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STRUCTURAL AND FUNCTIONAL STUDIES OF *MALASSEZIA SYMPODIALIS*-DERIVED ALLERGENS

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ABSTRACT

Atopic eczema (AE) is a chronic inflammatory skin disease characterised by pruritic (itchy) skin lesions. The pathogenic mechanisms underlying AE are still unclear, although several factors such as genetic predisposition, a dysfunctional skin barrier, exposure to environmental allergens and skin colonisation with microorganisms appear to be of importance. *Malassezia sympodialis* is a yeast which is part of our normal cutaneous flora. Approximately 50% of adult AE patients have serum IgE specific for *M. sympodialis* allergens or display immediate-type skin reactions against crude extracts of this yeast, while such reactivity is rarely observed in other allergic diseases, indicating that sensitization to this yeast is associated with AE. Ten allergens from *M. sympodialis* have been cloned to date, designated Mala s 1 and Mala s 5-13. Six of these exhibit sequence homology to known proteins whereas four do not. The aim of this thesis has been to gain knowledge of host-microbe interactions of allergens from *M. sympodialis* in the pathogenic mechanisms of AE and in healthy individuals. This has been accomplished by studying structural properties and cellular interactions, with a special focus on the two allergens Mala s 1 and Mala s 11.

Mala s 1 is a major allergen mainly localized in the yeast cell wall and exposed on the cell surface. Interestingly, Mala s 1 does not exhibit any significant sequence homology to known proteins. We have solved the crystal structure of Mala s 1 by single-wavelength anomalous dispersion techniques using selenomethionine-substituted Mala s 1. Mala s 1 folds into a six-fold β-propeller, a novel fold among allergens. The putative active site of Mala s 1 overlaps structurally with putative active sites in potential homologues, Q4P4P8 and Tri 14, from the plant parasites *Ustilago maydis* and *Gibberella zeae*, respectively. This resemblance suggests that Mala s 1 and the parasite proteins may have similar functions. In addition, we demonstrate that Mala s 1 binds to phosphatidylinositol (PtdIns) (3)-phosphate (P), PtdIns(4)P, and PtdIns(5)P, lipids possibly playing a role in the localization of Mala s 1 to the cell surface.

Mala s 11 displays a high degree of sequence homology to human manganese superoxide dismutase (hMnSOD). In AE patients sensitized to *M. sympodialis*, hMnSOD can elicit positive skin prick tests or atopy patch tests along with binding IgE, suggesting an autoimmune response. We report that Mala s 11 is able to inhibit IgE-binding to hMnSOD and *vice versa*, indicating that these two homologues share common IgE epitopes. We have also identified residues possibly involved in such cross-reactivity. In addition, we compared the influence of Mala s 11 and hMnSOD on human dendritic antigen presenting cells. Whereas rhMnSOD did not affect the phenotype of monocyte-derived dendritic cells (DCs), rMala s 11 up-regulated maturation markers and induced significantly higher levels of inflammatory cytokines in the culture supernatants. This suggests that DCs from healthy individuals possess the ability to distinguish between Mala s 11 and hMnSOD despite their high homology. Whether this is also the case for DCs in AE patients remains to be clarified.

In summary, we have determined a novel three dimensional structure not previously demonstrated among allergens. We demonstrate the ability of DCs to distinguish between proteins with high sequence homology and we provide a structural basis underlying the autoimmune response to hMnSOD in AE based on IgE-mediated cross-reactivity.

Thesis summary in Swedish – Svensk sammanfattning av avhandlingen

Strukturell och funktionell analys av allergen från jästsvampen *Malassezia sympodialis*

Atopiskt eksem (AE) är en kronisk hudsjukdom där torr hud och klåda är typiska symptom. Exakt vad som ligger bakom denna sjukdom är ännu inte känt, men faktorer som patientens genetiska bakgrund, en bristfällig hudbarriär, mikroorganismer på hudytan samt allergi anses ha en betydande roll. Vid en allergisk sjukdom reagerar kroppen på ofarliga proteiner i omgivningen genom att producera IgE-antikroppar. Dessa antikroppar kan binda till proteinerna, som då kallas allergen, och denna bindning ligger bakom de allergiska symptomen. Jästsvampen *Malassezia sympodialis* tillhör den normala hudfloran. Omkring 50% av vuxna AE patienter har IgE-antikroppar mot allergen från *M. sympodialis* eller positivt pricktest mot *M. sympodialis* extrakt. Dessa symptom ses sällan hos individer med andra allergiska sjukdomar, vilket tyder på att allergi mot *M. sympodialis* har en speciell koppling till AE. Hittills har tio allergen från *M. sympodialis* identifierats. Sex av dessa har likheter i DNA-sekvensen med tidigare kända protein, medan övriga fyra saknar detta. Målet med denna avhandling har varit att öka förståelsen kring interaktionen mellan *M. sympodialis* allergen och människa samt denna jästsvamps roll vid AE. Detta har gjorts genom att karakterisera den tredimensionella (3D) strukturen hos *M. sympodialis* allergen samt att studera hur dessa allergen påverkar celler i det mänskliga immunförsvaret. De två allergen som denna avhandling fokuserar på är Mala s 1 och Mala s 11. Allergenet Mala s 1 är lokaliserat i jästens cellvägg och är exponerat på dess cellyta. Detta allergen har ingen likhet i DNA-sekvens med något protein med känd funktion, och följaktligen är även funktionen hos Mala s 1 okänd. Vi har löst 3D strukturen för Mala s 1 och funnit en struktur som aldrig tidigare observerats hos ett allergen. Likheten mellan den del av strukturen som troligtvis innehåller aminosyror av störst vikt för funktionen av Mala s 1 och motsvarande område hos proteiner från växtparasiterna *Ustilago maydis* och *Gibberella zeae* tyder på att Mala s 1 och dessa proteiner har liknande funktion. Vi fann även att Mala s 1 binder till fosfatidylinositol, en lipid som kan ha en roll i att transportera Mala s 1 till cellytan.

Allergenet Mala s 11 har hög likhet i DNA-sekvens med det mänskliga enzymet mangansuperoxiddismutas (hMnSOD). Vi har visat att Mala s 11 kan hämma bindning av IgE-antikroppar till hMnSOD och vise versa, vilket tyder på att dessa två proteiner har strukturer som kan binda samma IgE-antikropp. Vi har även identifierat vilka aminosyror som skulle kunna vara inblandade i en sådan så kallad korsreaktivitet. Dessa resultat ger på molekylär nivå en trolig förklaring till den autoimmuna reaktionen mot hMnSOD som observerats hos AE patienter allergiska mot *M. sympodialis*. Vi har även jämfört effekten av Mala s 11 och hMnSOD på antigenpresenterande dendritiska celler (DCs), celler i immunförsvaret som tar upp främmande ämnen och sedan visar upp dem för andra celler i immunförsvaret. Våra resultat tyder på att DCs hos friska individer har förmågan att skilja mellan Mala s 11 och det kroppsegna hMnSOD trots den stora likheten mellan dessa proteiner. Det återstår att visa om DCs hos AE patienter har förlorat denna förmåga.

Sammanfattningsvis har dessa studier ökat kunskapen om strukturen hos *M. sympodialis* allergen. Resultaten har på molekylär nivå givit en förklaring till mekanismerna bakom en autoimmun reaktion på en kroppsegen substans med hög likhet med ett allergen samt påvisat förmågan hos DCs att skilja på proteiner med stor likhet i sekvens.

LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to in the text by their roman numerals:

I. Monica Vilhelmsson*, B. Martin Hallberg*, Omid Rasool, Arezou Zargari, Annika Scheynius and Adnane Achour. Crystallization and preliminary crystallographic study of the *Malassezia sympodialis* allergen Mala s 1.
Acta Cryst. 2006, F62:97-99.
*These authors contributed equally to this work.

II. Monica Vilhelmsson, Arezou Zargari, Reto Crameri, Omid Rasool, Adnane Achour, Annika Scheynius* and B. Martin Hallberg*. Crystal structure of the major *Malassezia sympodialis* allergen Mala s 1 reveals a β-propeller fold: A novel fold among allergens.
J Mol Biol. 2007, 369:1079-1086.
*Shared last authorship

III. Monica Vilhelmsson, Catharina Johansson, Gunilla Jacobsson-Ekman, Reto Crameri, Arezou Zargari and Annika Scheynius. The *Malassezia sympodialis* allergen Mala s 11 induces human dendritic cell maturation, in contrast to its human homologue manganese superoxide dismutase.
Int Arch Allergy Immunol. 2007, 143:155-162.

IV. Monica Vilhelmsson, Andreas G Glaser, Daniel Badia Martinez, Margit Schmidt, Catharina Johansson, Claudio Rhyner, Kurt D. Berndt, Annika Scheynius, Reto Crameri, Adnane Achour* and Arezou Zargari*. Mutational analysis of amino acid residues involved in IgE-mediated cross-reactivity between the *Malassezia sympodialis* allergen Mala s 11 and its human homologue manganese superoxide dismutase.
Submitted.
*Shared last authorship

Publications not included in the thesis:

1. Lisa Bandholtz*, Gunilla Jacobsson-Ekman*, Monica Vilhelmsson, Eva Buentke, Birgitta Agerberth, Annika Scheynius and Gudmundur H Gudmundsson. Antimicrobial peptide LL-37 internalized by immature human dendritic cells alters their phenotype.
   Scand J Immunol. 2006, 63:410-9.
   *These authors contributed equally to this work.

