Paper I, II, III), transformed with an adenovirus 12-SV40 virus hybrid from ATCC (American Type Culture Collection) and normal human bronchial epithelial (NHBE) cells (Paper I, II) from Clonetics were grown in serum-free bronchial epithelial cell basal medium (BEBM) with supplements (complete medium, BEGM) in tissue culture flasks coated with bovine plasma fibronectin, collagen and bovine serum albumin.

Alveolar cells

The human type II alveolar epithelial cell line A549 (Paper I, II) from ATCC was cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and 50 μg/ml gentamicin.

Monocytic U937 cells

The human monocytic leukemia cell line U937 (Paper I) from ATCC was cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and 50 μg/mL gentamicin.
All cells were maintained at 37°C in humidified atmosphere with 5% CO₂.

**Allergens**

The recombinant allergens rDer p 2, rLep d 2, rFel d 1 and rCan f 2 were expressed in *Escherichia coli* [110-113]. rEur m 2, LoTox™ rBet v 1 and LoTox™ rPhl p 5a and LoTox™ nFel d 1, as well as affinity-purified natural nDer p 2 and nDer f 2 were obtained from Indoor Biotechnologies. The endotoxin content of each allergen preparation is presented in *Table 2*.

<table>
<thead>
<tr>
<th>Allergen</th>
<th>Endotoxin Content (ng/mg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rDer p 2</td>
<td>176</td>
<td>Paper I, II</td>
</tr>
<tr>
<td>nDer p 2</td>
<td>632</td>
<td>Paper III</td>
</tr>
<tr>
<td>nDer f 2</td>
<td>89</td>
<td>Paper II, III</td>
</tr>
<tr>
<td>rEur m 2</td>
<td>12.7</td>
<td>Paper II</td>
</tr>
<tr>
<td>rLep d 2</td>
<td>31</td>
<td>Paper II</td>
</tr>
<tr>
<td>rFel d 1</td>
<td>4.5</td>
<td>Paper II</td>
</tr>
<tr>
<td>LoTox™ nFel d 1</td>
<td>&lt;0.5</td>
<td>Paper II, III</td>
</tr>
<tr>
<td>rCan f 2</td>
<td>17</td>
<td>Paper II</td>
</tr>
<tr>
<td>LoTox™ rBet v 1</td>
<td>&lt;0.5</td>
<td>Paper II</td>
</tr>
<tr>
<td>LoTox™ rPhl p 5a</td>
<td>&lt;0.5</td>
<td>Paper II</td>
</tr>
</tbody>
</table>

**siRNA transfection**

Pre-designed small interfering RNA (siRNA) from Ambion was used to down-regulate the expression of TLR2, TLR4 and MyD88 (*Paper III*). As a control nonspecific negative siRNA was used. BEAS-2B cells
were reverse transfected using siPORT™ NeoFX™ transfection agent and Opti-MEM media into 24-well culture plates. The principal of reverse transfection is that cells are transfected and seeded at the same time, thereby bypassing steps in the traditional pre-plating or forward transfection method.

**Analysis of proteins**

**ELISA**
The secreted amount of inflammatory mediators in cell free supernatants was measured using DuoSet enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Paper I, II, III). The principal of the sandwich ELISA used is that the antigen of interest is captured between two antibodies; a capture antibody immobilised in a microplate well and a biotin labelled detection antibody. The biotin of the detection antibody then binds to streptavidin conjugated to horseradish-peroxidase (HRP). The enzyme HRP converts the subsequently added substrate to a blue colour. The reaction is stopped by addition of sulphuric acid, turning the solution yellow (Figure 5). The colour development, which is directly proportional to the antigen concentration, is then measured spectrophotometrically.

![Figure 5](image)

*Figure 5.* A simplified illustration of the principles of sandwich ELISA.
**Bio-Plex**

Another method used to detect the presence of secreted mediators in cell supernatants was Bio-Plex assays from Bio-Rad (*Paper II*). The principle of this bead-based assay is similar to a sandwich ELISA in that the antigen is captured between a capture and detection antibody (*Figure 6*). The capture antibody is however not immobilised to the well, but rather soluble and bound to a colour-coded bead. The biotin of the detection antibody binds to streptavidin conjugated to the reporter dye phycoerythrin (PE).

