THE ROLE OF MALASSEZIA ALLERGENS AND MAST CELLS IN ATOPIC ECZEMA

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A quitter never wins and a winner never quits

Napoleon Hill
ABSTRACT

Atopic eczema (AE) is a chronic inflammatory skin disease, characterized by intense itching, dry skin, infiltration of immune cells and skin lesions. To date, the cause of AE, a disease affecting 15-30% of children and 2-10% of adults, remains unknown and the pathomechanisms are not fully understood. Many factors, however, have been implied to contribute to this complex disorder, such as genetic predisposition, skin barrier defects, environmental allergens and inappropriate immune responses to microorganisms. The skin commensal yeast Malassezia has been suggested to contribute to the eczema, since approximately 50% of adult AE patients have specific IgE or positive skin prick test and/or atopy patch test against the yeast. This thesis has focused on the effect of the pH of AE skin on the allergenicity of Malassezia as well as the yeast’s interaction with mast cells. In study I, we found that M. sympodialis produced, expressed and released enhanced amounts of allergens when cultured at a pH resembling AE skin compared to that of healthy individuals. One of the M. sympodialis allergens, designated Mala s 12, was selected for further investigation. In study II, we cloned, produced and characterized this allergen, which is expressed on the yeast’s cell surface. We could determine that Mala s 12 had 30-50% sequence similarity to the glucose-methanol-choline (GMC) oxidoreductase enzyme superfamily and that recombinant Mala s 12 could be recognized by serum IgE from 62% of M. sympodialis-sensitized AE patients, indicating that Mala s 12 is a major allergen in this patient group. In the last two studies of this thesis, we investigated the interaction between M. sympodialis and mast cells. An increased number of mast cells have been found in the upper dermis of lesional AE patients skin and some mast cells even occur in the epidermis. In study III we determined that M. sympodialis can activate mast cells. More specifically M. sympodialis extract can stimulate non-sensitized and IgE-sensitized mast cells to release inflammatory mediators, increase IgE mediated degranulation, influence MAPK activation and alter the IL-6 production by signaling through the TLR-2/MyD88 pathway. In study IV we found that mast cells from AE patients contain an increased amount of granule mediators compared to mast cells from healthy individuals. AE patient derived mast cells also showed an enhanced response to M. sympodialis extract compared to mast cells from healthy individuals and were unable to up-regulate the fungal recognition receptor Dectin-1 upon IgE-receptor cross-linking. These observed differences indicate a differential role for MCs in AE patients compared to healthy individuals.

In conclusion, M. sympodialis will release more allergens when cultured at a pH resembling that of AE skin, suggesting that the higher pH increases M. sympodialis allergenicity. Furthermore, mast cells can be activated by M. sympodialis and the activation is enhanced in mast cells from AE patients. Our findings will further help to elucidate the pathogenic mechanisms of AE and could contribute to the development of new treatment strategies for AE patients sensitized to M. sympodialis.
LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to by their Roman numbers:

I. Christine Selander, Arezou Zargari, Roland Möllby, Omid Rasool, Annika Scheynius.
Higher pH level, corresponding to that on the skin of patients with atopic eczema, stimulates the release of Malassezia sympodialis allergens.

II. Arezou Zargari, Christine Selander, Omid Rasool, Mahmoud Ghanem, Giovanni Gadda, Reto Crameri, Annika Scheynius.
Mal a s 12 is a major allergen in patients with atopic eczema and has sequence similarities to the GMC oxidoreductase family.

III. Christine Selander, Camilla Engblom, Gunnar Nilsson, Annika Scheynius, Carolina Lunderius Andersson.
TLR2/MyD88-dependent and -independent activation of mast cell IgE responses by the skin commensal yeast Malassezia sympodialis. Revised for J. Immunol.

Mast cells from patients with atopic eczema have enhanced levels of intrinsic granule mediators and do not up-regulate Dectin-1. Manuscript.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AE</td>
<td>Atopic eczema</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<td>APT</td>
<td>Atopy patch test</td>
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<td>ATCC</td>
<td>American type cell collection</td>
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<tr>
<td>BM MC</td>
<td>Bone marrow-derived mast cell</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CBMC</td>
<td>Cord blood-derived mast cell</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
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<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
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<tr>
<td>CTMC</td>
<td>Connective tissue mast cell</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
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<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
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<tr>
<td>FAD</td>
<td>Flavin adenine denucleotide</td>
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<tr>
<td>FcR</td>
<td>Fc receptor</td>
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<tr>
<td>GMC</td>
<td>Glucose-methanol-choline</td>
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<td>GOX</td>
<td>Glucose oxidase</td>
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<tr>
<td>HC</td>
<td>Healthy control</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL-</td>
<td>Interleukin-</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans’ cell</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MC</td>
<td>Mast cell</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
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<tr>
<td>MCTR</td>
<td>Mast cell containing tryptase</td>
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<tr>
<td>MCTC</td>
<td>Mast cell containing both tryptase and chymase</td>
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<tr>
<td>MMC</td>
<td>Mucosal mast cell</td>
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<tr>
<td>MoAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MyD88</td>
<td>Myeloid differentiation marker 88</td>
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<tr>
<td>M. sympodialis</td>
<td>Malassezia sympodialis</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
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<tr>
<td>NOD</td>
<td>Nuclear oligomerization domain</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
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<tr>
<td>PAR</td>
<td>Protease-activating receptor</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>PtdIns</td>
<td>Phosphatidylinositols</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>SCF</td>
<td>Stem cell factor</td>
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<tr>
<td>SPT</td>
<td>Skin prick test</td>
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<tr>
<td>TEWL</td>
<td>Transepidermal water loss</td>
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<tr>
<td>Th cell</td>
<td>T helper cell</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TNP</td>
<td>Trinitrophenyl</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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1 INTRODUCTION
1.1 THE BODY’S DEFENSE

Many defensive strategies have been developed to protect our body against harmful invaders throughout evolution. Our body is constantly exposed to microorganisms of different forms, including viruses, bacteria, fungi, protozoa and parasites. We need to cope with this pressure and find a balance between fighting off harmful invaders and cooperating with microorganisms that are beneficial for us. The outermost defense is the epithelial surface which forms a physical barrier to the environment and repels most mediocre attacks. Acting together with the epithelial surfaces, are the chemical barriers composed by enzymes found in tears, saliva and nasal secretions that can break down pathogens. Additionally, antimicrobial peptides and the acidic pH of sweat and gastric secretion can prevent growth of pathogens. Another important barrier is our normal flora of microorganisms present on the skin and in the gastrointestinal tract, which can prevent the colonization of bacteria by secretion of toxic substances or by competing with pathogens for nutrients. These barriers are very effective in preventing pathogen invasion, but pathogens that do succeed to breach these barriers will be efficiently removed by immune mechanisms, which function in the underlying tissues.

The immune system has been divided into the innate immune system and the adaptive immune system. The innate immune system, also termed the unspecific immune system, was the earliest form of defense that developed. It is the body’s first line of defense and has the ability of responding immediately with full force against a variety of pathogens. Once the infectious agents have penetrated the outer defense acute inflammation characterized by edema and recruitment of phagocytes, will initiate a defense against the invading pathogen. The humoral factors responsible for starting this acute inflammation are the proteins of the complement system and the coagulation system, interferons and lysozyme. The main line of defense in the innate immune system is the cellular response composed by neutrophils, macrophages, natural killer (NK) cells, mast cells (MCs) and eosinophils. The cellular response is initiated when pattern recognition receptors (PRRs) on the cells recognize pathogen-associated molecular patterns (PAMPs) on the invading microbes. There is a vast variety of PRRs including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), the mannose receptor and nuclear oligomerization domain (NOD) like receptors (Girardin et al., 2002). The TLRs, the most well studied family of PRRs, were first identified on the basis of sequence similarity with the Drosophila protein Toll and are an ancient family of proteins that includes related proteins in invertebrates and plants. Activation through PRRs will induce phagocytosis as well as secretion of cytokines and chemokines, which results in a strong inflammatory response. This activation can convert phagocytes into antigen presenting cells (APCs) as well as recruit professional APCs such as dendritic cells (DCs) to the site of infection with the ability of starting an adaptive immune response.

The adaptive immune system, unlike the innate immune system, has the ability of a tailored specific response and of generating immunological memory. However, this antigen-specific defense takes several days to develop. The adaptive and versatile response is made possible due to the ability of immunoglobulin (Ig)-like genes in B- and T-cells, which undergo rearrangements resulting in receptors that are able to recognize any molecule. Simply put, the adaptive response can further be described as
follows: upon recognition of antigen, the naïve B- or T-cell with the corresponding specificity will be primed. However, for activation to occur a second signal is also required, naïve B-cells need a signal from a T-helper cell whereas naïve T-cells need a costimulatory signal from an APC. When the necessary signals have occurred, a clonal expansion of T-cells and antibody-producing B-cells will be generated. The T-cells will thereafter differentiate into cytotoxic T-cells (if antigen has been presented on major histocompatibility complex (MHC) -I) or T-helper cells (if antigen has been presented on MHC-II). This activation will also lead to the development of an immunological memory. Once memory has been established, the adaptive immune response will immediately respond when re-challenged at a later time point. This mechanism of generating an immunological memory is used when vaccinating against various diseases. Although the immune system has been divided into two branches there is a lot of interplay between the two, and DCs and MCs have been shown to represent links between them (Janeway, 2004).

1.2 THE SKIN

The body’s largest organ is the skin, which as described above serves as an important defense against microbial invasion. Furthermore, it acts as a sensory organ to sense heat, cold, touch, itch and pain. It is comprised of two distinct layers: the epidermis, a surface layer of close packed epithelial cells, and the dermis, a layer of dense connective tissue (figure 1).