2. Sabine Zeller, Andreas G Glaser, Monica Vilhelmsson, Claudio Rhyner, and Reto Crameri. IgE-mediated reactivity to self antigens: a controversial issue.
   Int Arch Allergy Immunol. 2007, 145:87-93. *Review*.

3. Monica Vilhelmsson, Sergey Zelenin, Gunilla Jacobsson-Ekman, Annika Scheynius, Anita Aperia and Marina Zelenina. Nickel and copper decrease the plasma membrane permeability of human dendritic cells.
   Submitted.

CONTENTS

LIST OF ABBREVIATIONS

3D	three dimensional
AE	atopic eczema
APC	antigen presenting cell
APT	atopy patch test
BCR	B-cell receptor
CBA	cytometric bead array
CD	circular dichroism
DC	dendritic cell
ELISA	enzyme-linked immunosorbent assay
E. coli	*Escherichia coli*
FACS	fluorescence activated cell sorter
GM-CSF	granulocyte-macrophage colony stimulating factor
HLA-DR	human leukocyte antigen-DR
HPLC	high pressure liquid chromatography
iDC	immature dendritic cell
IDEC	inflammatory dendritic epidermal cells
IFN-	interferon
Ig	immunoglobulin
IL	interleukin
iMDDC	immature monocyte-derived dendritic cell
LC	Langerhans' cell
LPS	lipopolysaccaride
MDDC	monocyte-derived dendritic cell
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MnSOD	manganese superoxide dismutase
MR	molecular replacement
M. sympodialis	*Malassezia sympodialis*
NK cell	natural killer cell
PAMP	pathogen associated molecular patterns
PBMC	peripheral blood monocytes
PEG	polyethylene glycol
PRR	pattern recognition receptors
r	recombinant
SAD	single wavelength anomalous diffraction
S. aureus	*Staphylococcus aureus*
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis skin
Se-Met	selenomethionine-substituted
SIT	specific immunotherapy
SPT	skin prick test
TCR	T-cell receptor
TGF-β	transforming growth factor β
T_H cell	T helper cell

TLR	Toll-like receptor
T_{reg}	regulatory T cell
WT	wild type

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The human body encounters a diversity of potentially harmful pathogens. To protect us against these we have an immune system. The immune system consists of two interacting parts, innate and adaptive immunity [1]. Innate immunity is considered to be the first line of defence. Interfacial areas between host and the external environment such as the skin, respiratory and gastrointestinal tracts are protected against microbial entry by epithelia which provide a physical barrier against infection. Through the release of anti-microbial peptides the epithelia also provides chemical defence against microbes [2]. In addition, innate immunity involves neutrophils and macrophages which eradicate microbes via phagocytosis. Neutrophils and macrophages express germline-encoded receptors, also known as Pattern Recognition Receptors, (PRRs). These receptors recognize conserved structures that are shared by various classes of microbes, so-called Pathogen Associated Molecular Patterns (PAMPs). The PRRs can be exemplified by the Toll-like receptors (TLRs), which among others recognize substances such as lipopolysaccaride (LPS), a component of the cell wall of gram-negative bacteria. Innate immunity also involves the complement system which is a group of proteins activated when encountered with microbes. Complement proteins coat (opsonize) microbes, thereby stimulating phagocytosis, inflammation and the lysis of microbes. Through the release of cytokines and chemokines innate immunity stimulates inflammation, activates natural killer (NK) cells and macrophages and attracts antigen presenting cells (APCs).

Whereas innate immunity encompasses mechanisms that can deal with a microbe as soon as it is in contact with the host, adaptive immunity needs a longer time to mount a response. In this part of the immune system T and B lymphocytes are key players. T and B lymphocytes express antigen receptors that are produced by recombination of genes. Far more substances are therefore recognized in adaptive immunity than in innate immunity. It is of great importance that the immune system is able to distinguish between those antigens that are of potential danger to the host and those antigens that are harmless. Failure in doing so can lead to allergic disease. The immune system also needs to distinguish between antigens from the surroundings and substances from the host. When this is not done properly, autoimmune disease may be the outcome. Cells that have antigen receptors binding to harmless substances are eliminated. T and B lymphocytes recognize antigens in different ways. B lymphocytes have membrane bound antibodies as receptors. These can recognise a great diversity of antigens, whereas the receptors of T lymphocytes only can bind peptide antigens that are processed by and presented on the surface of APCs [1].

1.2 CELL-CELL COMMUNICATION VIA CYTOKINES

Cells have advanced mechanisms for communication. When cells of the immune system are not in direct contact with each other, signalling proteins called cytokines can be released. The cytokines bind to specific cytokine receptors on the cells they affect.

In addition to cytokines various cells release chemokines, chemoattractant proteins that are involved in the recruitment *e.g.* of cells involved in inflammation [1].

1.3 ANTIGEN PRESENTING CELLS

The task of APCs is the uptake of different types of antigens from the surroundings through various mechanisms. Inside the APC the antigen is processed and cleaved into fragments (peptides) and bound to major histocompatibility complex (MHC) class II molecules. At this point the APC plays an important role in deciding which parts of the secondary structure of the native antigen are worth mounting an immune response against. The MHC class II peptide complex is transported to the cell surface for presentation to naïve $CD4^+$ T lymphocytes (T lymphocytes that have not yet reacted with an antigen) via T-cell receptor (TCR). While MHC class II expression is mainly restricted to DCs, B cells and macrophages, MHC class I molecules are expressed on almost all cells [3]. MHC class I molecules present both endogenous and pathogen-derived proteins from the cytosol. Peptides on MHC class I molecules are presented to $CD8^+$ T cells (cytotoxic T-cells) [1].

1.4 DENDRITIC CELLS

Among APCs, DC are the most potent for T lymphocyte stimulation [4]. DCs derive from progenitors that reside in the bone marrow from where they migrate to peripheral tissues. At this stage the DCs are classified as being immature DCs (iDCs). In the immature state the DC constantly patrol peripheral tissues for antigens [5] and their characteristic shape with long dendrites enhances their ability to do so. Antigens can be taken up by iDCs via phagocytosis, macropinocytosis or by receptor-mediated uptake *e.g.* PRRs, mannose or scavenger receptors [6, 7]. After antigen uptake the DCs differentiate into a mature state. They loose their ability to take up antigens and their phenotype is now adjusted for antigen presentation. The antigens previously taken up are processed and bound to MHC molecules exposed on the outer side of the cell membrane for presentation to T lymphocytes. In addition, co-stimulatory molecules such as CD40, CD80 and CD86 which are needed for T lymphocyte activation, along with the maturation marker CD83 [8, 9], are up-regulated on the cell surface. Upon maturation the DCs migrate to lymphoid organs for antigen presentation [8]. The fact that DCs use mechanisms belonging to innate immunity for antigen uptake and thereafter present antigens to T lymphocytes makes them an important link between innate and adaptive immunity. However, it has become evident that DCs do not only function as transporters of antigens for presentation to T-cells, but also influence the nature of the T-cell response. Hence, the way DCs present antigens to T-cells affects whether tolerance or an active immune response to the antigen in question is mounted [10, 11].

1.5 DENDRITIC CELL SUBSETS

DCs constitute a heterogeneous cell population with several subsets. The different subsets in humans are suggested to derive from either common plasmacytoid (pDCs) or common myeloid (mDCs) progenitors [12]. pDCs and mDCs require different growth factors and transcriptional factors to develop, and can be distinguished on the basis of

their differences in expressed surface markers and cytokines produced. pDCs express low levels of CD11c [13] and high levels of CD123 [14]. mDCs express high levels of CD11c [13] and CD1a [14]. The development of different subsets is thought to be influenced by several factors including surrounding cytokines and types of pathogens present [15]. mDCs can be generated from monocytes *in vitro* through co-culture with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 [16].

Two subsets of mDCs found in the skin are the Langerhans' cells (LCs) and the inflammatory dendritic epidermal cells (IDEC) [14]. Typical for LCs are the Birbeck granules, which contain antigen-capturing Langerin [17]. LCs are involved in the initiation of an allergic response. IDECs are found in inflammatory skin diseases and enhance allergic inflammatory reactions [18]. Both LCs and IDECs can express the high affinity IgE receptor, FcεRI [14, 19]. FcεRI expressed on effector cells such as mast cells is of tetrameric form containing α-, β-, and two γ-chains. The FcεRI expressed on LCs and IDECs are different in that they lack the β-chain and is hence said to be trimeric [19]. The lack of the β-chain might have implications in the variable expression of FcεRI apparent on these cells.