*Figure 6.* A simplified illustration of the principles of Bio-Plex assay.

Since there are 100 specific color-coded beads, one advantage over a traditional sandwich ELISA is that several antigens in the supernatant can be detected simultaneously in a single well of a microplate, therefore being extremely time-saving if many mediators are analysed. Another benefit compared with ELISA is that a lot of data can be obtained from a small volume of e.g. cell supernatant.
**Flow cytometry**

The surface expression of cell adhesion molecules was analysed using PE-conjugated antibodies by flow cytometry (Paper I, II), which is a standard method to analyse proteins on or within a cell. Multiple characteristics of a single cell can be measured simultaneously. In principal, each cell intersects a laser beam and different detectors collect information about light scattering and fluorescence emission if cell components are labelled with fluorescent conjugated antibodies. The light scattering distinguishes cells depending on their size (Forward Scatter) and granularity (Side Scatter). The data is typically presented in dot-plots and histograms. Histograms are frequently used to display e.g. up-regulation of specific receptor expression when fluorescent conjugated antibodies are used. *Figure 7* shows an illustration of such a shift in fluorescence intensity between two samples.

![Flow cytometry histogram](image)

*Figure 7.* An illustration of a flow cytometry histogram with a shift in fluorescence intensity between sample A and B.

**Analysis of gene expression**

**TaqMan qPCR**

TaqMan gene expression assays from Applied Biosystems were used for analysing the mRNA expression of genes (Paper I, III). The
TaqMan principle relies on the cleavage of a dual-labelled probe by the Taq polymerase during hybridization. As long as the probe is intact, the quencher dye at the 3' end is in close proximity to the reported dye at the 5' end and inhibits fluorescence from it, but when the probe is cleaved, the fluorescence from the reporter dye permits quantitative measurements during the exponential stages of the PCR (Figure 8).

Figure 8. An illustration of the principles of TaqMan PCR.

The level of each target gene was normalised to the house-keeping gene 18S rRNA. Relative expression compared to the control was calculated using the ΔΔCT method [114], correcting for differences in PCR amplification efficiency of each primer and probe set.

Statistical analysis

The data in this thesis is considered normally distributed and therefore analysed using one-way-analysis of variance (ANOVA). Calculations were performed using SPSS. Data were considered significant at p<0.05.

Dunnett’s post hoc test was used to analyse statistical differences between groups (Paper I, II, III).
RESULTS

In this section the main results are presented. Further details are found in the corresponding paper.

**Paper I**

**Der p 2 activates bronchial epithelial cells**

The aim of this study was to examine the effect of Der p 2 on respiratory epithelial cells. Several studies by others show that the proteolytic HDM allergens Der p 1, Der p 3 and Der p 9 induce release of inflammatory mediators from respiratory epithelial cells [57-63]. While many major HDM allergens lack proteolytic activity and since their ability to directly activate epithelial cells has not been studied, the potential of Der p 2 to induce activation of human alveolar and bronchial epithelial cells *in vitro* was examined.

Exposure of bronchial epithelial cells BEAS-2B to recombinant (r)Der p 2 induced a dose-dependent increase in both protein secretion (*Paper I, Figure 2*) and mRNA levels (*Paper I, Figure 3*) of GM-CSF, IL-6, IL-8, MCP-1 and MIP-3α. 10 µg/ml of rDer p 2 was sufficient to induce a statistically significant response. The surface expression of ICAM-1 and secretion of soluble (s)ICAM-1 was also increased (*Paper I, Figures 4a, b and c*). The up-regulated expression of ICAM-1 was also associated with a subsequent enhanced adhesion of monocytic U937 cells (*Paper I, Figure 5a*).