![Figure 1. A schematic picture of the skin (modified from Hiroshi, 2007).](image)

The epidermis is composed of four cell types: keratinocytes, melanocytes, Langerhans’ cells (LCs), and Merkel cells (McGrath, 2004). Keratinocytes are the major celltype, constituting 95% of the epidermis (McGrath, 2004). The epidermal keratinocytes divide in the basal layer, produce keratins, differentiate and migrate to the upper layers forming the outer shield of dead cells called the stratum corneum (McGrath, 2004), all within in a period of 14 days (Hoath and Leahy, 2003). The stratum corneum serves as an important barrier function by protecting against environmental threats from the
outside and loss of water from the inside. However, the stratum corneum barrier is not absolute, since water permeation is possible and the normal movement of water through the stratum corneum into the atmosphere is known as transepidermal water loss (TEWL). Lipids that limit the permeability barrier of the stratum corneum consist of a mixture of ceramides (45-50%), cholesterol (25%), free fatty acids (10-15%) and less than 5% of several other lipids (Madison, 2003). Since the epidermis is the body's physical barrier against the environment, APCs such as LCs are commonly localized in the basal layer of the epidermis, where they efficiently take up antigens and act as initiators of the adaptive immune response (Merad et al., 2002).

Directly below the epidermis is the thicker dermis, which is composed of two layers, the papillary and reticular dermis (Hiroshi, 2007). The main functions of the dermis are to regulate temperature and to supply the epidermis with nutrient-saturated blood. Embedded within the dermis are sweat glands, sebaceous glands, hair follicles, nerve endings, lymph nodes and blood vessels (Hiroshi, 2007). Cell types found in the dermis are fibroblasts, macrophages, plasma cells and MCs (Hiroshi, 2007).

### 1.3 MAST CELLS

MCs are primarily found in the tissues exposed to our environment (Metcalf et al., 1997), particularly beneath the epithelial surfaces and in close contact with blood vessels, nerves, smooth muscle cells, mucus glands and hair follicles (Galli et al., 2005). MCs were first described by Paul Ehrlich in the late 1870s as granular, large sized cells, resident in the connective tissue with reactivity to aniline dyes (Ehrlich, 1878). MCs have thereafter been shown to arise from hematopoietic cells in the bone marrow (Kumamoto et al., 2003). Immature MC progenitors, leave the bone marrow and enter the blood circulation in small numbers. Upon recruitment into the tissues, they mature into MCs under the influence of SCF and other specific growth factors (Metcalf et al., 1997). However, MC progenitors have also been found in mouse hair follicles, suggesting a local source for skin MCs (Kumamoto et al., 2003). Mature MCs are a heterogeneous population and have in humans been divided based on their protease content into MCs containing tryptase (MC_T) and MCs containing both tryptase and chymase (MC_TC) (Irani et al., 1986). MC_T are mainly localized in mucosal tissues such as the lung and the intestinal epithelium and correspond to the rodent mucosal MCs (MMC) (Metcalf et al., 1997). In contrast, MC_TC have mainly been observed in the skin and their rodent equivalent are the connective tissue MCs (CTMC) (Metcalf et al., 1997). A distinction between the two types of MCs cannot be made solely based on their different distribution in tissues, since both cell types can be found in most tissues (Kaliner, 1993). When the MCs have matured in the tissues they can reside there for a long time, as for example rodent intestinal MCs that have a half life of around 40 days (Enerbäck and Löwhagen, 1979). MCs have the possibility of influencing many tissue processes due to their location and ability of releasing a vast array of different mediators. The different mediators can be divided into preformed mediators stored in secretory granules that upon activation can be rapidly released, de novo synthesized lipid-derived mediators, cytokines, chemokines, growth factors, free radicals and other mediators such as substance P (Galli et al., 2005) (summarized in figure 2).
Upon activation, MCs have been shown to, depending on the type of stimuli, selectively release mediators that can affect both innate and adaptive immune functions. MCs can be activated in many different ways, among them for example through the well studied cross-linking of the high affinity IgE receptor (FcεRI) in allergy and activation through PRRs by microbial compounds. MCs are thought to be involved in host defense against pathogens, since they mediate a variety of antimicrobial activities (Dawicki and Marshall, 2007). Based on their ability to rapidly respond to activation they may also be important for the early recruitment of effector cells, such as neutrophils to the site of infection. MCs have further been shown to directly kill bacteria through phagocytosis (Arock et al., 1998; Feger et al., 2002; Malaviya et al., 1994; Wei et al., 2005) and new data indicates that MCs also can kill bacteria by entrapping them in extracellular structures composed by DNA, histones, tryptase and LL-37 (von Köckritz-Blickwede et al., 2008). In vivo studies in MC deficient mice have further shown the importance of MCs in the immune response against bacteria, since after reconstitution with MCs the ability to clear bacterial infections, such as Helicobacter felis, Citrobacter rodentium, Pseudomonas aeruginosa and Klebsiella pneumoniae, was restored (Malaviya et al., 1996a; Siebenhaar et al., 2007; Wei et al., 2005; Velin et al., 2005). Additionally, MCs are well known for their role in the defense against parasites, through IgE-mediated and innate immune responses (Metz et al., 2008). In response to a viral infection MCs can release mediators that recruit T-cells and NK cells, an MC activation distinctly different from that against bacteria (Dawicki and Marshall, 2007). Furthermore, MCs might also be involved in the defense against fungi, however, this has not been extensively investigated in vivo. In vitro experiments have shown that zymosan (a polysaccharide prepared from the cell wall of Saccharomyces cerevisiae) can induce leukotriene release from human MCs (Olynych et al., 2006). Besides being important cells in innate immunity MCs have been shown to be involved in the adaptive immune response where they can present antigen on both MHC-I and MHC-II (Frangjé et al., 1993; Malaviya et al., 1996b), express co-stimulatory molecules (Frangjé et al., 1996; Gauchat et al., 1993) and thus interact with DCs, T- and B-cells.

All these mentioned features of MCs make them highly interesting to study in the context of both health and disease. MCs have so far been associated with body homeostasis, bacterial and parasitic clearance. They have also been linked to allergic diseases such as allergic rhinitis, atopic eczema (AE), atopic asthma and some food allergies (Kalesnikoff and Galli, 2008). MCs are also thought to be involved in autoimmune diseases like rheumatoid arthritis, bullous pemphigoid and multiple sclerosis (Gregory and Brown, 2006). Furthermore, increased numbers of MCs have
been observed in many forms of cancer and they have been shown to be involved in basal cell carcinoma, colonic epithelial tumors and Hodgkin lymphoma (Diaconu et al., 2007; Molin et al., 2002; Wedemeyer and Galli, 2005). Additionally, there is an abnormal increase in MC numbers that accumulate in several organs in the disease mastocytosis (Metcalfe, 2008).

1.4 FUNGI

Fungi are eukaryotic organisms that lack chlorophyll and vascular tissue. They come in a variety of shapes and sizes, from single celled organisms to large chains of cells such as the 0.15 km² mushroom Armillaria bulbosa one of the largest known living organisms on Earth today (Smith, 1992). They can be found in nearly all environments and particularly in moist areas. A fungus acquires food outside its body by secreting strong digestive enzymes into the food generating small organic molecules which can then be absorbed. Many fungi produce compounds with biological activity and a variety are used by the industry today, such as detergent enzymes and antibiotics like penicillin and cephalosporin (Campbell, 1999). Two well established cultural uses of fungi are in bread baking and fermentation of alcoholic beverages. The responsible fungus here is the most important domesticated fungus, the yeast Saccharomyces cerevisiae.

Yeasts are single celled fungi that inhabit liquid or moist habitats, including humans, animals and plants. They reproduce asexually by budding of daughter cells or sexually by forming asci or basidia. Most yeasts are opportunists and coexist with its host without any negative consequences. However, under certain conditions they can cause infections in humans and animals, for example, due to pH changes or immunsuppression (Campbell, 1999). Opportunistic human fungi that become pathogenic are an increasing problem, mostly since advantages in science have led to the survival of more immunosuppressed patients. The virulence of opportunistic fungi often involves morphological changes, ability to grow at elevated temperatures and pH, adherence and alteration in the composition of the commensal microflora (van Burik and Magee, 2001). Some yeasts that can become pathogenic in humans are: Malassezia species, Candida albicans, Cryptococcus neoformans and Saccharomyces cerevisiae.

1.4.1 Malassezia

The fungus Malassezia is a lipophilic yeast that belongs to the normal commensal skin microflora of humans and other warm-blooded animals (Ashbee and Evans, 2002). Malassezia is classified as being dimorphic since it has been found to exist in both yeast and mycelial phases (Ashbee and Evans, 2002). Previously these two phases of Malassezia were thought to be two different organisms and the yeast form was named Pityrosporum and the mycelial form Malassezia. However, in 1986 they were recognized to be two forms of the same organism and were thus collectively named Malassezia (Cannon, 1986). Malassezia has been placed into the phylum Basidiomycota (Batra et al., 2005) and reproduce asexually by budding from a broad base (Chen and Hill, 2005). However, sequencing of the genomes of two Malassezia species, M. globosa and M. restricta, revealed the presence of mating genes with the indication that Malassezia may also be capable of sexual reproduction (Xu et al., 2007). The shape of the yeast cells is round, oval or cylindrical and may vary in size from 1 to
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8 μm in diameter (Keddie, 1966). The cell wall of Malassezia is very thick (0.12 μm) and multilayered, consisting mainly of sugars (70%), lipids (15-20%) and proteins (10%) (Ashbee and Evans, 2002). All Malassezia species except M. pachydermatis requires an exogenous source of long chain fatty acids in order to grow, since they are unable to synthesise C14–C16 saturated fatty acids (Shifrine and Marr, 1963). Due to their lipid requirement, Malassezia species preferably colonizes the scalp, face, neck, upper chest and back of the body where the sebaceous glands are abundant. During puberty, the sebaceous glands become more active in producing lipids and Malassezia colonization increases. The colonization reaches its maximum during the third decade of life and thereafter decreases with increasing age, probably due to reducing lipid content of the skin (Bergbrant and Faergemann, 1988). So far ten species of Malassezia have been isolated from human skin: M. dermatitidis, M. furfur, M. globosa, M. obtusa, M. pachydermatis, M. restricta, M. slooffiae, M. sympodialis, M. japonica and M. yamatoensis (Sugita et al., 2005). The normally harmless species of Malassezia are able to cause several human skin diseases as well as systemic diseases in immunodeficient humans and dermatitis in animals (Ashbee and Evans, 2002). In fact, Malassezia was first described in 1846 by Eichstedt as yeast-like cells in the stratum corneum of patients with the skin disease pityriasis vesicolor. Other skin diseases where Malassezia have been implicated are seborrhoeic dermatitis, folliculitis and dandruff (Gupta et al., 2004). Furthermore, Malassezia appears to be an important trigger factor in AE, especially in adolescent and adult patients with head and neck AE (Gupta et al., 2004). M. sympodialis (figure 3), the species studied in this thesis, is recognized as the most common Malassezia species recovered from both healthy individuals and AE patients according to studies conducted in Sweden, Russia and Canada (Ashbee, 2007).