1.6 T LYMPHOCYTES

T-cells form in the bone marrow and migrate to the thymus. Through gene rearrangement a variety of T-cells with different TCR specificity are formed and undergo positive selection. During this process T-cells that are able to bind MHC molecule-antigen complexes with appropriate affinity receive a survival signal while those who fail to do so die by apoptosis. After this process the T-cells also go through negative selection in which most cells that react too strongly against self peptides are eliminated as a mechanism to prevent autoreactivity. Following positive and negative selection the T lymphocytes re-circulate the lymphoid organs. Upon activation by APCs they differentiate into subset cells with different functions [1].

1.6.1 $CD4^+$ T lymphocyte subsets

Peptides on MHC-II molecules are presented to naïve $CD4^+$ T lymphocytes which stimulate the differentiation into a number of different subsets. The best defined subsets are type 1 helper T-cells (T_H1 cells) and type 2 helper T-cells (T_H2 cells). The T_H1 cells stimulate phagocyte-mediated killing of microbes (cell-mediated immunity) by production of the cytokine interferon-γ (IFN-γ). T_H2 cells stimulate the production of IgE antibodies by producing the cytokines IL-4 and IL-13 (humoral immunity) [1]. Another group of T lymphocytes that has received a lot of attention lately are the so called regulatory T-cells (T_{reg}). This subset regulate or suppress the function of other T lymphocyte types, through the release of cytokines such as IL-10 and TGF-ß [20]. Within this subset, different variants referred to as Tr1, Th3 and naturally occurring T_{regs} have been identified. The Tr1 and Th3 are thought to differentiate from naïve T-cells and are therefore said to be "adaptive" T_{regs} while the naturally occurring T_{regs} differentiate from thymocytes expressing the T_{reg} marker Foxp3 [20]. T_{regs} are suggested to have implications in the development of allergy and several autoimmune diseases [21, 22]. The most recently discovered T lymphocyte subset is the T_H17. These

differentiate from naïve T-cells in the presence of TGF-β and IL-6 [23] and are thought to be of importance in the defence against bacteria and fungi e.g. *Candida albicans* [24]. T_H17 has been suggested to play a role in autoimmune conditions [25, 26].

1.6.2 $CD8^+$ T lymphocytes

$CD8^+$ T lymphocytes, also referred to as cytotoxic T-cells, kill cells infected with pathogens of various kinds by binding with their TCRs to MHC class I-antigen complexes. Through the release of cytotoxins such as perforin and granulysin, pores are made in the plasma membrane of the target cell, leading to cell death [1].

1.6.3 T-cell epitopes

The part of an antigen to which the receptor of a T-cell or a B-cell is specific is referred to as an epitope. T-cells can only recognize antigen peptides that have been processed by APCs and that are presented by MHC class II molecules in the case of $CD4^+$ T-cells and MHC class I molecules in the case of $CD8^+$ T-cells. T-cell epitopes are therefore always linear and usually comprise 8-11 amino acid residues [3].

1.7 B LYMPHOCYTES

B lymphocytes also form and develop in the bone marrow. As with T lymphocytes they undergo receptor gene rearrangement and negative selection to eliminate those cells recognizing self antigens. B lymphocytes mediate protection against pathogens via antibodies. The process of antibody production from B lymphocytes is initiated by the binding of antigens to B cell receptors (BCR) which are membrane-bound antibodies, along with direct interaction with T lymphocytes and with cytokines released from T lymphocytes [1]. Initially, a B lymphocyte expresses immunoglobulin M (IgM) or IgD. During the process of maturation B-cells undergo isotype switching to other classes of antibodies such as IgG, IgA or IgE.

1.7.1 B-cell epitopes

There are vital differences between B- and T-cell epitopes which can be explained by the route they recognize antigens. While T-cells are restricted to the recognition of processed antigen peptides presented on MHC molecules, BCR recognize antigens in their native form. As a consequence, B-cell epitopes can either be linear or conformational. The amino acids comprising a conformational epitope are not necessarily localized close to each other in the primary sequence of the protein but can form a specific conformational epitope in the folded protein [27, 28]. Hence antibodies raised against such a conformational epitope may fail to recognise an unfolded protein. Structural studies indicate that most B-cell epitopes display a surface area of 650-900 Å [28] and contain 15-22 residues, of which 5-6 are of great importance for IgE binding [29]. It is likely that a typical 20 kDa antigen could bind between 5-to-10 antibodies simultaneously [30].

1.8 ALLERGY

It is essential for the immune system to distinguish between antigens that can represent a danger to the host and antigens that are harmless. Failure in doing so can lead to development of hypersensitivity diseases such as IgE-associated allergy. Atopy is the hereditary tendency to produce IgE antibodies to harmless environmental proteins, *e.g.* pollen [31]. Common symptoms of IgE-associated allergy are rhinitis, rhinoconjunctivitis, atopic eczema (AE) and asthma. IgE-associated allergies have increased rapidly in industrialized countries [32], affecting more than 25% of the population [33]. Allergy has increased rapidly in these areas, suggesting that life-style factors in addition to genetic factors are involved.

A restrictive use of antibiotics and vaccinations among individuals leading an anthroposophic lifestyle is associated with a lower risk of developing allergic disease [34-36]. This supports the theory that life-style factors are of importance in the development of allergic diseases. The observation that growing up on a farm reduces the risk of developing allergy [35, 37] further supports this theory and is in line with "the hygiene hypothesis". According to the "hygiene hypothesis" exposure to microbes early in life will reduce the risk of developing allergic diseases and represents one plausible explanation for the increase in allergic diseases connected with the life-style in industrialized countries [32]. However, recent studies indicate that the rapid increase in allergy prevalence might be stabilized [38, 39]. In addition to environmental factors, several genes have been linked to allergic diseases, *e.g.* genes encoding the classical T_H2 type cytokines IL-4 and IL-13 [40].

In the pathogenic mechanisms of allergy a series of immunological events lead to unwanted IgE-mediated responses to harmless antigens. Initially, antigens are taken up by iDCs. This event induces maturation of the DC which then stimulates differentiation of naïve T lymphocytes into T_H2 cells. The cytokines IL-4 and IL-13 produced by T_H2 cells will stimulate isotype switching in B lymphocytes to produce IgE antibodies. IgE is released from B lymphocytes and binds to three types of receptors; the high affinity IgE receptor (FcεRI), the low affinity IgE receptor (FcεR2/CD23) and the epsilon binding protein (epsilonBP). Upon the next encounter with the same allergen, the allergen can bind directly to the IgE on the surface of various cells. The binding of antigen to IgE on mast cells will stimulate immediate hypersensitivity reactions with the release of mediators and proinflammatory cytokines, causing a rapid increase in vascular permeability and smooth muscle contraction. The immediate hypersensitivity reactions are followed by an intense inflammatory reaction termed the late-phase response. During this phase DCs with IgE bound to IgE receptors on their surface capture allergens for presentation to T-cells. This leads to migration of T_H2 cells, eosinophils and basophils to the site of inflammation. The mechanisms of IgE-associated allergy are summarised in Figure 1 [33].

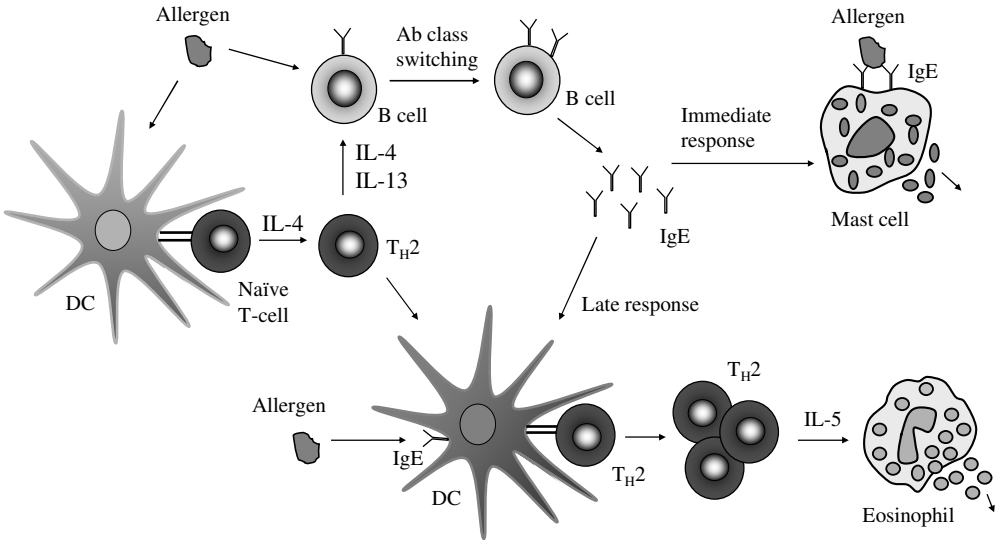

Figure 1. The mechanisms of IgE-associated allergy. Allergens are taken up by antigen presenting cells such as DCs for presentation to naïve T-cells, which in the presence of IL-4 differentiate into T_H2 cells. T_H2 cells in contact with allergen presenting B-cells in the presence of IL-4 and IL-13 promotes a switch to IgE production. In the immediate response in a sensitized individual, allergens bind to and cross link IgE bound to IgE receptors on mast cells. This induces degranulation and release of inflammatory mediators. During the late phase allergens are presented to T-cells, leading to the recruitment of eosinophils to the site of inflammation. (Modified from [33]).