Using inhibitors of the MAPK and NF-κB signal transduction pathways the Der p 2-induced activation was shown to be heavily dependent on NF-κB. BAY 11-7082, which blocks IκB phosphorylation and subsequently inhibits NF-κB activation, almost
results
completely blocked secretion of the studied cytokines and chemokines (Paper I, Figure 4c and 7) as well as ICAM-1 secretion, expression and the subsequent adhesion of U937 cells (Paper I, Figure 4c, 5b and 7).

The MAPK inhibitors did not affect the Der p 2-induced expression of ICAM-1, but especially the p38 (SB203580) and JNK (SP600125) inhibitors did, to varying degrees, block the secretion of inflammatory mediators (Paper I, Figure 7).

The results showing Der p 2-induced mediator release from BEAS-2B cells were verified in primary NHBE cells (Paper I, Figure 2). Secretion of the previously mentioned mediators, except IL-6, was significantly increased. In contrast, the alveolar A549 epithelial cells were unaffected by allergen exposure.

In conclusion, these result show that Der p 2, in addition to its ability to provoke specific adaptive immune responses, may augment innate immune responses by direct activation of epithelial cells.

Paper II
Activation of bronchial epithelial cells by Der f 2, Eur m 2, Lep d 2, Fel d 1 and Can f 2

The aim of this study was to examine the effect of a larger panel of group 2 mite allergens from house dust and storage mites, namely Der p 2, Der f 2, Eur m 2 and Lep d 2, on alveolar and bronchial epithelial cells. The effects of the structurally dissimilar non-proteolytic Fel d 1 from cat, Can f 2 from dog, Bet v 1 from birch and Phl p 5a from timothy were also studied.
RESULTS

All these group 2 mite allergens were found to activate bronchial BEAS-2B and NHBE cells. The secretion of G-CSF, GM-CSF, IL-6, IL-8, MCP-1, MIP-3α was significantly increased from BEAS-2B (Paper II, Figures 1 and 3) as well as the expression of ICAM-1 (Paper II, Fig 2). Increased secretion of G-CSF, IL-6 and IL-8 was measured from NHBE cells (Paper II, Fig 4).

The cat allergen Fel d 1, both recombinant and natural, as well as the dog allergen rCan f 2 did induce secretion of the same mediators from BEAS-2B cells as the mite allergens (Paper II, Figure 1) and expression of ICAM-1 (Paper II, Figure 3), while the response from NHBE cells was somewhat weaker (Paper II, Figure 4). The two pollen allergens rBet v 1 and rPhl p 5a, on the other hand, did not have an effect on either the alveolar A549 or bronchial BEAS-2B cells.

Paper III
Der p 2 and Der f 2-induced activation of bronchial epithelial cells is mediated through TLR4 and MyD88 signalling

The aim of this study was to examine if Der p 2 and Der f 2-induced activation of BEAS-2B cells is mediated through TLR4 and its main adaptor protein MyD88, since the group 2 mite allergens are structurally related to MD-2 [88, 89] and it has been shown that Der p 2 can functionally mimic MD-2 [95]. The potential role of TLR2 was also studied, since it has been reported that Der p 2 activates airway smooth muscle cells by mechanisms involving TLR2 and MyD88 [115]. The cat allergen Fel d 1 was used as a structurally dissimilar control. In this study the affinity purified natural allergens nDer p 2, nDer f 2 and nFel d 1 were used.
Down-regulation of TLR4 and Myd88 expression in BEAS-2B cells by siRNA transfection did significantly inhibit both nDer p 2 and nDer f 2-induced activation (*Paper II, Figures 2 and 3*). In contrast, no major effect of TLR2 silencing was observed.