1.5 ALLERGY

Although the immune system usually defends us against diseases, it can, when in imbalance, also be the cause of disease. An over reactive immune system can for example result in severe diseases like autoimmunity and allergy. Allergy is an overreaction of the immune system to otherwise harmless environmental antigens, so called allergens. Type I allergy or IgE-mediated allergy (Janeway, 2004) is characterized by the occurrence of allergen-specific IgE antibodies (Ishizaka and Ishizaka, 1967; Johansson et al., 1968). A hereditary tendency to produce IgE antibodies against allergens is termed atopy. Disorders associated with atopy are asthma, rhinoconjunctivitis and eczema (Johansson et al., 2004). The IgE mediated allergic response first requires a sensitization to the allergen that will provoke the allergic response (Akdis and Akdis, 2007). Sensitization occurs when the antigen first enters the body through the mucosal surfaces where APCs take it up, process it into peptides and presents it on MHC-II molecules (Kay, 2001). Upon recognition of the MHC-II peptide complex in IL-4 milieu, specific T-helper two cells (Th2) are activated to expand and produce the cytokines IL-4 and IL-13. B-cells that have also taken up the
antigen and then presented its peptides on MHC-II, will upon encountering the primed Th2 cells become activated to proliferate and differentiate into IgE producing plasma cells. The secreted IgE attaches via its constant region to FcεRI on MCs and the individual is now sensitized to the antigen. When exposed to the antigen for a second time, the antigen will crosslink the IgE on the MCs resulting in degranulation and release of preformed mediators such as histamine, tryptase, chymase, prostaglandin and leukotrienes (Hansen et al., 2004). Typical symptoms of this immediate reaction are itching, swelling and edema (Galli et al., 2008; Kay, 2001). These immediate reactions may be followed by a late-phase reaction occurring 6-9 h after exposure. During the late phase reaction inflammatory cells like Th2 cells, neutrophils, eosinophils and basophils are recruited to the site of inflammation in the airways resulting in smooth muscle contraction, edema and airway hyperreactivity (Galli et al., 2008; Kay, 2001). In figure 4 the IgE mediated allergic reaction is summarized.

![Figure 4. The IgE-mediated allergic reaction (modified from (Valenta, 2002)).](image)

The prevalence of allergic diseases has increased considerably in Western societies during the last decades (Beasley et al., 2000). However, there are now indications that the increase in incidents have ceased (Braun-Fahrlander et al., 2004; Grize et al., 2006), although AE may be an exception (Grize et al., 2006). Allergy is a very complex disease and we neither have a clear picture of what causes the onset of the disease nor the increased prevalence of allergies. However, a family history of allergic disease (Wright, 2004), the Western lifestyle and environmental factors (Galli et al., 2008) are thought to be important for the development of allergy. In 1989, the hygiene hypothesis was introduced suggesting that reduced family size and cleaner homes, with the implication of lower microbial exposure during childhood, could explain the increase in allergic disease (Strachan, 1989). This theory was further supported by the finding that an anthroposophic lifestyle, with restrictive use of antibiotics and vaccinations, was associated with a decreased prevalence of developing allergies (Alfve'n et al., 2006; Alm et al., 1999; Flöistrup et al., 2006). Furthermore, living on a farm has also been shown to protect against development of allergies (von
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Mutius and Radon, 2008). Although there is support for the hygiene hypothesis, there is currently no definitive proof that a reduced microbial burden is the cause of the increasing prevalence of allergy (Yazdanbakhsh et al., 2002). Environmental factors such as pollutants have also been suggested to affect the development of allergies (Riedl and Diaz-Sanchez, 2005).

Apart from lifestyle factors, many susceptibility genes have been associated with allergic diseases. Chromosomes 2, 5, 6, 11, 12 and 13 have been linked to atopy (Vercelli, 2008). Particularly of interest are chromosome 5, where the genes encoding for the Th2 cytokines IL-4, IL-5 and IL-13 can be found, and chromosome 11 that harbors the gene encoding for the FcεRI (Vercelli, 2008).

1.6 ATOPIC ECZEMA

AE is one of the most common allergies in the industrial world. It is a chronic relapsing inflammatory disease of the skin, which causes intense itch. The skin of AE patients is characteristically red, dry and crusted. As the prevalence of other allergies, the occurrence of AE has increased during recent years, now affecting 15-30% of children and 2-10% of adults (Bieber, 2008). The cause of AE is not known but contributing factors are thought to be genetic predisposition, life style and environmental factors, defects in the skin barrier as well as an influence of microorganisms (Akdis et al., 2006; Bieber, 2008).

After the latest revision of the nomenclature the term AE should now only be used where allergen specific IgE and/or positive skin test has been determined (Johansson et al., 2004) (figure 5). The diagnosis of AE is based on clinical features assessed by the severity scoring of atopic dermatitis (SCORAD) index (Severity scoring of atopic dermatitis, 1993), exclusion of other types of eczema, serological tests of allergen specific IgE as well as skin prick test (SPT) and atopy patch test (APT) (Akdis et al., 2006).

After the latest revision of the nomenclature the term AE should now only be used where allergen specific IgE and/or positive skin test has been determined (Johansson et al., 2004) (figure 5). The diagnosis of AE is based on clinical features assessed by the severity scoring of atopic dermatitis (SCORAD) index (Severity scoring of atopic dermatitis, 1993), exclusion of other types of eczema, serological tests of allergen specific IgE as well as skin prick test (SPT) and atopy patch test (APT) (Akdis et al., 2006).

1.6.1 Genetics of AE

AE has a high hereditary occurrence, suggesting the presence of susceptibility genes specific for AE (Bieber, 2008). Several pathophysiological genes have been linked to AE (Akdis et al., 2006). Similar to other allergic diseases, the Th2 cytokine genes (IL-3, -4, -5 and -13) on chromosome 5 and the gene encoding for the β-chain of the FcεRI on chromosome 11 have been associated with AE. Since there exists a subgroup of non-atopic eczema patients (Akdis et al., 2006) (formerly called intrinsic or nonallergic AE) who have the same clinical features as other AE patients, except for having low IgE levels and no detectable specific allergen sensitization, some genes unrelated to IgE can be expected to be associated with AE (Leung and Bieber, 2003). The development of AE has also been linked to primary defects in the structure and function of stratum corneum, most commonly based on hereditary defects in filaggrin production (Elias et al., 2008). However, the genetic background alone can not explain the observed increase in AE in recent times (Novak et al., 2003).
1.6.2 Lifestyle and environmental factors contributing to AE

Lifestyle and environmental factors have been proposed as risk factors for developing AE in genetically susceptible individuals (Novak et al., 2003). The influence of the environment as early as in utero may skew the immune system of the fetus towards a Th2 profile (Novak et al., 2003). Furthermore, as proposed by the hygiene hypothesis, decreased family size, increased use of antibiotics and decreased bacterial stimulation due to improved personal hygiene may during the early years of life influence the development of AE. Stress has been suggested as another risk factor for increasing the severity of AE, but the mechanism of interaction between the nerve and the immune system is not well understood (Akdis et al., 2006). The increased levels of nerve growth factor and substance P detected in plasma of AE patients that correlate with disease severity support this theory (Toyoda et al., 2002).

1.6.3 Skin barrier in AE

The skin of AE patients is dry and fragile and several dysfunctions in the skin have been observed (Akdis et al., 2006). Well known features of AE skin is increased TEWL, decrease in stratum corneum lipids, decrease in antimicrobial peptides and increased pH (Elias et al., 2008). Recently, loss-of-function mutations were detected in the filaggrin gene in AE patients (Ekelund et al., 2008; Palmer et al., 2006; Weidinger et al., 2006). Deficiency in filaggrin functions can result in increased TEWL and disruption of the extracellular lamellar bilayers of the stratum corneum. Furthermore, decreased filaggrin has been connected to increased stratum corneum pH (Elias et al., 2008). Serine protease activity has been shown to increase upon an enhanced skin pH resulting in decrease in stratum corneum lipids, reduced water-retaining ceramides and increased levels of IL-1α and β (Elias et al., 2008). On the other hand, the immune system has also been shown to be able to modulate the barrier of the skin, since the Th2 cytokine IL-4 recently was shown to be able to suppress ceramide synthesis and inhibit filaggrin expression (Howell et al., 2007). Together, these defects result in a disrupted skin barrier with increased susceptibility to antigen penetration.

1.6.4 Itch and AE

Intense itch is a predominant symptom of AE, which significantly affects the quality of life of these patients (Bieber, 2008). The urge to scratch in order to diminish the sensation of itch often results in more pruritus generating further scratching and the patient enters a negative itch-scratch cycle, which further damages the fragile skin barrier and enables the entry of more allergens resulting in increased itch. It has been demonstrated that pain stimulation can inhibit itch, however, in AE patients this inhibition is not efficient (Ishiuji et al., 2008). Injection of acetylcholine (a major neurotransmitter) has been shown to cause itch instead of pain in patients with AE (Yosipovitch and Papoiu, 2008), highlighting the complexity of the mechanisms causing itch in AE. However, many mediators have been proposed to mediate AE itch: tryptase, histamine, IL-2, IL-31, NGF, β-endorphin, acetylcholine, prostanoids and substance P (Yosipovitch and Papoiu, 2008).
1.6.5 Host-microbe interactions in AE

AE patients are easily infected by microorganisms that can worsen the eczema (Bieber, 2008), this is probably due to the defect skin barrier of AE, which is characterized by a ruptured skin and decrease in protective antimicrobial peptides. The increased microbial burden has also been suggested to be due to defects in PRRs. NOD2 polymorphisms are associated with AE, which could be a disadvantage since NOD2 signaling has been shown to result in antimicrobial production in keratinocytes (De Benedetto et al., 2009). TLR-2 has also been suggested to be involved in the disease and mutations in the TLR-2 gene has been connected to AE, however, this connection of TLR-2 to AE is debated (De Benedetto et al., 2009). The bacteria Staphylococcus aureus has been shown to infect most AE patients causing increased inflammation through the activation of T-cells and macrophages by superantigens (Akdis et al., 2006). AE patients are also more susceptible to viral infections such as Herpes simplex or Vaccinia (Bieber, 2008). As stated previously, the yeast Malassezia is considered to be a contributing factor in AE, since sensitization to the yeast can be detected in patients with AE (Schmid-Grendelmeier et al., 2006).