1.9 ALLERGENS

As described in Figure 1 an allergen has the capacity to induce the production of specific IgE during the sensitisation phase along with eliciting clinical symptoms during the subsequent immediate and late phase responses. Typical allergens are proteins or glycoproteins with a molecular weight ranging from 5 to 80 kDa [41]. Over a thousand allergens have been sequenced [42] from various sources such as pollen, animal dander, mites, food, insects and fungi. Information about these can be retrieved from databases solely dedicated to keeping track of identified allergens [42, 43]. Allergens are normally referred to by the first three letters of the genus followed by the first letter of the species and last, a number indicating the chronological order of allergen identification [44]. Hence the first allergen identified from cat (*Felis domesticus*) is called Fel d 1. When more than 50% of the individuals sensitized to allergens from one certain source are sensitized to one particular allergen, this allergen is termed a major allergen as opposed to a minor allergen [45].

1.9.1 What makes an antigen an allergen?

In order to better understand the mechanisms underlying allergic disease and in order to establish strategies for prevention and treatments, more knowledge about the properties of allergens is required. Efforts have been made in trying to elucidate why some antigens can elicit an allergic response whereas others do not seem to have this ability. Initially one needs to consider how an allergen is first exposed to the immune system. The allergen dose, solubility and stability are all important parameters [46]. Additional

substances present at the time of exposure, either from the allergen source itself, *e. g.* lipids from pollen grains [47], or from another source such as air pollutants or host response factors during an infection, can also influence the immune response to allergens [48].

Although structural and functional studies of allergens have failed to determine any properties shared by all allergens, a few interesting observations have been made which could at least partially explain the allergenicity of some antigens. Many allergens are enzymes, and it seems that enzymatic activity can trigger an allergic immune response [49]. This can be exemplified by the proteolytic activity of the major dust mite allergen Der p 1 which enhances IgE production [50]. However, the mechanisms through which enzymatic activity contributes to the allergenicity of an antigen are likely to differ between allergens with different enzymatic activities. Several allergens have grooves or cavities [51-53]. Binding of ligands within such cavities could probably contribute to the stability of these allergens. Many allergens are glycosylated [49], although it is unclear to what extent and via which mechanisms this could contribute to allergenicity. It appears that allergens can be grouped into relatively few protein families, indicating that some protein classes have a higher probability to render an allergic response [52, 54, 55]. However, since many allergens can also be found outside of these general protein families, allergenicity can not be fully explained by specific structural features.

1.9.2 Cross-reactivity

The similarity between an endogenous substance and a foreign substance, leading to autoreactive T- or B-cells, is also referred to as cross-reactivity or molecular mimicry. This can be exemplified by an allergen and another protein sharing common amino acids on the surface creating B-cell epitopes. IgE can then cross-react between the two proteins [56]. Through this mechanism it is possible to elicit an allergic response toward a different protein than the one involved in the sensitization phase [57]. Both T- and B-cell mediated cross reactivity have been observed among allergens. The most studied cross-reactive allergen is probably the major birch pollen Bet v 1 [58]. This allergen does not only share homology with other pollen allergens [59], but also with allergens from apple [60], peanut [61] and cherry [62] among others [63-65]. Cross-reactivity is also evident among other allergenic sources such as mites [66, 67] and fungi [68, 69]. Interestingly, allergens from birch [70] and fungi [71-76] have been demonstrated to cross-react with human homologues.

1.10 ATOPIC ECZEMA

The chronic inflammatory skin disease AE is thought to affect 10-20% of children and 1-3% of adults in industrialised countries [77]. The increase in prevalence observed for other allergic diseases also applies to AE [78, 79]. The disease is characterised by severely itchy (pruritic), red, dry and crusted skin (Figure 2). Due to constant rubbing and itching, chronic thickening of the skin (lichenification) forms with time. Additionally, loss of the surface of the skin (excordation) occurs. In infants the affected areas are mostly the cheeks and extensor parts of the extremities. During childhood (2-12 years), flexural areas are affected. Among adults the head, flexural areas, hands and feet are commonly affected [78]. Different strategies to diagnose AE exist. SCORAD

provides a standardized index taking both the severity and the extent of the eczema into account [80]. In addition to SCORAD, excluding other forms of eczema along with serological tests of allergen specific IgE along with SPTs and APTs are important parts of the diagnosis [81].

Many factors, both genetic and environmental, are likely to be of importance in allergic pathogenesis. AE has been suggested to be the start of the "atopic march" later leading to asthma and allergic rhinitis [82]. Common features between AE and asthma such as the increase of prevalence, shared candidate genes and immunological features, has been highlighted in support of this theory [82]. However, it is debated that AE might have a stronger genetic linkage to other skin diseases and that patients having both AE and asthma represent a separate phenotype [37]. Around 80% of AE patients display elevated serum levels of IgE, sensitization against aeroallergens and food allergens, and/or allergic rhinitis and asthma [81]. AE in this subgroup of patients is referred to as being of the extrinsic or atopic type. The remainder are said to be the intrinsic or non-atopic type [31, 83, 84]. Yet another subgroup of AE patients encompasses those with IgE-mediated reactivity to endogenous substances [85, 86]. Such a response can be mediated by IgE cross-reactivity between allergens and endogenous substances [74, 75]. However, this only partially explains this phenomenon since reactivity to endogenous substances without homology with allergens has been observed in AE patients [85].

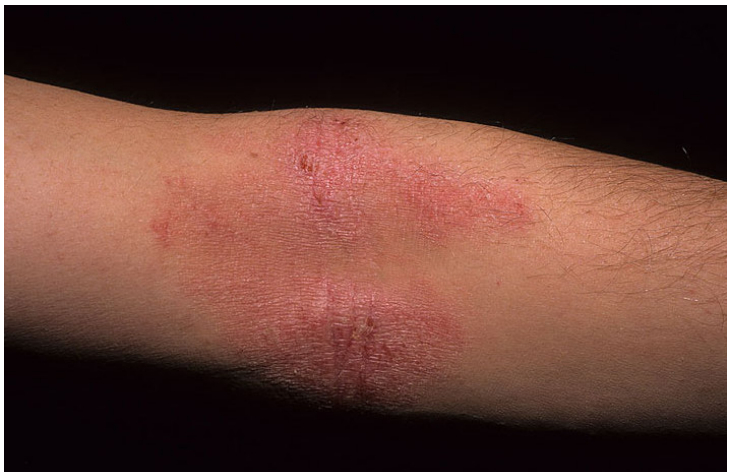

Figure 2. Atopic eczema on an arm of an adult patient [87].

1.10.1 Genetics of AE

Parental AE is significantly associated with manifestations and severity of AE in children [81]. A number of genes may play a role in the disease [81, 88]. Given that the typical clinical manifestation of AE is a dysfunctional skin barrier, as further discussed below, it is not surprising that some of these genes are related to the function of the epidermis [89-91], *e.g.* filaggrin [92-94]. Other genes with a possible implication in AE are involved in immunological responses [88]. Among these, genes for the cytokines IL-4 [95, 96], GM-CSF [97] and TGF-ß1 [98] are found along with the gene for the ß-chain of the high affinity IgE receptor, FcεRIß [99].

1.10.2 Environmental triggers of AE

In addition to allergens, stress, possibly mediated by neuropeptides and irritant factors such as woolly clothing and chemicals, can worsen the symptoms in the skin [81]. AE patients commonly suffer from skin infections with *Staphylococcus aureus* which activates T-cells and macrophages via the release of superantigens [100]. Allergic sensitisation to the fungi *Malassezia sympodialis* [101] (discussed further below) is evident in about 50% of adult AE patients and is probably facilitated by the dysfunctional skin barrier [101].

1.10.3 Skin barrier functions in AE

Several properties are altered in AE skin, in both lesional and non-lesional areas; a reduced number of water-retaining ceramides [81], alkaline pH [102] possibly affecting lipid metabolism [103], overexpression of chymotrytic enzyme [91], decreased IgA production [104], lower levels of antimicrobial peptides [105] and increased expression of molecular adhesion of *S. aureus* [106]. These factors, along with the genetic background, probably contribute to a dysfunctional skin barrier function in AE, failing to provide sufficient protection against antigens of various kinds.

1.10.4 Immunological responses in AE

The initial phase of AE lesions in the skin is associated with elevated levels of the T_H2 biased cytokines IL-4 and IL-13 [107]. During the subsequent chronic phase levels of the T_H2 cytokine IL-5 [107] along with T_H1 biased cytokines IL-12 and IL-18 [81] and cytokines suggested to be involved in skin remodeling such as IL-11, IL-17 and TGF-β1 [108] are all elevated. Recently, IL-31 has also been suggested to be a cytokine of importance in AE [109]. Various types of cells such as T lymphocytes, NK-cells, keratinocytes, eosinophils, mast cells and DCs are thought to be involved in the pathogenic mechanisms [81, 110-113]. The DC subtypes LCs and IDECs from AE patients have much higher levels of the high affinity IgE receptor, FcεRI [19] and hence are believed to contribute to allergic inflammation in the skin. In addition, the lower number of pDCs observed in the skin of AE patients in comparison to healthy controls might lead to a higher susceptibility to viral infections [14, 81].