Down-regulation of TLR4 had no major effect on nFel d 1-induced cell activation, whereas silencing of TLR2 or MyD88 did down regulate the secretion of all mediators (*Paper II, Figures 4*).
DISCUSSION

Cell activation by group 2 mite allergens

The structural similarities between group 2 mite allergens and MD-2 suggest that these allergens are lipid-binding proteins [88, 89]. Although lipid binding is recognised as a common characteristic in more than 50% of major allergens [18], the potential of group 2 mite allergens to activate lung epithelial cells has not previously been studied. In contrast, the effect of several proteolytic mite allergens has been studied extensively. Der p 1, Der p 3 and Der p 9 have been shown to induce release of proinflammatory mediators from respiratory epithelial cells [57-62]. Proteolytic activity of HDM extracts also increase the permeability of the epithelial barrier by disrupting tight junctions [116-120].

The results in this thesis show that the major group 2 allergens Der p 2, Der f 2 and Eur m 2 from HDMs, as well as the storage mite allergen Lep d 2, in addition to their ability to provoke adaptive immune responses, may also augment in innate immune responses by inducing mediator release in bronchial epithelial cells. Although a broad spectrum of inflammatory mediators was studied using the Bio-Plex system no differences in their response patterns were observed. Since these allergens are structurally homologous [80] it was not unexpected that the responses they induced were similar.

The mechanisms behind accumulation and activation of inflammatory cells in the lung are complex, involving many factors operating in concert. These steps are orchestrated by a number of chemokines and cytokines released in the local microenvironment from epithelial, as well as other cells.
The mediators that were induced by the group 2 mite allergens indicate that activation of bronchial epithelial cells by group 2 mite allergens may augment in recruitment, activation and in prolonging the survival of many different inflammatory cells. Both an eosinophilic and neutrophilic response may be promoted by secretion of IL-8, since in addition to being a key molecule in neutrophil recruitment, IL-8 may also participate in the recruitment of eosinophils to the lung [121]. GM-CSF enhances the survival of eosinophils [122] whereas MCP-1 takes part in their activation [123].

In addition to participating in innate inflammatory responses, group 2 allergens may also contribute to specific immune responses by enhancing activity of dendritic cells. MIP-3α and MCP-1 are important chemoattractants for dendritic cell precursors, whereas GM-CSF takes part in their proliferation and maturation [124].

Interestingly, the results in paper II also show that Fel d 1 and Can f 2 induce similar responses, representing two additional non-proteolytic aeroallergens capable of direct activation of bronchial epithelial cells. In contrast, Bet v 1 and Phl p 5a did not activate the cells. Both birch and timothy pollen extracts have been shown to induce secretion of proinflammatory cytokines from nasal and respiratory epithelial cells, but which components of the extract that caused the effect is unknown [119, 125].

**Activation through TLR4 by Der p 2 and Der f 2**

The role of TLR4 on respiratory epithelial cells in group 2 mite- as well as HDM allergy has become a pressing issue recently. The results in paper III indicate that the Der p 2 and Der f 2-induced activation
of BEAS-2B cells is mediated through the TLR4/MyD88 pathway, since a 50% down-regulation of either TLR4 or MyD88 mRNA expression by siRNA transfection, resulted in a significantly decreased reactivity to both allergens.

These results support the hypothesis that that the structural relationship of group 2 mite allergens with MD-2 [88, 89] may provide these allergens with the ability to also functionally mimic this protein. This has been shown in an animal model of allergic asthma [95]. Both MD-2 and Der p 2 contain a tyrosine residue at a site important for interaction with TLR4 and if Der p 2 was mutated at this site it failed to induce Th2 inflammation [95]. Results from other animal studies have also shown that expression of TLR4 and MyD88 is necessary for HDM-induced asthma development [93, 94].

In contrast, the results in paper III show that down-regulation of TLR2 expression did not affect the cell activation, which contradicts another study suggesting that Der p 2-induced activation of airway smooth muscle cells is mediated through TLR2 pathways [115].