1.6.6 Malassezia and AE

In 1983, Clemmensen and Hjort observed that AE patients with head and neck distribution of the eczema and sensitization to Malassezia improved clinically after oral anti-fungal treatment (Clemmensen, 1983). Since then, researchers have studied the role of the commensal yeast Malassezia as a trigger factor in AE. Approximately 50% of adolescent and adult AE patients demonstrate positive skin prick test (SPT), atopy patch test (APT) and/or specific serum IgE against Malassezia (Scheynius et al., 2002; Schmid-Grendelmeier et al., 2006). Furthermore, anti-fungal treatment, besides from decreasing eczema severity, also lowers the specific Malassezia IgE as well as total IgE levels (Bäck and Bartosik, 2001; Bäck et al., 1995; Lintu et al., 2001). Malassezia extracts contain a wide range of IgE-binding proteins. To date, the genes for 13 allergens from two different Malassezia species have been cloned (Table 1). Of these, ten genes have been cloned from M. sympodialis (ATCC strain no. 42132) and produced as recombinant allergens designated Mal a 1, and Mal a 5-13. The other three allergens (Mal f 2-4) were cloned from M. furfur (CBS strain no. 2782). The crystal structure of Mal a 1, 6 and 13 have been obtained, providing insight into the function of these allergens (Glaser et al., 2006; Limacher et al., 2007; Vilhelmsson et al., 2007). Autoreactivity caused by cross-reactivity between Malassezia allergens and human proteins has been suggested in AE, since the allergens Mal a 6, 10, 11 and 13 show high homology to human proteins (Andersson et al., 2004; Limacher et al., 2007; Lindborg et al., 1999). Cross-reactivity has been shown between Mal a 1, Mal a 6, Mal a 11 and Mal a 13 and their human homologues cyclophilin, thioredoxin and manganese superoxide dismutase, respectively (Andersson et al., 2004; Schmid-Grendelmeier et al., 2005; Vilhelmsson et al., 2008). Two of the major M. sympodialis allergens, Mal a 1 and Mal a 12, are localized in the cell wall of M. sympodialis (Zargari et al., 1997) and have been suggested to be released into culture medium and thus probably also onto the skin. Prior to the study presented in paper I and II of this thesis, the influence of host milieu on the M. sympodialis allergen release as well as the characterization of the Mal a 12 allergen had not been assessed.
Several studies have investigated the cellular and humoral immune response to *Malassezia*. The cellular interaction with the yeast has so far mainly been investigated in T-cells, keratinocytes, DCs, NK cells, melanocytes and dermal fibroblasts (Ashbee, 2006). The study of *Malassezia*’s interaction with the cellular immune system was first assessed by stimulation of peripheral blood mononuclear cells (PBMC). PBMCs from AE patients with IgE reactivity to *M. sympodialis* have been shown to have a higher proliferative response to *M. sympodialis* compared to PBMCs from healthy controls (Tengvall Linder et al., 1998). Further research attempted to investigate the effect of *M. sympodialis* on different cell populations and Tengvall Linder et al. demonstrated that *M. sympodialis* specific T-cell clones derived from lesional skin or peripheral blood of AE patients had a Th2-like cytokine profile (Tengvall Linder et al., 1998; Tengvall Linder et al., 1996).

The impact of the thick lipid capsule of *Malassezia* on the host-microbe interaction has been investigated by Thomas et al. (Thomas et al., 2008). They found that de-capsulated *Malassezia* caused an increased release of the inflammatory cytokines IL-1α, IL-6, IL-8, TNF-α as well as a decrease of the anti-inflammatory cytokine IL-10 from human keratinocytes (Thomas et al., 2008). Furthermore, the IL-8 release from keratinocytes upon stimulation with *M. furfur* has been shown to be TLR-2/MyD88 dependent (Baroni et al., 2006). In contrast, *Malassezia* cells with an intact capsule suppressed the production of the above mentioned inflammatory cytokines and caused an increased IL-10 release from keratinocytes (Thomas et al., 2008). This observed difference in activation due to the presence/absence of the lipid capsule of *Malassezia* suggests that a local variation in lipid availability on the skin could result in thinning of the lipid capsule of *Malassezia*. This could change the immune tolerance against *Malassezia* and cause inflammation. As mentioned above, AE patients have lower lipid skin content compared to healthy individuals, thus *Malassezia* present on AE skin is likely to have a thinner capsule, which would favor an inflammatory response to *Malassezia*.

LCs, that represent a subset of immature DCs present in the epidermis, have a highly efficient antigen uptake and can take up allergens diffusing through a ruptured epidermis (Banchereau and Steinman, 1998). Human immature DCs have
been shown to take up whole M. sympodialis yeast cells or allergen components of the yeast in vitro, resulting in DC maturation and release of TNF-α, IL-1β and IL-18 (Buentke et al., 2001). M. sympodialis activation exclusively caused up-regulation of IL-8, CD54, CD83, IL-1R and monocyte-derived chemokine (MDC) in DCs from AE patients, but not in DCs from healthy individuals (Gabrielsson et al., 2004), indicating an inherent difference between DCs from AE patients compared to HC. Malassezia has also been suggested to influence the interaction between DCs and NK cells, since a close contact between DCs and NK cells has been demonstrated in M. sympodialis ATP-test-positive skin of AE patients (Buentke et al., 2002). Furthermore, pre-incubation of DCs with M. sympodialis resulted in a decreased susceptibility to NK cell-induced cell death and an up-regulation of the co-stimulatory molecule CD86 (Buentke et al., 2004). Combined, these findings indicate that Malassezia under certain conditions could augment an inflammatory response in AE.

1.6.7 Mast cells in AE

The number of MCs is increased in the lesional skin of AE patients (Damsgaard et al., 1997; Horsmanheimo et al., 1994), making it particularly interesting to study their potential interaction with Malassezia and its contribution to AE. MCs can produce a broad spectrum of inflammatory mediators and thus have the ability to contribute to the pathomechanism of AE. MCs may also support the Th2 polarization in the skin of AE patients by production of IL-4 and IL-13 (Horsmanheimo et al., 1994; Obara et al., 2002). The observation that MCs in AE skin lesions express IL-8 (Fischer et al., 2006), known to be involved in recruitment of inflammatory cells, further supports the involvement of MCs in the disease. In AE lesions, the MCs have been found to concentrate in the upper dermis close to the epidermis (Järvikallio et al., 1997) and some MCs have also been found in the epidermis (Groneberg et al., 2005). The main MC type in human skin is MC TC, however, in the upper dermis of lesional AE, Järvikallio et al. found that 80% of the MCs were of MC TC type compared to 100% in normal skin (Järvikallio et al., 1997), indicating an increased infiltration of MC TC in AE skin.

Several studies have reported an association between polymorphisms of the chymase gene and AE (Mao et al., 1996; Tanaka et al., 1999; Weidinger et al., 2005). However, both inflammatory properties (Terakawa et al., 2008) and inhibitory effects (Järvikallio et al., 1997) of chymase have been suggested in the context of AE. Terakawa et al. observed that application of a chymase inhibitor decreased the skin swelling and amount of inflammatory cells in a mouse model of AE (Terakawa et al., 2008). Furthermore, links between chymase and pruritus in AE have been postulated, since injections of chymase into human skin results in itch (Hagermark et al., 1972). In contrast, Järvikallio et al. showed a significant decrease in the percentage of chymase containing MCs in AE skin and the authors suggested an inhibitory function of chymase regulating the tryptase activity (Järvikallio et al., 1997). The MC protease tryptase has also been suggested to be involved in the pathomechanism of AE through activation of the proteinase-activated receptor-2 (PAR-2) on nerve cells resulting in itch (Steinhoff et al., 2003). However, prior to the studies presented in paper III and IV of this thesis the role of MCs in the host-microbe interaction of AE had not been investigated.
2 AIMS OF THE THESIS

The general aim of this thesis was to obtain knowledge of the interaction between Malassezia and the host in AE. More specifically I aimed to elucidate the role of M. sympodialis allergens and MCs in the pathomechanisms of the disease AE.

The specific aims of the individual papers were to:

**Paper I.** Investigate whether the production and release of M. sympodialis allergens are influenced by pH conditions mimicking those observed in AE and healthy skin.

**Paper II.** Find the complete sequence encoding for the M. sympodialis allergen Mala s 12, and to characterize the protein biochemically and immunologically.

**Paper III.** Investigate if M. sympodialis can activate MCs, and if so, to study the interaction in terms of mechanism of activation, MC degranulation and cytokine release.

**Paper IV.** Study MCs derived from HC and AE patients and compare their phenotypes as well as pattern of activation when stimulated with M. sympodialis.
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3 MATERIALS AND METHODS

This section is an overview of the materials and methods used in paper I-IV. A more detailed description is given in the respective “Materials and methods” sections of the individual papers.

3.1 SUBJECTS

In paper I, II and IV we used serum samples and/or skin biopsies as well as generated mast cells from patients with AE, diagnosed according to the UK working party criteria (Williams et al., 1994). The patients were Phadiatop® positive (Phadia AB) with elevated total plasma or serum IgE and specific plasma or serum IgE to M. sympodialis (ImmunoCAP™ m70, Phadia AB). As controls in paper II and IV we used serum samples and/or skin biopsies as well as generated mast cells from healthy controls with no personal history of skin disorders and a negative Phadiatop®. The studies were approved by the Regional Ethics Committee in Stockholm, Sweden.