1.10.5 Pruritus

The strong pruritus in AE individuals has a big impact on the patients' nightly rest and overall quality of life [81]. Although the mechanisms underlying pruritus development are poorly understood, it seems to be triggered by allergen and irritant exposure, changes in humidity, sweating and stress [81]. It can not solely be explained by the release of histamine from mast cells since anti-histamine treatment is not efficient [114]. Inflammatory cells along with substances such as cytokines have been suggested to play a role in this process [81]. In addition, the involvement of neurotrophins suggests a link between the immune system and nervous system [115, 116].

1.11 *MALASSEZIA*

The yeast *Malassezia*, previously denoted *Pityrosporum*, is part of the normal human skin flora [117]. It colonizes areas of the stratum corneum (the outermost layer of the epidermis that consists of dead cells), with high concentrations of sebum (oily secretion from the sebaceous glands) [118]. The yeast cells are 1-8 μm, round, oval or cylindrical and divide by budding (Figure 3) [117, 119]. The cell wall is very thick and mainly consists of carbohydrates, proteins and lipids [120].

Malassezia belongs to the fungal phylum Basidiomycota [121] which contains well known plant and animal pathogens [122]. Though usually harmless, *Malassezia* can act as a pathogen and cause skin diseases in humans such as pityriasis versicolor, seborrhoeic dermatitis, folliculitis and dandruff [123]. In addition, *Malassezia*-related diseases have been observed in dogs [124]. As further discussed below, allergy to *Malassezia* has been observed in AE patients. To date, ten species of *Malassezia* have been isolated from human skin; *M. dermatis*, *M. furfur*, *M. globosa*, *M. obtusa*, *M. pachydermatis*, *M. restricta*, *M. slooffiae*, *M. sympodialis, M. japonica*, and *M. yamatoensis* [125], and three additional variants from animals; *M. nana*, *M. caprae* and *M. equine* [126]. All but *M. pachydermatis* are lipid-dependent [118]. While *M. sympodialis* has been suggested to be the species most frequently isolated from AE patients and healthy individuals in studies performed in Sweden, Russia and Canada, *M. furfur* seems to be the most frequent in Japan [117, 118, 127]. Recently the whole genome for *M. globosa* and *M. restricta,* species that are mainly associated with dandruff and seborrhoeic dermatitis, were sequenced [128, 129]. This information will hopefully lead to a better understanding of the pathogenic mechanisms in *Malassezia*-related diseases.

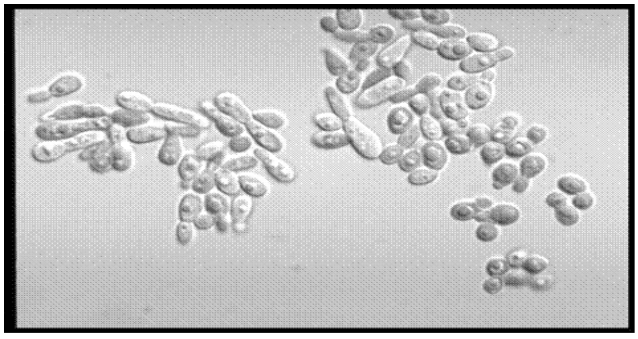

Figure 3. Light microscopy picture of *Malassezia sympodialis* [130].

1.12 *MALASSEZIA* AND ATOPIC ECZEMA

The first association between *Malassezia* and AE was observed in 1983, when it was discovered that treatment with the anti-fungal drug ketoconazole improved AE [131]. In addition, treatment with ketoconazole decreased the severity of eczema and lowered the levels of total IgE and *M. sympodialis*-specific IgE [117]. Around 50% of adult AE patients demonstrate immediate skin type reactions or have specific IgE against *M. sympodialis* [101], while this is very rare in other allergic diseases [132]. This suggests that an allergic response to this yeast is associated with AE. An increased release of

allergens from *M. sympodialis* has been observed at the alkaline pH characteristic of AE skin in comparison to the pH of healthy skin [133].

1.13 *MALASSEZIA* ALLERGENS

Malassezia extracts are currently used for detection of IgE antibodies in serum, for skin prick test (SPT) and atopy patch test (APT). As for allergen extracts in general, the use of *Malassezia* extracts can be problematic due to the variation of allergen content between batches. Factors such as culture time influence the concentration of IgE-binding components in the extract [134]. The problem with batch variations can be overcome by the use of recombinant allergens and hence the identification of allergenic components from *Malassezia* is important for diagnostic purposes [135]. In a study including 156 AE patients IgE reactivity to three recombinant *M. sympodialis* allergens was compared to that to *M. sympodialis* extract [136]. It was reported that 55% of the patients had IgE antibodies to any of the three recombinant *M. sympodialis* allergens, whereas only 47% had IgE antibodies to *M. sympodialis* extract, indicating that identification of AE with recombinant allergens is preferable. The identification of allergens from *Malassezia spp.* is also of importance for understanding the mechanisms involved in an immune response to a particular allergen.

At the present time the genes of 13 allergens (Table 1), ten from *M. sympodialis* and three from *M. furfur*, have been identified, cloned and produced as recombinant proteins. Four of the *M. sympodialis* allergens identified so far, Mala s 1 and Mala s 7 – 9 are unknown in terms of function and might present novel proteins. Structural and functional studies of these allergens are essential in order to determine their function in *Malassezia* and their role in the pathogenic mechanisms of AE. One of these four allergens lacking sequence homology to known proteins, Mala s 1, is of particular interest since it is localized in the cell wall [137] and hence should be easily accessible to cells of the immune system.

The sequence of four *M. sympodialis* allergens, namely Mala s 6, 10, 11 and 13, reveals high homology to human endogenous proteins [75, 138, 139]. The demonstrated cross-reactivities of Mala s 6 and Mala s 13 with human cyclophilin and thioredoxin, respectively, suggest that autoimmunity due to molecular mimicry could play a role in the pathogenesis of AE. The major allergen Mala s 11 displays sequence homology to manganese superoxide dismutase (MnSOD) [139]. MnSOD has been defined as an allergen in *Aspergillus fumigatus* and is denoted Asp f 6 [71]. *A. fumigatus* is a fungus implicated in pulmonary diseases [71]. Asp f 6 can cross-react with human MnSOD (hMnSOD) at B- and T-cell levels [140]. Additionally, a comparison of the crystal structures of Asp f 6 and hMnSOD revealed patches of identical amino acid residues that are displayed on the surface of both enzymes and could therefore constitute cross-reactive IgE-binding epitopes [71]. hMnSOD can bind IgE, induce T-cell reactivity and positive APTs in AE patients sensitized to *Malassezia* [141]. This makes Mala s 11 particularly interesting to study since the observed autoimmune response to hMnSOD could be due to cross-reactivity between Mala s 11 and hMnSOD.

Table 1. Allergens cloned from *Malassezia*

Allergen*	Size (kDa)	Function/sequence similarity	Reference
Mala s 1	36	Unknown	[142]
Mala f 2	20	Peroxisomal protein	[143]
Mala f 3	21	Peroxisomal protein	[143]
Mala f 4	35	Mitochondrial malate dyhydrogenase	[144]
Mala s 5	18	Peroxisomal protein	[138]
Mala s 6	17	Cyclophilin	[138]
Mala s 7	16	Unknown	[145]
Mala s 8	18	Unknown	[145]
Mala s 9	14	Unknown	[145]
Mala s 10	86	Heat shock protein	[139]
Mala s 11	22	Manganese superoxide dismutase	[139]
Mala s 12	67	Glucose-methanol-choline oxireductase family	[146]
Mala s 13	12	Thioredoxin	[75]

* *Malassezia* allergens are referred to by the first four letters in the genus (Mala) instead of only the first three, which is normally the case for allergens, in order to avoid confusion with apple (*Malum* = Mal) allergens.

1.14 CELLULAR INTERACTIONS OF *MALASSEZIA* AND *MALASSEZIA* ALLERGENS

Human immature monocyte-derived dendritic cells (iMDDCs) can bind and internalize *M. sympodialis* yeast cells and allergic components from the yeast [147]. *M. sympodialis* has also been shown to stimulate maturation of DCs in terms of up-regulation of the maturation marker CD83 and the co-stimulatory molecules CD80 and CD86 [148], along with an increased release of the cytokines TNF-α and IL-1β [148]. These data suggested that sensitization to *M. sympodialis* in AE can be mediated by iDCs in the skin, and that this can contribute to the inflammatory reactions. DCs from AE patients also appear to have an increased capacity to react with *M. sympodialis* allergens since *M. sympodialis* up-regulated genes coding for the maturation marker CD83 and the inflammatory cytokine IL-8, among others, in MDDCs from patients with AE but not in MDDCs from healthy individuals [149]. At the T-cell level, AE patients appear to have stronger responses to *M. sympodialis* than do healthy individuals [150, 151]. The T_H2 cell-like cytokines produced by these T lymphocytes suggested that *M. sympodialis* might contribute to an IgE-mediated inflammation in the skin of AE patients [150, 151]. Further studies on DC interaction with *Malassezia*-derived allergens and with their human endogenous homologues will help us to understand the breaking of immunological tolerance to *M. sympodialis,* leading to an autoimmune response against endogenous allergen homologues such as hMnSOD.