**Intracellular MAPK and NF-κB signalling**

It is well established that both MAPKs, which integrate signals from various receptors and translate these signals into cell functions, and NF-κB, as a potent transcription factor responsible for transcription of several proinflammatory molecules, are important in inflammatory diseases such as asthma [126]. Studies using HDM extracts and Der p 1 have also indicated that the activation of airway epithelial cells involves NF-κB and MAPK signalling [60, 127].
The results in *paper I* indicate that the Der p 2 induced activation involves NF-κB and MAPK pathways. The inflammatory mediators are differently regulated. Both the expression of ICAM-1 and the secretion of mediators showed a strong dependency on NF-κB, since activation was almost completely abolished when NF-κB was blocked. Whereas none of the MAPK inhibitors affected ICAM-1 expression, the secretion of mediators was down-regulated to different extents by inhibition of p38 and JNK activation. Inhibition of ERK did not down-regulate the secretion of any of the mediators. Secretion of both GM-CSF and IL-8 showed a broad dependency on MAPKs, especially JNK. MCP-1 was also dependent on JNK, while the secretion of IL-6 and MIP-3α was slightly decreased when activation of p38 was inhibited.

Taken together these results show that NF-κB is essential for Der p 2-induced activation of BEAS-2B cells and that MAPK pathways also are involved to some extent. It is, however, not possible to draw any definite conclusions regarding precise signalling pathways from the experiments using the MAPK inhibitors, since the degree of cross-regulation is high and inhibition of ERK and JNK signalling even augmented the Der p 2 induced secretion of some mediators.

**Cell lines**

Immortalised cell lines differ from primary cell cultures, which is likely to affect their responsiveness to allergen exposure. In both *paper I* and *paper II* the main results found in BEAS-2B cells were verified using primary NHBE cells. The observed higher background level of secreted mediators from NHBE cells and the differences in
magnitude might thus be explained by the immortalised phenotype of BEAS-2B. The difference in response magnitude is also consistent with results from a previous study [128].

**Allergen preparations and LPS**

The use of recombinant allergens raises the question of bacterial contaminants in the allergen preparation and whether these might influence the cellular response. Since LPS in theory might be able to activate respiratory epithelial cells, extensive control experiments were conducted using *E. coli* LPS (*Paper I* and unpublished data). BEAS-2B cells were not responsive to LPS at concentrations present in the allergen preparation. A relatively high dose of LPS was needed to achieve a cell response above background level, which has also been shown by others [129, 130]. Respiratory epithelial cells have been shown to express little or no MD-2 [131], which can explain their low responsiveness to LPS. Moreover, co-exposure of cells with Der p 2 and the potent LPS inhibitor Polymyxin B did not inhibit the activation. In addition, the responses induced by the group 2 mite allergens were of similar magnitudes despite the fact that the LPS content ranged from 89 ng/mg for nDer f 2 up to 632 ng/mg for nDer p 2 (*Paper III*).

In a mouse model of asthma, Der p 2 has been shown to induce a T\(_{\text{H}2}\) inflammation in the presence of an extremely low dose of LPS, usually associated with induction of tolerance [95]. The T\(_{\text{H}2}\) responses were moreover observed only in wild-type and MD-2-deficient mice, not in TLR4-deficient mice. Similarly, in a mouse model of HDM-induced asthma, HDM extracts induced T\(_{\text{H}2}\) responses via TLR4 when
contaminating LPS levels were far below doses known to promote T\textsubscript{H}2 responses, whereas LPS alone did not induce similar responses [94].

Taken together, it is highly unlikely that the responses seen by the group 2 allergens in this thesis are solely due to endotoxin contamination. Since the group 2 mites belong to the MD-2 like lipid binding group of proteins, a small lipid cargo may, however, be needed to provide these allergens with their intrinsic adjuvant activity.
CONCLUDING REMARKS

Taken together, the results in this thesis show that the major non-proteolytic group 2 allergens Der p 2, Der f 2 and Eur m 2 from the HDM species *D. pteronyssinus*, *D. farina* and *E. maynei* as well as Lep d 2 from the storage mite *L. destructor* may activate bronchial epithelial cells by adjuvant-like mechanisms. The activation is mediated through TLR4 and its downstream adaptor protein MyD88, most likely due to the allergens resemblance with MD-2. Activation of TLR4/MyD88 pathways does subsequently lead to activation of MAPKs and NF-κB, which lead to increased secretion of mediators and expression of adhesion molecules. This activation may thus in many ways contribute to innate immune reactions in the lung and subsequently asthma pathogenesis.