3.2 MICE

In paper III we generated bone marrow-derived mast cells (BMMCs) from C57BL/6 mice, gene deleted for TLR-2, TLR-4, MyD88 and Dectin-1, respectively, and wild type control mice. The study was approved by the Regional Ethics Committee for animal welfare in Stockholm, Sweden.

3.3 METHODOLOGY

Confocal laser scanning microscopy (CLSM) [I] Microscopic technique enabling scanning through samples in sections of the z-plane.


Enzyme-linked immuno sorbent assay (ELISA) [III, IV] Quantitative technique used here to detect IL-6, IL-8, MCP-1, cysteinyl leukotrienes and histamine release from mast cells.


Human mast cell generation [IV] CD34+ peripheral blood cells were cultured in the presence of requisite growth factors and cytokines to differentiate mast cells.

Immunocytochemistry [I, IV] A technique used to assess the presence of a specific protein in cells by use of a specific antibody.

Immunohistochemistry [IV] A technique to localize proteins in a tissue section through the use of specific antibodies.

Lipid binding assay [II] A protein of interest is incubated with membranes spotted with lipids.
Mouse bone marrow-derived mast cells (BMMC) [III]

Cells derived from mouse bone marrow were cultured in IL-3 conditioned medium to obtain mast cells.

M. sympodialis extract production [III, IV]

A protein based extract produced from M. sympodialis (ATCC strain 42132).

M. sympodialis specific IgE measurement [I, II, IV]

Quantitative measurement of IgE specific for M. sympodialis in plasma or serum analyzed with ImmunoCAP™ (m70, Phadia A B).

N-acetyl-β-D-hexosaminidase release assay [III, IV]

Degranulation assay, which involves measurement of an enzyme stored in the mast cell granules released in the same fashion as histamine.

Phadiatop® measurement [IV]

Detection of 11 common aeroallergens, measured with the ImmunoCAP™ method (Phadia A B).

Quantitative real-time PCR [I, IV]

Isolation of RNA from cells followed by conversion of RNA into cDNA. Thereafter PCR quantification of specific genes in real time using a florescent dye.

Recombinant protein production [II]

Cloning and expression of recombinant Mala s 12 in Escherichia coli and Saccharomyces cerevisiae.

Statistical analysis [III, IV]

Wilcoxon’s matched pairs test was employed for comparing paired samples and the Mann Whitney U-test for comparisons between the groups.

Total plasma or serum IgE measurement [I, II, IV]

Quantitative measurement of total IgE in plasma or serum with the ImmunoCAP™ method (Phadia A B).

Tryptase quantification [IV]

Detection of tryptase by ImmunoCap™ assay.

Western blotting (Immunoblotting) [I, II, III]

Gel electrophoretic separation of proteins and transfer to protein binding membranes for enzyme-conjugated antibody detection.
4 RESULTS AND DISCUSSION

4.1 THE ENHANCED PH OF AE SKIN STIMULATES RELEASE OF M. SYMPODIALIS ALLERGENS (PAPER I)

The skin pH of AE patients is augmented (pH 5.90±0.76), compared to that of normal healthy skin (pH 5.36±0.5) which is one of the characteristics of the disturbed skin barrier in AE (Sparavigna et al., 1999). This observed enhanced pH has been connected to the severity of itching and an increased microbial colonization in patients with AE (Rippke et al., 2004; Schmid-Wendtner and Korting, 2006). Since the commensal yeast M. alassezia can act as a trigger factor in AE (Scheynius et al., 2002), we were interested in examining the effect of increased host pH on the allergenicity of the yeast. We therefore investigated whether higher pH mimicking that of AE skin may stress M. sympodialis to release more allergens into the environment. To assess this question we cultured M. sympodialis in Dixon broth medium at different pH. Initially a tube cultivation was set up and the yeast was cultured at pH 6.1 or pH 5.5 for up to 15 days. However, during the culture period we observed a decrease of the culture pH, and therefore we performed large scale fermentor experiments at pH 6.1 and pH 5, where we could control the pH as well as obtain more material for further analysis. Thereafter, the culture supernatants were analyzed for the presence of IgE-binding components by using immunoblotting. The analysis of the culture supernatants revealed that allergen release by M. sympodialis was enhanced in the culture supernatants with the higher pH (figure 6), thus suggesting that the skin of AE patients provides an environment where M. sympodialis will be triggered to release augmented amounts of allergens. Since the viability during the culture period was 100% at both pH conditions, it is not likely that the observed enhanced allergen release is due to release from dead cells. However, another observed difference was that in the pH 6.1 culture the cell number was four times lower compared to that of pH 5, indicating that the higher pH reduces the growth rate of M. sympodialis. This might provide an explanation for the lower number of M. sympodialis cells isolated from the skin of AE patients compared to healthy controls (Gupta et al., 2001; Sandström Falk et al., 2005). Despite that the cells cultured at pH 5 grow four times better, still more allergens were detected in the culture of pH 6.1.

![Image](image_url)
The observed pattern of increased allergen release after culture at the skin pH of AE patients was particularly true for a 67-kDa allergen, designated Mala s 12. Interestingly, Mala s 12 is present in the cell wall of *M. sympodialis* (Zargari et al., 1997), thus exposed for interaction with the environment. To determine if the increased release of the allergen also was reflected on the cell surface, *M. sympodialis* cells cultured at pH 5 or pH 6.1 were immunocytochemically stained for the presence of Mala s 12. The staining intensity was examined using confocal laser scanning microscopy (CLSM) and we could determine that *M. sympodialis* cultured at pH 6.1 for 24 h expressed approximately three times more Mala s 12 on their cell surface compared to cells cultured at pH 5 (figure 7). After 48 h of culture cells from both pH 6.1 and pH 5 expressed similar amounts of the Mala s 12 allergen on their cell surface. However, one should keep in mind that no Mala s 12 release was detected from *M. sympodialis* cultured for 48 h at pH 5 (figure 6). This indicates that although an equal expression of Mala s 12 was detected on the cell surface of *M. sympodialis* cultured for 48 h at pH 5 compared to pH 6.1, this does not reflect the amount of Mala s 12 released.

We went further to investigate if the enhanced pH of AE skin also could influence the transcription of Mala s 12. To assess this question, cDNA was derived from *M. sympodialis* cells cultured in either pH 6.1 or pH 5 and the expression of Mala s 12 mRNA was determined using quantitative PCR. An increased Mala s 12 mRNA expression was found in cells cultured at pH 6.1 compared to pH 5 (figure 8), suggesting that the pH of AE skin has an effect on *M. sympodialis* on the transcriptional level as well.
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The cause of the increased skin pH of AE is not known, but studies suggest that urocanic acid might influence skin acidity (Schmid-Wendtner and Korting, 2006). Urocanic acid is produced in the skin from histidine by the enzyme histidase (Kröner and Kermici, 2000). Interestingly, there is a significant decrease in urocanic acid in AE skin, indicating a reduced histidase activity (Schmid-Wendtner and Korting, 2006). Additionally, deficiency in the filaggrin gene has been suggested to contribute to increased skin pH in AE patients (Elias et al., 2008).

A connection between the skin microbial flora and skin pH has been proposed. Microorganisms grow at different rates at different pH, thus (Schmid-Wendtner and Korting, 2006) an increased pH may result in changes of the microflora. Another yeast, Candida albicans, has been shown to be more invasive when applied on healthy skin in suspension of pH 6 compared to pH 4.5 (Runeman et al., 2000). Notably, AE patients are also more frequently colonized by Candida species (Faergemann, 2002), however, in contrast to Malassezia, the involvement of Candida species in AE pathology is questionable (Schmid-Grendelmeier et al., 2006).

The findings presented in this study indicate that it would be beneficial for AE patients who are allergic to M. sympodialis to use acidic soaps and creams. Although this has to be verified, one can speculate that acidic pH might result in decreased allergen release in vivo on the skin. Treatment with either a pH 5 cream or topical application of acidic electrolytic water has been shown to significantly improve the eczema of AE patients (Rippke et al., 2004). The use of conventional soaps with high pH have been shown to increase the pH of the hands of healthy individuals approximately three times and the pH was not completely normalized even after 90 min (Schmid-Wendtner and Korting, 2006). On the other hand, soaps have been used by humans for many thousands of years, although less frequently compared to recent decades. However, during the last 30 years, it has become more common to use synthetic cleaning agents (Schmid-Wendtner and Korting, 2006). The use of soaps with acidic pH have also been shown to reduce both bacterial infection and viral transmission compared to alkaline pH soaps (Schmid-Wendtner and Korting, 2006).

Collectively, we demonstrated that higher pH triggers an augmented allergen release from M. sympodialis. The observed difference was especially true for the Mala s 12 allergen, which is produced, expressed and released to a greater extent when cultured at a pH resembling that of AE skin. This suggests that Malassezia on the skin of AE patients will release more allergens, which would trigger inflammation.

4.2 CHARACTERIZATION OF THE ALLERGEN MALA S 12 (PAPER II)

After having observed that the Mala s 12 allergen was one of the most prominent allergens released from M. sympodialis, cultured at a pH resembling AE skin, we aimed to express it as a recombinant protein to be able to characterize Mala s 12 both biochemically and immunologically. A truncated form of the allergen had previously been isolated by screening IgE-binding clones of a M. sympodialis phage display cDNA library. With the help of this truncated form we succeeded in obtaining the complete sequence encoding Mala s 12. We proceeded to express the cDNA encoding Mala s 12 as a His6-tagged protein in E. coli and thereafter purified it from both the soluble fraction and inclusion bodies. The obtained recombinant protein could be recognized by our MoAb (designated 9G9C8) directed against native-Mala s 12 (Zargari et al., 1994).
The obtained complete sequence for *M. ala s 12* is composed of 1857 bp, which contains an open reading frame of 618 amino acids and has a calculated molecular mass of 66.286 kDa. The *M. ala s 12* sequence also show a similarity of 30-50% to the glucose-methanol-choline (GMC) oxidoreductase enzyme superfamily. This enzyme family is a group of flavoenzymes and one of them, glucose oxidase (GOX) has been derived from the fungus *Aspergillus niger* (Frederick et al., 1990), known to be involved in the allergic respiratory disease aspergillosis (Tillie-Leblond and Tonnell, 2005). Interestingly, GOX converts glucose and oxygen into gluconolactone and the corrosive hydrogen peroxide, the latter potentially an irritant if released onto the skin.