M. sympodialis might also influence the interaction between NK-cells and DCs in the skin since MDDCs pre-incubated with this yeast are less susceptible to NK-cell induced cell death [113]. The role of NK-cells in the immune response mounted against this yeast is also indicated by the fact that their presence further increases the expression of CD86 on MDDCs pre-cultured with *M. sympodialis* [112]. Human keratinocytes upregulate the expression of the antimicrobial peptide β-defensin-2 when

exposed to *Malassezia,* thereby limiting further uptake of this yeast [152]. This mechanism is hampered in AE patients for whom a decreased level of antimicrobial peptides is apparent [105]. These results provide an explanation as to why AE patients are more susceptible to the entry of microbes (such as *Malassezia*) in addition to the role of a dysfunctional skin barrier.

2 AIMS OF THE THESIS

The overall aim of this thesis was to gain knowledge regarding the host microbe interactions of allergens from *M. sympodialis* in the pathogenesis of AE and in healthy individuals. This was achieved through studying their structural properties and cellular interactions.

The specific aims of the individual papers were to:

I. Establish crystallization conditions for the *M. sympodialis* allergen Mala s 1.

II. Determine the crystal structure of Mala s 1.

III. Study the effects of the *M. sympodialis* allergen Mala s 11 and its human homologue MnSOD on MDDC differentiation in terms of the expression of maturation markers, signalling in terms of cytokine production, and interaction with lymphocytes.

IV. Determine whether an IgE-mediated cross-reactivity occurs between Mala s 11 and its human homologue MnSOD and if so, to map amino acid residues involved in such cross-reactivity.

3 METHODOLOGY

Methods used in paper I – IV are described in detail in the respective "Materials and methods" sections. Here follows an overview of the methods that were used in this thesis with reference to the papers in which they were applied:

Circular dichroism (CD) (IV)	Structural analysis of the secondary structure of proteins.
Cytometric bead array (CBA) (III)	Flow cytometry-based detection of cytokines in cell culture supernatants by antibody-coated capture beads.
Enzymatic activity test (IV)	Assessment of MnSOD reactivity of rMala s 11 and rhMnSOD.
Flow cytometry (III)	Detection of surface markers on DCs by fluorochrome-conjugated antibodies.
Hanging drop vapour diffusion (I-II)	Technique to generate protein crystals for use in X-ray crystallography.
High pressure liquid chromatography (HPLC) (IV)	Purification of affinity-tagged recombinant proteins.
iMDDC generation (III)	Isolation of $CD14^+$ PBMCs by positive selection using magnetic activated cell sorter (MACS) followed by culturing in the presence of IL-4 and GM-CSF.
Isolation of peripheral blood monocytes (PBMCs) (III)	Ficoll separation of peripheral blood.
ImmunoCAP (III-IV)	Diagnostic assay for measurement of allergen specific IgE in sera or plasma.
Lipid binding assay (II)	Method to study protein-lipid binding by incubating membranes spotted with lipids with protein in solution.
Molecular modelling (IV)	Computational modelling of protein structures using homologous structures as templates.
Molecular replacement (MR) (II)	X-ray crystallography method using diffraction data from crystals of the protein in question in combination with the already

	solved crystal structure of a homologue for structural determination.
PBMC proliferation (III)	Measurement of PBMCs division by [^{3}H]-thymidine incorporation into DNA.
Protein refolding (IV)	Refolding of recombinant proteins by dialysis buffer exchange.
Recombinant protein production (I-IV)	Cloning and expression of recombinant Mala s 11 (rMala s 11) and recombinant human MnSOD (rhMnSOD) in *Escherichia coli.*
Single wavelength anomalous diffraction (SAD) (II)	X-ray crystallography method for structural determination based on diffraction data from crystals containing anomalous scatterers.
Site-directed mutagenesis (IV)	Method to introduce mutations at specific sites in plasmid DNA.
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (I-IV)	Analysis of protein size and purity.
Solid phase- and inhibition enzyme-linked immunosorbent assay (ELISA) (IV)	Detection of allergen specific IgE in sera or plasma (solid phase ELISA). Measurement of inhibition of specific IgE binding to the allergen in question (inhibition ELISA).
Statistical analysis (III)	Non-parametric Friedman two-way analysis of variance followed by Friedman multiple comparison tests. Wilcoxon matched pairs test.

4 RESULTS AND DISCUSSION

4.1 CRYSTALLIZATION AND STRUCTURAL DETERMINATION OF MALA S 1 (PAPERS I-II)

The major allergen Mala s 1 was the first to be identified from *M. sympodialis* [142, 153]. It is of particular interest for several reasons. Firstly, a number of studies report IgE and/or positive SPT or APT to Mala s 1 in AE patients [132, 136, 142, 150, 153-155]. Interestingly, Mala s 1 is present in the cell wall, exposed on the surface of the yeast cells [137] and can be released into the surrounding environment (unpublished data, Christine Selander, Karolinska institutet). This probably facilitates interaction with the human innate and acquired immune systems. DNA sequence comparison indicated that other *Malassezia* species contain sequences with high homology to Mala s 1, suggesting that these species express a Mala s 1 protein homologue [156]. However, Mala s 1 does not exhibit sequence homology to any protein with known function. We aimed to solve the three dimensional (3D) structure of Mala s 1. In doing this we hoped to determine the nature of Mala s 1, both with regard to the function that Mala s 1 might have in the yeast and in regard of properties that might explain the allergenicity of Mala s 1.

4.1.1 Production and crystallization of Mala s 1

The expression of allergens as recombinant proteins has the advantage of being an easy procedure that results in high yields to a relatively low cost in comparison to the isolation of native allergens from extracts [157]. Since the content of different allergens may vary between extracts, recombinant allergens may also be a better alternative for use as standardized diagnostic tools. Allergens are commonly produced as recombinant proteins in *E. coli.* However, recombinant proteins produced in this prokaryotic system might lack structurally important post-translational modifications such as glycosylation.

Recombinant Mala s 1 (rMala s 1) has previously been produced in *E. coli* in our laboratory [154]. The rMala s 1 protein was pure and soluble at a high concentration. It was concluded that only a small fraction of Mala s 1 is glycosylated in its natural form and, importantly, it exhibited IgE-binding capacity similar to that of native Mala s 1 in the extract [154]. We therefore decided to use rMala s 1 produced in *E. coli* as previously described [154] for structural studies of Mala s 1. The expressed protein comprises of 334 amino acid residues with a calculated molecular weight of 36812.8 Da (EMBL accession number X96486) [142]. Following purification both rMala s 1 and Se-Met rMala s 1 migrated as single bands in agreement with the predicted size when analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

X-ray crystallography is an experimental method based on the diffraction of X-rays by protein crystals and has previously been employed to solve the crystal structure of over 40 allergen structures [53, 74, 75]. Within a crystal high numbers of protein molecules are packed together in a homogenous fashion. The X-ray diffraction from a single protein molecule would be too weak to detect, but with a homogenous protein crystal it is possible to amplify this signal. The induced formation of protein crystals is not trivial, however. The protein in question must be of rather high concentration and it has to be relatively pure. The protein is mixed with reagents that lower the solubility of the

protein at concentrations close to spontaneous precipitation. The protein is then slowly concentrated to the point where crystals might start to form. It is important that only a few crystals start to form, so that there is enough soluble protein around in order for them to grow to sufficient size. Normally a number of different reagents have to be tested in order to identify good conditions for crystal growth. In addition, parameters such as protein concentration, pH, temperature, volume of the crystallization sample and pressure all affect crystal formation.

In this study we used the most common technique for generating protein crystals, hanging drop vapour diffusion. In this technique around 1-5 µl of protein in solution and around equal volume of crystallization buffer is placed on a glass cover slide. The cover slide is placed over a reservoir containing around 1 ml of crystallization buffer. The drop now has lower concentration of crystallization buffer than the reservoir. Water will therefore evaporate from the drop, causing the protein concentration in the drop to slowly increase until the point where crystals might form. The best crystallization conditions for rMala s 1 were determined to be 25% polyethylene glycol (PEG) 8K, 0.2M $(NH_4)_2SO_4$. Typically, 1 µl of 3 mg ml^{-1} of protein were mixed at a 1:2 ratio with the crystallization buffer and the drop was allowed to equilibrate at room temperature. Crystals formed after a few weeks and were improved in terms of quality and size by streak seeding using cat whiskers. Since the interactions holding protein molecules together are much weaker than those in mineral crystals, they call for gentle handling. During diffraction data collection the crystal is placed in a strong X-ray beam. One way to make the crystal handle this harsh treatment better is to cool it down before diffraction data collection, and we therefore soaked the crystals in cryoprotectant (80% crystallization buffer and 20% glycerol) before freezing them in liquid nitrogen.