One important and interesting aspect is what differs between the innate immune responses after HDM allergen exposure in non-atopic and atopic individuals, since both may be exposed to equal amounts of allergens, but still only the atopic patients develop an allergic inflammation. Primary cultures of bronchial epithelial cells derived from allergic asthmatics have been shown to secrete increased amounts of inflammatory mediators both at baseline as well as after Der p 1 exposure, compared to cells from healthy individuals [63]. Since bronchial epithelial cell lines were used in this thesis, it would in the future be interesting to compare the responses induced by group 2 mite allergens in epithelial cells derived from non-atopic with atopic subjects.
Förekomsten av allergiska luftvägssjukdomar, såsom astma och hösnuva, har ökat under de senaste decennierna, speciellt i västvärlden. Exponering för luftburna allergener är otvivelaktigt en betydande riskfaktor för utvecklandet av dessa sjukdomar. Exponering för allergener kan provocera immunsystemet hos sensibiliserade individer genom att aktivera ett klassiskt adaptivt immunvar med aktivering av T-lymfocyter och utsöndring av IgE-antikroppar från B-lymfocyter. Andra inflammatoriska mekanismer spelar dock också en viktig roll för hur symptom uppstår efter inandning av allergener. Lungans epitelceller, som utgör en viktig barriär för att förhindra olika partiklar från att ta sig in i kroppen, kan även aktivt delta i dessa inflammatoriska reaktioner genom att till exempel utsöndra inflammatoriska mediatorer som i sin tur kan delta i rekrytering och aktivering av inflammatoriska celler till lungan.

Proteiner härstammande från dammkvalster tillhör de vanligast förekommande luftburna inomhusallergenerna i stora delar av världen och exponering för dessa är starkt förknippat med uppkomsten av allergiska sjukdomar. Man räknar med att 50-85% av alla astmatiker är sensibiliserade mot olika typer av kvalsterallergener, globalt sett. Eftersom kvalster trivs i varma och fuktiga miljöer är kvalster och kvalsterallergi inte vanligt förekommande i de nordiska länderna, här härstammar istället de dominerande allergenerna från katt, hund och pollen.

Ett stort antal allergener har identifierats från kvalster och dessa är indelade i ett 20-tal grupper med avseende på storlek och strukturella likheter mellan olika kvalsterarter. Allergenerna i grupp 1, 3 och 9 är proteolytiska enzymer och det är ett vedertaget faktum att dessa kan
klyva proteinreceptorer på ytan av lungans epitelceller. Detta leder i sin tur till att cellerna kan utsöndra ett flertal inflammatoriska mediatörer och på så sätt aktivt delta i det medfödda immunförsvaret. Grupp 1 och 2 allergenerna anses vara de mest betydande, eftersom över 80% av individerna sensibiliserade för kvalster är känsliga för allergener som tillhör dessa grupper. Till skillnad från allergenerna i grupp 1, saknar allergenerna i grupp 2 emellertid proteolytisk aktivitet, men inga studier där grupp 2 allergeners möjliga effekt på lungepitelceller undersöks är gjorda.

Syftet med denna avhandling var därför att utreda om och hur betydande grupp 2 kvalsterallergener från olika kvalsterarter interagerar med humana lungepitelceller. Effekterna av de strukturellt besläktade Der p 2, Der f 2 och Eur m 2 från dammkvalsterarterna *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* respektive *Euroglyphus maynei* och Lep d 2 från förrådskvalstret *Lepidoglyphus destructor* studerades i cellförsök. Därutöver studerades även kattallergenet Fel d 1 (*Felis domesticus*), hundallergenet Can f 2 (*Canis familiaris*), björkallergenet Bet v 1 (*Betula verrucosa*) och timotejallergenet Phl p 5a (*Phleum pratense*), som även de är betydande icke-proteolytiska allergener, men strukturellt skilda från grupp 2 kvalsterallergenerna.