To further investigate the observed similarity to the GMC oxidoreductase enzyme superfamily we determined if r*M. ala s 12* contained flavin like the other members of the GMC oxidoreductase enzyme superfamily. We could determine that the purified protein contained flavin and, with MALDI-TOF mass spectrometry, we observed that it specifically contained flavin adenine denucleotide (FAD). To test if r*M. ala s 12* possessed enzymatic activity, a number of alcohols, which are common substrates for other members of the GMC oxidoreductase enzyme superfamily, were tested as possible substrates for r*M. ala s 12*. The enzymatic activity was first tested at pH 7, at which no enzymatic activity could be detected. Next we also examined the enzymatic activity at pH 5.5, which resembles the pH of normal skin and thus the normal habitat of *Malassezia*, however, we were unable to detect any enzymatic activity at this pH. These findings propose that *M. ala s 12* either utilizes a yet untested alcohol as a substrate or, despite having sequence similarity with the enzymes of the GMC oxidoreductase enzyme superfamily, *M. ala s 12* does not possess enzymatic activity. Posttranslational modifications in *E. coli* could affect the enzymatic activity of r*M. ala s 12* and we therefore also expressed it in the yeast *S. cerevisiae*. However, no enzymatic activity could be detected. Current evidence suggests that enzymatic activity is not required for inducing allergy (Wymann et al., 1998), and this is supported by the finding that many allergens, including cat, birch pollen and Der p 2, are not enzymatically active (Sehgal et al., 2005).

*M. ala s 12* has, as previously stated, been shown to be localized at the cell surface of *M. sympodialis* (Zargari et al., 1997). To further investigate the properties of *M. ala s 12*, we investigated the ability of the protein to interact with membrane lipids, known to take part in membrane transport. We found that r*M. ala s 12* strongly associated with phosphatidic acid (PA), phosphatidylinositol-4-phosphate (P) and PtdIns(4,5)P2, which all are associated with membrane transport (Haucke and Di Paolo, 2007; Roth, 2004). This suggests that these lipids might be involved in the transport of r*M. ala s 12* to the plasma membrane of *M. sympodialis*.

To evaluate the IgE-binding ability of r*M. ala s 12* compared to native *M. ala s 12*, we performed inhibition immunoblotting using *M. sympodialis* extract as the source of native *M. ala s 12*. We found that r*M. ala s 12* possesses the same allergenicity as the native *M. ala s 12*, since *M. sympodialis* extract inhibits the binding of AE serum IgE to the r*M. ala s 12* and r*M. ala s 12* significantly inhibited the IgE-binding to the native *M. ala s 12* in the *M. sympodialis* extract. To further evaluate the IgE-binding capacity of r*M. ala s 12*, sera from 21 AE patients sensitized to *M. sympodialis* and 5 healthy control sera were used to detect IgE-binding to the r*M. ala s 12*. 62% of the sera
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contained IgE to rMal a s 12, thus defining it as a major allergen of M. sympodialis sensitized AE patients.

In this study we have characterized the molecular and immunological properties of Mal a s 12. It is important to understand the properties of an allergen as resistance to degradation, binding capacity, sequence homology and biological functions, since they are indicators of allergenicity (Sehgal et al., 2005). The sequence homology of Mal a s 12 to the GMC oxidoreductase enzyme superfamily indicates that Mal a s 12 might function as an enzyme, although this has to be further verified since we were unable to show that Mal a s 12 possesses enzymatic activity. We could, however, also determine that Mal a s 12 is probably transported to the cell wall of M. sympodialis through the interaction with membrane lipids. This characteristic of binding lipids has also been noted among other allergens such as food allergens (Breiteneder and Clare Mills, 2005). One can speculate that since Mal a s 12 is expressed on the cell surface of M. sympodialis (Zargari et al., 1997) and can be released into the culture medium (paper I), it is likely that Mal a s 12 would also be released in vivo, onto the ruptured skin of AE patients. Following release onto the skin, Mal a s 12 could probably come into contact with immune cells, such as LCs and MCs. These cell types both express the FcRI for IgE and are suggested to be involved in the pathogenesis of AE (Bieber, 2008; Kalesnikoff and Galli, 2008). Since we herein demonstrate that Mal a s 12 can be recognized by IgE in a majority of AE patients’ serum, it is likely that these immune cells can be activated by Mal a s 12.

In summary, we have cloned a major allergen from M. sympodialis, Mal a s 12, and the resulting protein can be grouped into the GMC oxidoreductase enzyme superfamily. These characteristics of Mal a s 12 demonstrate that it has the potential of contributing to the inflammation in AE skin.

4.3 M. SYMPODIALIS CAN ACTIVATE MAST CELLS (PAPER III)

The number of MCs is increased in the lesional skin of AE patients (Damsgaard et al., 1997; Horsmanheimo et al., 1994) and can thus be suspected to contribute to the chronic inflammation. Due to the ruptured skin barrier in AE, it is likely that compounds from Malassezia can come into contact with MCs in the skin. We were therefore interested in exploring this proposed interaction and hypothesized that Malassezia can activate MCs.

In order to assess this question we decided to analyze the effect of M. sympodialis extract on bone marrow-derived MCs (BMMCs) from C57BL/6 mice. We chose to work with MCs derived from mice, since we then could use gene deleted mice as a well-established model system to explore possible activation mechanisms. The yeast extract was chosen as a tool to investigate the possible interaction between Malassezia and MCs, since it contains a variety of allergens known to be recognized by serum from AE patients sensitized to M. sympodialis. Furthermore, we find it more likely that the MCs in the skin would come in contact with components from Malassezia diffusing through a ruptured epidermis rather than the intact yeast. The extract was first tested for its ability to activate non-sensitized Wt BMMCs and we analyzed signs of activation as increased degranulation, release of cysteinyl leukotrienes and production of the inflammatory cytokine IL-6 and the chemokine MCP-1. We could not observe any degranulation or release of IL-6 and MCP-1 from non-sensitized Wt BMMCs following addition of M. sympodialis extract. We found,
however, that the extract stimulated non-sensitized Wt BMMCs to release cysteinyl leukotrienes in a dose-dependent manner. Olynych et al. have previously shown that the fungal product zymosan can induce release of cysteinyl leukotrienes from MCs, by signaling through the fungal recognition receptor Dectin-1 (Olynych et al., 2006). In contrast, in our study the observed release of cysteinyl leukotrienes was independent of Dectin-1, since Dectin-1 knockout BMMCs released cysteinyl leukotrienes in a similar fashion to Wt BMMCs following culture with M. sympodialis extract. Since Dectin-1 recognizes carbohydrates, and the M. sympodialis extract used in this study mainly is composed of proteins and only low amounts of carbohydrates, this could provide a possible explanation for the independence of Dectin-1 in our study.

We next investigated the effect of M. sympodialis extract on IgE-sensitized MCs, since MCs in the skin of AE patients express IgE on their surface (Grabbe et al., 1996). BMMCs from Wt mice were sensitized with IgE-anti-trinitrophenyl (TNP) and thereafter treated with 0.01-100 µg/mL M. sympodialis extract. The highest concentration of M. sympodialis extract induced MC degranulation, release of MCP-1 but no IL-6 production. Chemokine production in MCs is mediated through MAPK signaling (Wong et al., 2006), and we therefore investigated if M. sympodialis could cause activation of MAPK in IgE-sensitized MCs. We determined that IgE-sensitized Wt BMMCs, treated with the highest concentration of M. sympodialis extract, activated phosphorylation of the MAPK ERK 1/2, indicating that M. sympodialis can activate MAPK signaling in IgE-sensitized MCs. This enhanced MAPK activation may be connected to the MCP-1 release from IgE-sensitized MCs cultured with M. sympodialis extract. Additionally, M. sympodialis extract induced release of cysteinyl leukotrienes from IgE-sensitized MCs in a dose-dependent manner. Interestingly, the levels of cysteinyl leukotrienes released were increased in IgE-sensitized MCs compared to non-sensitized MCs. This indicates that the IgE-sensitization increases the MCs’ susceptibility to release cysteinyl leukotrienes in response to M. sympodialis activation. These findings concord with a study by Genovese et al., which demonstrates that the interaction between IgE on MCs and bacterial antigens results in an enhanced release of cysteinyl leukotrienes (Genovese et al., 2000). In agreement with our results from non-sensitized MCs, the release of cysteinyl leukotrienes from IgE-sensitized MCs cultured with M. sympodialis extract, was not dependent on signaling through Dectin-1.

As BMMCs express several TLRs (Marshall, 2004) we explored if M. sympodialis could interact with MCs through a TLR-dependent pathway that mediates degranulation and MCP-1 release. BMMCs from MyD88 knockout mice were generated, since MyD88 is a protein involved in the signaling pathway of most TLRs (Takeda et al., 2003). IgE-sensitized MyD88 knockout BMMCs were after culture with M. sympodialis extract activated in a similar fashion to Wt BMMCs, indicating a MyD88-independent activation of M. sympodialis-exposed IgE-sensitized MCs. This observed antigen-independent activation of MCs suggests that some components of the M. sympodialis extract acts on IgE-anti-TNP-sensitized MCs through what might be described as an ‘IgE-superantigen-like’ effect. Proteins from bacteria have previously been demonstrated to bind to FceR1-bound IgE and thereby act as IgE superantigens (Genovese et al., 2003).

It has been reported that E. coli can interfere with MC responses and can negatively affect IgE-mediated activation (Kulka et al., 2006). We therefore studied the
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Effect of M. sympodialis extract on the FcεRI expression of Wt BMMC. We noted similar FcεRI expression after 24 h of incubation with or without M. sympodialis extract. We further analyzed if M. sympodialis would affect MC activation induced by aggregation of IgE receptors. IgE-anti-TNP-sensitized Wt BMMC were therefore activated by addition of TNP-BSA together with increasing amounts of M. sympodialis extract. The M. sympodialis extract significantly enhanced the IgE-mediated degranulation and modified the IL-6 release in a dose-dependent manner, however, no increase in release of cysteinyl leukotrienes or MCP-1 was detected in IgE-receptor cross-linked MCs upon addition of M. sympodialis extract.