The X-rays are diffracted by the electron cloud of the protein molecules after which the diffracted X-rays are collected by a detector. The crystal is rotated in order to collect the diffraction pattern in all crystal directions. It is of the greatest importance that the crystal is not damaged during data collection since data from all possible crystal directions are required in order to solve the structure. The resolution and hence the quality of the structural model that can be built based on the diffraction data is limited by the quality of the crystal. After data processing the diffraction data is presented as diffraction spots of various intensities. The diffraction data can be described as the Fourier transform of the electron density of the protein molecules. Whereas the intensities of these Fourier transforms are given from the diffraction data, calculating the phase requires additional information. Several approaches to calculate the phases exist. The easiest is molecular replacement (MR) and can be used when a structural homologue of the protein in question is known [71]. This information combined with X-ray diffraction data can be used to solve the 3D structure. For Mala s 1, however, no such homologue with known crystal structure exists. In cases like this one has to use more advanced methods. Here we used single wavelength anomalous diffraction (SAD) in which strong anomalous scatters are added to the protein. This is commonly obtained by exchanging normal methionine residues with methionine residues coupled with selenium, so-called selenomethionine (Se-Met) [158]. By collecting diffraction data on Se-Met substituted protein crystals, the phase of the Fourier transform can be calculated and sufficient data is generated to calculate the electron density of the protein molecules. Se-Met rMala s 1 was produced in *E. coli* using the same clone as for rMala s 1 [154] and was crystallized using the same conditions as for rMala s 1.

Diffraction data used for structural determination was collected at beamlines i711 (Max-lab, Lund, Sweden) and ID29 (ESRF, Grenoble, France). In addition to these datasets diffraction data from the crystals was also collected at BESSY, Berlin, Germany and at NSLS, New York, USA. The structure of Mala s 1 was solved using a SAD dataset collected on a Se-Met rMala s 1 crystal which diffracted to 1.8 Å. By performing MR using a 1.35 Å diffraction dataset collected on a rMala s 1 crystal and the structural model determined with the SAD dataset, a structure with higher resolution was achieved.

4.1.2 Overall structure

The 3D structure of Mala s 1 was determined to a resolution of 1.35 Å. Mala s 1 forms a relatively compact (42x44x46 Å), cup like β–propeller consisting of six blades, arranged cyclically around a central pore (Figure 4). This represents a novel fold, never before observed for an allergen. Some β-propeller proteins, such as proteases, use their central pore for substrate selection and in some cases to maintain their catalytic function without dissociating from the substrate between each catalytic step [159]. Localized beneath the putative active site within the N-terminal crater, is the side chain of residue K259. It should be noted that although the side chain of K259 closes the pore in the present crystal structure, other side-chain rotamers of lysine could result in the partial opening of the pore, suggesting a regulatory and/or gating function for residue K259 (Figure 4b and 4c).

4.1.3 Putative active site of Mala s 1

The active sites involved in substrate recognition or catalysis on β-propeller folded proteins are often localized within the centre of the N-terminal cup [160]. In Mala s 1, this site has two closely positioned glutamate (E38 and E258) as well as two phenylalanine (F111 and F327) residues (Figure 4b and 4c). In addition, the positively charged residues K106, R206, and K259, also point into this potential active site. The entry of the active site is lined with aromatic residues, suggesting that glycosidic bond breakage might be the function of Mala s 1. However, standard enzymatic tests for glycosidase activity were negative in both acidic and alkaline conditions when using various Mala s 1 concentrations. We can not exclude that the reaction conditions used might not be optimal in order to reveal the putative enzymatic activity of Mala s 1.

4.1.4 Structural homologues of Mala s 1

As previously mentioned, Mala s 1 does not display primary structure homology to any protein with known function. Having solved the 3D structure of Mala s 1 we aimed to find structural homologues. A number of proteins were identified to share a similar overall homology to Mala s 1, but did not display any resemblance to the hypothetical active site. However, upon threading all protein sequences deposited in SwissProt on the crystal structure of Mala s 1, two potential homologues; the hypothetical proteins Q4P4P8 (NCBI accession number XM_755969) and Tri114 (NCBI accession number XM_383719), both with unknown functions, were found. These two proteins derive from the maize parasite *Ustilago maydis* [161, 162] and the wheat parasite *Gibberella zeae* [163], respectively. Tri14 is a protein part of the mycotoxin (trichothecene) synthesis gene cluster largely responsible for the pathogenicity of *G. zeae* as demonstrated by its severely impaired virulence in knockout strains [164].

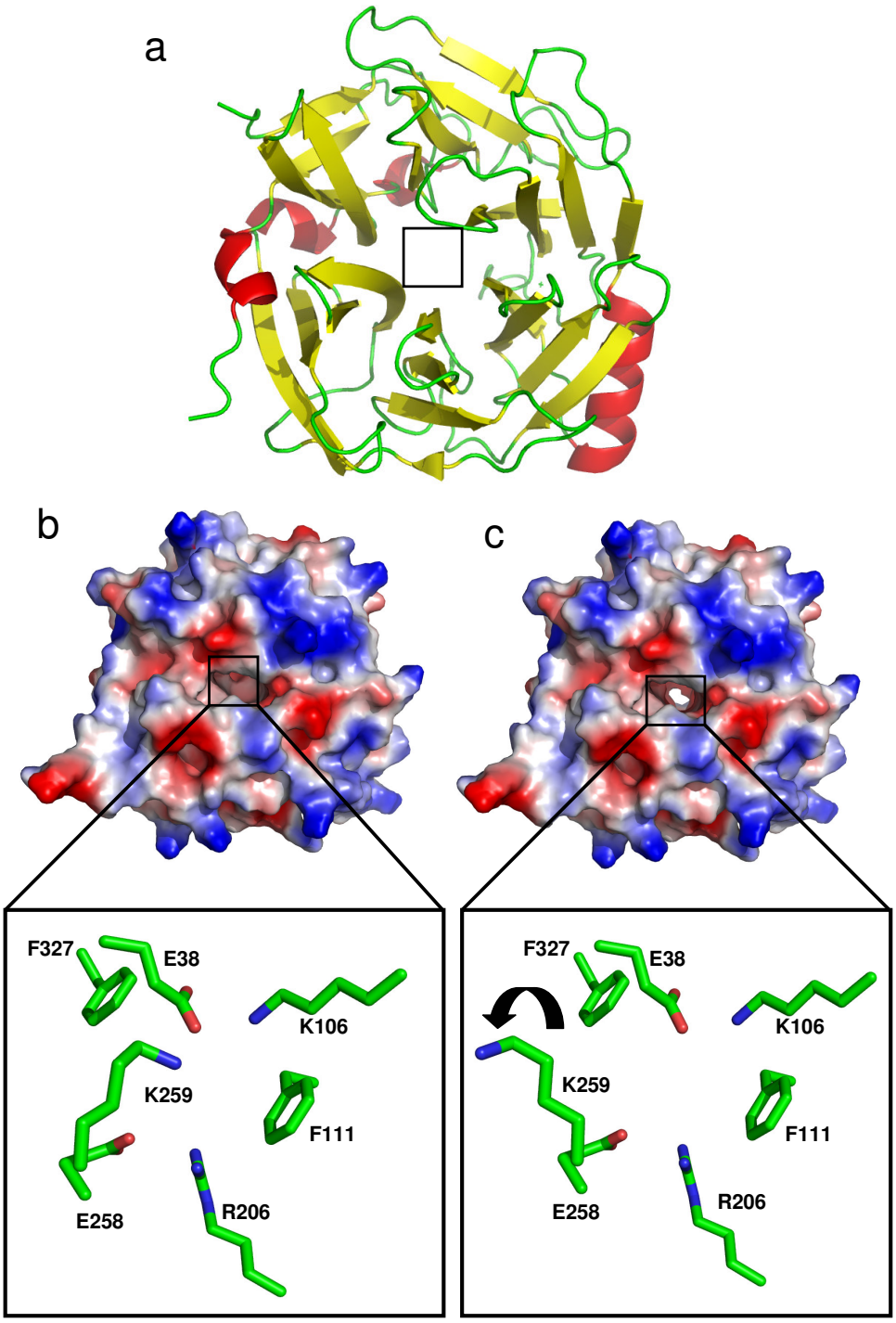

Figure 4. Crystal structure of Mala s 1. (**a**) Ribbon diagram of the overall structure of Mala s 1 with β-sheets, α-helix and random coil coloured in yellow, red and green, respectively. The position of the putative active site is indicated with a black square. Figures (**b**) and (**c**) show surface representation of Mala s 1 with negatively and positively charged areas coloured in red and blue, respectively. A magnification of the putative active site suggests that different conformations of residue K259 may either close (**b**) or open (**c**) access to the pore of Mala s 1.