Resultaten i denna avhandling visar att grupp 2 kvalsterallergener aktiverar bronkiala epitelceller, vilket mättes som en ökad utsöndring av ett flertal inflammatoriska mediatörer. Kattallergenet Fel d 1 och hundallergenet Can f 2 inducerade också utsöndring av samma mediatörer och utgör således två ytterligare icke-proteolytiska allergenstrukturer som direkt kan aktivera bronkiala epitelceller. De
två studerade pollenallergenerna gav däremot inte upphov till någon aktivering av celler.

Sammantaget bidrar resultaten i denna avhandling till förståelsen av hur icke-proteolytiska grupp 2 kvalsterallergener, utöver att aktivera ett specifikt adaptivt immunförsvar, även kan medverka i det icke-specifika immunförsvarvet genom att aktivera lungans epitelceller. Dessa allergener kan således på detta sätt bidra till att förvärra den pågående luftvägsinflammationen vid allergisk astma.
ACKNOWLEDGEMENTS

Det är många som på ett eller annat sätt hjälpt mig och gjort den här avhandlingen möjlig. Ett särskilt tack vill jag ge till:

Min huvudhandledare Anders Bucht för att jag rätt självständigt har fått jobba med ett projekt som jag verklig tyckte om, men att du även hjälpt till då jag behövt det.

Min biträdande handledare Thomas Sandström för din positiva uppmuntran angående både avhandlingen och andra projekt. Tack också för alla bra kliniska synpunkter på ramberättelsen.


Ett extra tack till Lina T, Bettan, Barbro och Sofia för att ni alla tog er tid att läsa kappan och kom med bra förslag till ändringar!

Tack även till alla andra trevliga människor på FOI, utan er hade tiden på jobbet varit trålig!

Kollegorna i lungans forskningsgrupp på universitetet, speciellt Maria, Yamshid, Ester och Ann-Britt för roligt sällskap på resor, kurser och labbet.

Mina medförfattare Guro och Hans i Stockholm för ni kan allt om framställningen av rekombinanta allergener och att jag har fått tillgång till dem.

Lena och Kerstin på universitetet för all administrativ hjälp samt Maria och Kjerstin på FOI för alla artiklar ni snabbt letat reda på.
Ett stort tack också till alla vänner på båda sidorna om kvarken, både forskande och icke-forskarande. Jag har inte haft så mycket tid för er på sistone, men jag lovar att jag ska bli mera social efter detta! Jag är skyldig rätt många middagar, så det är bara att boka in er nu.

Perhe Hämäläinen, kiitos osoittamastanne mielenkiinnosta työtäni kohtaan sekä mukavista hetkistä aurinkoisella mökkilaiturilla.

Mommo, Fammo och Faffa, är så glad för de stunder vi hinner ses. Tack för allt, inte minst för alla bär som ni har försett mig med. Ni har kanske inte riktigt förstått vad jag har hållit på med, men nu har jag äntligen ”studerat” klart!


Mamma Bernice och pappa Ralf för att ni alltid trott på mig och hjälpt till med allt mellan himmel och jord. Även om jag uppskatat ert intresse för min forskning har jag uppskatat den ”kvalsterallergenfria zonen” hemma ännu mera.

Tack också till resten av min förträffliga släkt!

Till sist min egen lilla familj. Lasse, Frida och bebisen i magen för att ni gett mig motivation och energi. Lasse, för allt stöd, vet att du också har fått jobba hårt. Tack också för den grundliga språkgranskningen. Främst vill jag ändå tacka er för att ni påmint mig om att det finns viktigare saker i livet än en avhandling! Älskar er och ser så mycket fram emot livet efter disputationen!

Tack!

Camilla

The studies in this thesis were supported by grants from the Swedish Ministry of Defence, the Swedish Armed Forces, the Vårdal Foundation, the Swedish Asthma and Allergy Association, the Swedish Heart-Lung Foundation and the J C Kempe Memorial Fund.
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