To determine if activation through PRRs could be the cause of the increased degranulation, we assessed the activation of BMMC from Dectin-1, TLR-2, TLR-4 and MyD88 knockout mice, respectively, following their co-activation with TNP-BSA and M. sympodialis extract. However, all the deficient BMMC exhibited equivalent degranulation reactivity to Wt BMMC. Thus, the cause of the observed increase in degranulation can not be explained by signaling through either Dectin-1, TLR-2, TLR-4 or MyD88.

As stated above M. sympodialis extract affected IgE-receptor cross-linked BMMC to alter their IL-6 production in a dose-dependent manner. Addition of low concentrations of M. sympodialis extract led to a significant increase in the IL-6 production and high concentrations led to a significant decrease. A synergistic activation through FcεRI and either of TLR-2 or TLR-4 has been reported to aggravate IL-6 production in BMMC (Qiao et al., 2006), and we thus proceeded to investigate how M. sympodialis extract influenced IgE-receptor cross-linked BMMC from TLR-2, TLR-4 and MyD88 knockout mice, respectively. The IL-6 release from TLR-4 knockout BMMC was influenced in a similar fashion as Wt BMMC by addition of M. sympodialis extract. In contrast, M. sympodialis extract exerted no significant effect on IL-6 production in IgE-receptor cross-linked BMMC derived from TLR-2 or MyD88 knockout mice, respectively. This indicates a dependency on signaling through the TLR-2/MyD88 pathway and a possible synergistic effect between TLR-2 and FcεRI. These findings corroborate with the work of Baroni et al., which showed that human keratinocytes increase their gene expression of TLR-2 and MyD88 following activation with M. furfur (Baroni et al., 2006).

Since cytokine production in MCs has been shown to require MAPK signaling (Qiao et al., 2006), we next studied if M. sympodialis extract influenced activation of MAPK in IgE-receptor cross-linked Wt BMMC cultured with or without M. sympodialis extract. We found that higher concentrations of M. sympodialis extract inhibited phosphorylation of the MAPK ERK 1/2, indicating that the modulation of the IL-6 release might be mediated through the ERK 1/2 pathway.
Figure 9. A schematic model for activation of MCs by M. sympodialis extract. Non-sensitized MCs release cysteinyl leukotrienes after activation with M. sympodialis extract (A). IgE-sensitized MCs release cysteinyl leukotrienes, degranulate and produce MCP-1 but not IL-6 upon addition of M. sympodialis extract, as indicated in bold letters (B-C). M. sympodialis extract enhances the degranulation of IgE-receptor cross-linked MCs and by signaling through TLR-2 modifies their IL-6 production, as indicated in bold letters (D-F).

Taken together, our results demonstrate that M. sympodialis extract causes release of cysteinyl leukotrienes from non-sensitized MCs. In IgE-sensitized MCs, the extract induced release of cysteinyl leukotrienes, degranulation, MCP-1 production and activation of the MAPK ERK 1/2. Moreover, M. sympodialis extract enhanced IgE-dependent MC activation, inhibited activation of the MAPK ERK 1/2 and altered IL-6 production in a dose-dependent manner through the TLR-2/MyD88 pathway. Our findings (summarized in figure 9) imply that the effects of Malassezia on MCs might exacerbate the inflammation in AE.

4.4 MAST CELLS FROM PATIENTS WITH AE SHOW AN ENHANCED ACTIVATION TO M. SYMPODIALIS COMPARED TO MAST CELLS FROM HC (PAPER IV)

After discovering that mouse MCs can be activated by M. sympodialis extract (paper III), we wanted to investigate if these results also could be verified in the human system. Therefore, we aimed to study the interaction between M. sympodialis extract and MCs derived from AE patients, with M. sympodialis sensitization, compared to
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MCs from healthy controls (HC). Studies on human MCs have mostly been conducted using cord blood-derived MCs (CBMCs) (Saito et al., 1995), since peripheral blood-derived progenitors have a low proliferation rate, thereby limiting the possibilities of investigating adult human MCs. However, a protocol was recently established for generating high numbers of skin-like MCs from human peripheral blood (Lappalainen et al., 2007), facilitating more extensive studies of the role of MCs from adult individuals in different human diseases. We used this protocol in order to generate MCs from both AE patients and HC, and the obtained MCs were thereafter exposed to M. sympodialis extract.

Both types of MCs generated showed homogenous cell growth, viability and morphology throughout the culture period of 9 to 11 weeks. Despite this, enhanced amounts of the granule mediators’ β-hexosaminidase, histamine, and tryptase were found in MCs from AE patients as compared to HC. Following IgE-receptor cross-linking or culture with M. sympodialis extract, however, similar levels of the mediators were released from MCs of both HC and AE patients. One can speculate that upon a amplified activation, the MCs from AE patients may retain an augmented response to degranulate compared to MCs from HC. In vivo data support this, by augmented levels of histamine in skin and plasma of AE patients (Ring and Thomas, 1989). The enhanced levels of histamine and tryptase in AE patients’ MCs could have an impact on disease severity in the AE skin, since histamine recruits effector cells into tissues and affects their maturation, activation and polarization, thereby contributing to chronic inflammation (Jutel and Akdis, 2007). The observed enhanced granule content of tryptase in MCs derived from AE patients might also contribute to AE-related itch, since evidence has emerged for the role of tryptase in the itch mechanism of AE, through activation of the proteinase-activated receptor-2 (PAR-2) on nerve cells (Steinhoff et al., 2003).

To further investigate the reactivity of MCs derived from AE patients compared to HC we determined their ability to release inflammatory cytokines following stimulation with M. sympodialis extract. Interestingly, only MCs derived from AE patients responded to M. sympodialis extract by releasing the inflammatory cytokine IL-6. This cytokine has been demonstrated to modulate the Th1/Th2 differentiation of CD4+ T cells by promoting Th2 differentiation (Diehl and Rincon, 2002), an imbalance that has been associated with AE pathology (Akdis et al., 2006). In addition, IL-6 has been reported to support human MC growth (Kinoshita et al., 1999) and to increase MC histamine content (Kinoshita et al., 1999). Collectively, these data suggest that Malassezia compounds diffusing through the ruptured skin of AE patients, might, by inducing the release of IL-6 from activated MCs, augment the inflammatory response and may further enhance MC density as observed in the lesional skin of AE patients (Damsgaard et al., 1997).

In comparison to IL-6, M. sympodialis extract enhanced the IL-8 production of IgE-receptor cross-linked MCs from both AE patients and HC. These results indicate that M. sympodialis could aggravate an already ongoing allergic response in AE. Although the MCs from HC and AE patients responded with IL-8 release in a similar manner, one should keep in mind that elevated levels of IL-8 positive MCs have been noted in AE skin compared to in healthy skin (Fischer et al., 2006), which may result in an increased IL-8 release in AE compared to in HC.
Furthermore, IgE-receptor cross-linking is more likely to occur in patients with AE, who are sensitized to a variety of allergens (Akdis et al., 2006).

To elucidate the mechanism of Malassezia activation, we further investigated the presence of the fungal recognition receptors TLR-2 and Dectin-1 on MCs derived from HC and AE patients. These two receptors were chosen, since we in paper III demonstrated that M. sympodialis can activate murine BMMCs to produce IL-6 through the TLR-2/MyD88 pathway, and it has previously been shown that the fungal product zymosan can induce MC activation through the Dectin-1 receptor (Olynych et al., 2006). No increase in TLR-2 mRNA expression was evident after MC activation, whereas, following IgE-receptor cross-linking the Dectin-1 receptor was exclusively up-regulated in MCs from HC. This finding could indicate that MCs from AE patients are unable to increase their Dectin-1 mRNA expression upon activation, and this would generate a defect response against fungal infection.

Dectin-1 has also been suggested to stimulate proliferation and activation of T-cells (Kimberg and Brown, 2008), which suggests a decreased ability of MCs in AE skin to activate T-cells. The role for the observed Dectin-1 mRNA up-regulation upon IgE-receptor cross-linking in MCs from HC could possibly function as an increased defense mechanism during a parasitic infection with the purpose of increasing T-cell recruitment.

In order to verify that Dectin-1 is also present on MCs in the skin, we stained skin sections from AE patients and HC. As previously shown by others (Damsgaard et al., 1997; Horsmanheimo et al., 1994), we could confirm an increase of tryptase-positive MCs in the AE lesional skin compared to HC skin. We also found that a majority of tryptase-positive MCs in both HC and AE patients’ skin expressed Dectin-1. To our knowledge, the expression of Dectin-1 has not been investigated in human skin prior to this study.

The observed differences in our study between MCs derived from AE patients and HC suggests that MCs from patients with AE may have a different gene expression profile compared to MCs from HC. This suggestion concord with the findings of Gabrielson et al. who described a difference in gene expression between in vitro monocyte-derived dendritic cells from AE patients compared to HC (Gabrielson et al., 2004).

In conclusion, the findings presented in this study suggest that MCs derived from AE patients and HC are different regarding the amount of preformed granule mediators, their IL-6 secretion in response to M. sympodialis, and in their ability to up-regulate the fungal recognition receptor Dectin-1 upon IgE-receptor cross-linking. These observed differences in MC activity provide new insights into the pathogenic mechanisms underlying AE and indicate an inherent difference between MCs from HC and AE patients.
5 CONCLUSIONS

**Paper I.** In this study we demonstrated that the yeast *M. sympodialis* produced, expressed and released an enhanced amount of allergens when cultured at a pH resembling that of AE patient skin. This was particularly true for the *Mala*s 12 allergen. These findings suggest that *M. sympodialis* on the skin of AE patients could release more allergens and thus contribute to the inflammation.

**Paper II.** We cloned, produced and characterized the *Mala*s 12 allergen. The amino acid sequence of *Mala*s 12 showed 30-50% similarity to the glucose-methanol-choline (GMC) oxidoreductase enzyme superfamily. The r*Mala*s 12 did not show any enzymatic activity, suggesting that it either utilizes an untested alcohol as substrate or that it lacks enzymatic activity. Furthermore, *Mala*s 12 was found to be a major allergen in AE.

**Paper III.** Here we investigated if the yeast *M. sympodialis* can activate MCs. We demonstrated that *M. sympodialis* can indeed activate MCs, since *M. sympodialis* extract causes release of cysteiny1 leukotrienes from nonsensitized MCs. In IgE-sensitized MCs, the extract induced release of cysteiny1 leukotrienes, degranulation, MCP-1 production and activation of the MAPK ERK 1/2. Moreover, *M. sympodialis* enhances MC IgE-dependent activation, inhibits MAPK activation and alters the IL-6 production in a dose-dependent manner through the TLR-2/MyD88 pathway.

**Paper IV.** In this study we examined the phenotype and pattern of activation of MCs derived from AE patients compared to HC. We determined that MCs from AE patients sensitized to *M. sympodialis* differ from MCs from HC. MCs from AE patients have increased intrinsic levels of granule mediators and release enhanced amounts of IL-6 when activated by *M. sympodialis*. Furthermore, MCs from AE patients may be unable to up-regulate their Dectin-1 receptor expression upon activation. These observed differences in MC activity indicate an inherent difference between MCs from HC and AE patients.

In summary, the work included in this thesis has contributed to the understanding of the host-microbe interaction of *M. sympodialis* in AE. We provide evidence indicating that *M. sympodialis* on the skin of AE patients will be triggered by the elevated skin pH to increase the release of allergens. Furthermore, we demonstrate that MCs can be activated by *M. sympodialis* and that AE patients' MCs differ from MCs from HC. Our findings could contribute to the development of new treatment strategies for AE patients sensitized to *M. sympodialis*. 
FUTURE PERSPECTIVES

The work presented in this thesis has provided new insights into the host-microbe interaction of *Malassezia* in AE, and the role of MCs in this interaction. These findings also generated new questions and in the following section I will elaborate on what would be interesting to explore in the future.

In paper I, we demonstrated that a pH resembling that of AE skin stimulated an enhanced release of multiple allergens from *M. sympodialis*. We subsequently investigated the effect of the enhanced pH on one of the allergens, namely *Mala s 12*. The effect of the increased pH on a number of other *M. sympodialis* allergens remains to be characterized. In doing so a broader understanding of the reason for the yeast to release these proteins could be obtained. Changes in pH can transform an opportunistic microorganism into becoming pathogenic (Campbell, 1999) and it would therefore also be interesting to determine the effect of the increased pH of AE skin on the other microorganisms that are part of the human microflora. One obvious question after discovering that the pH of AE skin triggers allergen release from *M. sympodialis* in vitro is: does this also occur in vivo? Previous work in our laboratory has attempted to stain *Malassezia* allergens on AE skin, however, this proved to be technically difficult. Given that an optimal detection method could be developed one possibility to verify this would be to analyze skin biopsy specimens from AE and HC skin for the presence of *Malassezia* allergens.

In paper II, we cloned, produced and investigated the characteristics of the *M. sympodialis* allergen *Mala s 12*. From this study we obtained data showing that *Mala s 12* has sequence similarities to the GMC oxidoreductase enzyme superfamily and that *Mala s 12* can interact with membrane lipids. It would in my opinion be worth to investigate if *Mala s 12* also could interact with lipids on human skin, since our results in paper I suggest that *Mala s 12* could be released onto the skin. Moreover, to come closer to discovering the function of *Mala s 12* in the yeast, one approach would be to generate *Mala s 12* knockout *M. sympodialis* and to study the properties of the resulting yeast. The fact that the sequence for the whole *M. sympodialis* genome is not available has hampered the research on understanding the allergic properties of *M. sympodialis* in AE. However, the genomes of two other *Malassezia* species, *M. globosa* and *M. restricta*, were recently sequenced (Xu et al., 2007) providing valuable information on the host-microbe interactions of these species. Obtaining the full sequence also for *M. sympodialis* might thus help us to further understand the features of *M. sympodialis* and its contribution to AE inflammation.

In paper III and IV, we demonstrated that *M. sympodialis* can activate MCs, which had not been investigated prior to our studies. Many questions remain to be answered regarding the mechanisms of activation. For example one puzzling question from our investigation of mice MCs: what is the mechanism by which *M. sympodalis* extract causes activation of IgE-sensitized MCs? In paper III, we speculate that this activation could be mediated through an ‘IgE-superantigen-like’ effect. To find out more about this interaction, I would like to proceed by determining the component(s) in the *M. sympodialis* extract responsible for activating the IgE-sensitized MCs. The *M. sympodalis* extract used to activate MCs in paper III and IV is mainly composed of proteins. To more thoroughly determine the role of *M. sympodalis* in AE, the effect of other components of the yeast, such as lipids or carbohydrates, on MC
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activation should be investigated. We are currently in the process of determining the carbohydrate composition of *M. sympodialis* and have indications of the presence of (1-6)-β-glucan, mannan and chitin (Kruppa et al. Abstract submitted to the American Society for Microbiology 109th General Meeting, Philadelphia, Pennsylvania, USA, May 17-21, 2009).

We have preliminary data indicating that the ‘IgE-superantigen-like’ activation can also be observed in human CBMCs sensitized with myeloma IgE. In contrast, this ‘IgE-superantigen-like’ activation could not be observed in human MCs derived from adult individuals (paper IV). This may, however, be explained by the use of different sources of myeloma IgE. The next step would thus be to use the same myeloma IgE as was used to sensitize CBMCs for the sensitization of MCs derived from adult individuals, to see if we then could detect the ‘IgE-superantigen-like’ effect also in MCs derived from adult individuals.

Another question remaining to be clarified is the mechanism responsible for the increased degranulation from IgE-receptor cross-linked MCs upon stimulation with *M. sympodialis* extract. In paper III we determined that this enhanced activation was not mediated through the co-stimulation of FceRI and the following PRRs: TLR-2, TLR-4, MyD88 and Dectin-1. The enhanced degranulation could possibly be due to the interaction of *M. sympodialis* extract components with IgE or directly with its receptor FceRI, which could result in strengthening of the FceRI activation.

We observed MC activation by the yeast *M. sympodialis*, and therefore it would also be interesting to study a possible MC activation by other yeasts like Cryptococcus and Candida. The colonization of both of these yeasts is increased on atopic eczema skin compared to healthy skin (Schmid-Grendelmeier et al., 2006).

In the last study of this thesis we obtained data indicating that MCs derived from AE patients and HC are inherently different. To investigate the underlying cause of this difference a future gene expression microarray analysis could be made of MCs derived from AE patients compared to HC. Furthermore, it would be interesting to determine if this difference between MCs derived from AE patients compared to HC could be due to epigenetic regulation. In paper IV we also demonstrate that MCs from HC up-regulate their Dectin-1 mRNA expression after IgE-receptor cross-linking, whereas MCs derived from AE patients fail to do so. The function in HC of Dectin-1 up-regulation following IgE-receptor cross-linking could, as we speculate in paper IV, be a beneficial mechanism for the host during a parasitic infection, since Dectin-1, besides being a fungal recognition receptor, also can stimulate T-cell recruitment and activation. Thus, further research should attempt to determine the connection between IgE-receptor cross-linking in MCs and the expression of Dectin-1.

The overall goal of the work presented in this thesis is to improve the understanding of how Malassezia can go from being a harmless yeast of the commensal flora to promoting allergy in AE patients. Yet, a more complete understanding of the triggers of AE is needed, an understanding which ultimately could be used for the development of novel treatment strategies.
7 POPULÄRVETENSKAPLIG SAMMANFATTNING


Atopi är en individuell och/eller årtlig benägenhet att bilda IgE-antikroppar mot harmlösa antigen (allergen), ofta proteiner. Den allergiska individens immunsystem reagerar mot allergen, som t.ex. pollen, med hjälp av dessa IgE-antikroppar. Det ger upphov till symtom som klåda, snuva och svullnad. Patienter med atopiskt eksem har oftast IgE-antikroppar mot allergen i omgivningen som kan förvärra eksemet. Jästsvampen *Malassezia* som alla människor normalt har på huden anses förvärra atopiskt eksem. IgE-antikroppar mot *M. sympodialis* allergen har hittats hos omkring 50 % av vuxna patienter med atopiskt eksem. När patienter med *M. sympodialis*-allergi behandlats med svampdödande medel förbättras eksemet och IgE-nivåerna i blodet minskar.

Det övergripande syftet med min avhandling har varit att utforska interaktionen mellan *M. sympodialis* och dess värd människan i samband med atopiskt eksem. Mer specifikt så ville vi undersöka hur det förhöjda pH-värdet på atopisk eksemhud påverkar jästsvampen *M. sympodialis*‘s förmåga att utsöndra allergen, samt undersöka om *M. sympodialis* kan aktivera mastceller.


Vi ville därför i den sista studien undersöka om dessa fynd också gällde mastceller hos människa och om mastceller från patienter med atopiskt eksem är annorlunda än mastceller från friska individer. Resultaten visar att även mastceller från människa kan aktiveras av *M. sympodialis*. Mastceller från patienter med atopiskt eksem reagerar starkare på *M. sympodialis*-aktivering som jämfört med mastceller från friska individer. Vi kunde dessutom visa att mastceller från patienter med atopiskt eksem innehöll mer inflammationsframkallande ämnen i sina granula jämfört med mastceller från friska individer.

Sammantaget har studierna som presenteras i den här avhandlingen bidragit till en ökad förståelse av samspelet mellan *M. sympodialis* och dess värd människan. Resultaten visar att det förhöjda hud-pH:t hos patienter med atopiskt eksem stimulerar *M. sympodialis* till att frisätta mer allergen och att *M. sympodialis* kan aktivera mastceller till att starta en inflammation. Dessa observationer tyder på att lokal behandling med surt pH skulle vara fördelaktigt för patienter med atopiskt eksem och *M. sympodialis* allergi. Eftersom vi fann att mastceller från patienter med atopiskt eksem reagerar mer kraftfullt mot *M. sympodialis* skulle behandling med mastcellsstabiliserare vara en möjlig strategi för att mildra eksemet.
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Tack Christine.
9 REFERENCES


The role of *Malassezia* allergens and mast cells in atopic eczema


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