Interestingly, Tri14 has been suggested to be involved in host-cell recognition or in cell-wall processes involved in plant cell invasion by the parasite. This would concord with our finding that Mala s 1 is localized in the cell wall. A comparison of the putative active site of Mala s 1 with the corresponding region in Q4P4P8 and Tri114 indicated that a phenylalanine residue (F111) and a glutamic acid residue (E38) are conserved between all three proteins at this site. Furthermore, two additional residues, K106 and R206, are also conserved between Mala s 1 and Q4P4P8. The conserved residues within the potential active sites might suggest similar functions for Mala s 1 in the yeast *M. sympodialis* and for the two related proteins of the plant fungal parasites. Recently, the genomes of the *Malassezia* species *M. globosa* and *M. restricta* were sequenced [128]. The resemblance between *Malassezia* and *Ustilago maydis* is clearly not limited to Mala s 1 and Q4P4P8 since DNA sequence comparison indicated *Ustilago maydis* as the fungus most closest related to *Malassezia* among all fungi with complete genome sequences [129].

4.1.5 Lipid binding

Mala s 1 is mainly localized in the cell wall and is exposed to the cell surface [137]. Since phosphatidylinositols are lipids involved in membrane trafficking [165] we investigated the ability of Mala s 1 to bind such lipids. The results indicate that Mala s 1 is able to bind phosphatidylinositol (PtdIns) (3)-phosphate (P), PtdIns(4)P and PtdIns(5)P. Although the nature of this binding has to be investigated further, these results suggest that PtdIns are possibly involved in the transport of Mala s 1 to the cell wall.

4.1.6 Implications for Mala s 1 in AE

Mala s 1 has the capacity to induce the production of IgE and IgG [132, 136, 166] along with eliciting positive SPTs or APTs [132, 150] in AE patients. Mala s 1 can induce proliferation of PBMCs *in vitro*, both in AE patients and in healthy individuals [150]. *In vivo*, it is likely that cells of the immune system such as DCs come into contact with Mala s 1 since this particular allergen is expressed on the surface of the yeast cells [137] and can be released into the culture medium (unpublished data, Christine Selander, Karolinska Institutet). We here present a novel protein fold, never before observed among allergens. The overall fold of Mala s 1 as such, can therefore not explain its allergenic properties. Instead, it supports the hypothesis that allergenicity can not be predicted solely by the overall structure. However, the 3D structure of Mala s 1 provides some suggestions as to why Mala s 1 belongs to the group of antigens that can elicit an allergic response. A compact 3D structure is one property related to allergens [46] and is what we observed for Mala s 1. Although Mala s 1 lacks cysteine residues and hence does not form disulfide bonds, binding of a ligand to the putative active site of Mala s 1 might contribute to the stability of the protein. We demonstrate that Mala s 1 bind lipids, a property observed among allergens [46, 48].

4.2 THE EFFECT OF MALA S 11 AND HUMAN MNSOD ON MDDCS (PAPER III)

Whereas Mala s 1 does not exhibit sequence homology to any known proteins, Mala s 11 has high homology to manganese superoxide dismutase (MnSOD) [139]. The finding that this allergen has an IgE-binding frequency of 75% to 28 sera from patients

with AE having IgE-antibodies to *M. sympodialis* indicates that this is a major allergen [139]. Human MnSOD (hMnSOD) can bind IgE, induce T-cell reactivity and positive APTs in AE patients sensitized to *Malassezia* [141]. This makes Mala s 11 particularly interesting to study since the observed autoimmune response to hMnSOD could be due to cross-reactivity between Mala s 11 and hMnSOD.

Given that DCs are faced with the task of discriminating between self and non-self, we hypothesized that Mala s 11 and hMnSOD might affect DCs generated from healthy individuals differently. To this end we studied their effects on DCs in their immature state, reflecting the properties of DC subsets such as Langerhans´ cells in the skin. Since our experimental set-up necessitated a high number of cells and isolation of DC directly from blood or skin might not lead to sufficient numbers of DCs in their immature state, we generated DCs from peripheral blood monocytes *in vitro*. This was accomplished by culturing monocytes in the presence of IL-4 and GM-CSF for six days according to previously established protocols [16, 148].

4.2.1 Alteration of DC phenotype

To study the effect on surface marker expression, generated immature MDDCs (iMDDCs) were co-cultured with rMala s 11 or rhMnSOD. In parallel, iMDDCs cultured in medium alone or in the presence of LPS were used as negative and positive controls, respectively. After 24 h the MDDCs stimulated with rMala s 11 or LPS displayed a mature phenotype with a statistically significant higher expression of the maturation marker CD83 and co-stimulatory molecules CD40, CD80 and CD86 in comparison to MDDCs cultured in medium alone or co-cultured with rhMnSOD. Our observations suggest that Mala s 11 can affect the immune system by up-regulating DC maturation markers and co-stimulatory molecules, whereas hMnSOD does not. In addition they indicate that Mala s 11, as opposed to hMnSOD, induces DCs with a higher T-cell stimulatory capacity through up-regulation of HLA-DR.

4.2.2 Release of cytokines

The maturation state of DCs are associated with the release of various cytokines influencing the immune system [10, 11]. We therefore collected supernatants from MDDCs co-cultured with rMala s 11, rhMnSOD, LPS or cultured in medium alone, and analysed these for TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12p70 contents using a cytometric bead array (CBA). Using this technique all the above cytokines can conveniently be measured simultaneously in one sample (detection limit 20 pg/ml). After 4 h there was a significant increase with a further increase at 24 h in the levels of TNF-α, IL-6 and IL-8 in supernatants from MDDCs co-cultured with rMala s 11 in comparison to supernatants from MDDCs cultured in medium alone. Significantly higher levels of IL-10 and IL-12p70 were observed after 24 h in supernatants from MDDCs co-cultured with rMala s 11 in comparison to MDDCs cultured in medium alone. The cytokine levels from MDDCs co-cultured with rMala s 11 were at both time points for all cytokines analysed in the range with that of MDDCs co-cultured with LPS. For MDDCs co-cultured with rhMnSOD, the cytokine concentrations were at the levels of MDDCs cultured in medium alone. TNF-α is known to induce DC migration [8, 167]. Hence, Mala s 11 might induce DC migration whereas hMnSOD does not. Although IL-6 has earlier been connected with a T_H2 differentiation of naïve T lymphocytes [168], recent studies suggest IL-6 to promote Th17

differentiation in the presence of TGF-β while inhibiting T_{reg} differentiation [24]. Our results therefore indicate that Mala s 11 might promote a T_H17 differentiation, in the case TGF-β is present, along with suppression of T_{reg} differentiation. The chemoattractant cytokine IL-8 mediates inflammation through recruitment of neutrophils and T-cells [169]. Hence our results suggest that in contrast to hMnSOD, Mala s 11 could be of importance for the recruitment of inflammatory cells. IL-12 production from DCs induces a T_H1 differentiation while IL-10 can inhibit T_H1 differentiation via inhibition of IL-12 production and promote T_{reg} differentiation in the presence of IL-10 [24, 170]. Since we here observed a simultaneous release of IL-10 and IL-12 from six out of the nine donors tested, we can not, however, draw any conclusions about T_H1- or T_H2-biased differentiation based on the production of these cytokines. The levels of IL-1β remained unaltered in all MDDC culture conditions.

Previously, it has been demonstrated that *M. sympodialis* whole yeast cells induce maturation of human MDDCs from healthy individuals, in terms of up-regulated levels of CD80, CD83 and CD86 along with elevated levels of TNF-α [148], which is in agreement with our present data using only its component Mala s 11. In contrast, IL-10 and IL-12p70 levels released from MDDCs from healthy donors co-cultured with *M. sympodialis* remained unaltered [148]. The difference between the present study in which increased levels of these two cytokines were observed in response to Mala s 11 could be due to a later time point (46 h) for the cytokine measurements compared to the previous study, in addition to a different method for cytokine detection, besides the use of whole yeast cells [148].

4.2.3 Stimulation of allogeneic lymphocytes

After having analysed the phenotypic differences in MDDCs stimulated with rMala s 11 and hMnSOD we investigated their ability to induce lymphocyte proliferation. MDDCs pre-cultured with rMala s 11, rhMnSOD or LPS or cultured in medium alone were studied in an allogeneic lymphocyte proliferation assay. iMDDCs pre-cultured with medium alone generated a MDDC/PBMC ratio-dependent proliferative response in allogeneic CD14 depleted PBMCs. This proliferation was further enhanced by the exchange of MDDCs pre-cultured with rhMnSOD, rMala s 11 or LPS. However, the lymphocyte stimulating capacity was significantly higher ($P < 0.05$) for MDDCs stimulated with rMala s 11 than with rhMnSOD. In four out of five experiments performed, MDDCs pre-stimulated with rMala s 11 induced a proliferative response in the range of that of MDDCs pre-stimulated with LPS. The maximum background proliferation for PBMCs alone ($2x10^5$ cells/well) and MDDCs alone (10^4 cells/well) never exceeded 1416 ± 32 cpm and 399 ± 78 cpm (mean ± SEM, n = 5), respectively. Thus these results, in addition to the phenotype analysis, suggests that DCs of healthy individuals possess the ability to discriminate between the exogenous microbial component Mala s 11 protein and its human homologue hMnSOD, despite their high degree of sequence homology. The presence of TLR-ligands such as LPS has been suggested to be one way in which DCs discriminate between microbial and self-antigens [171]. In the present study, the ability of MDDCs to discriminate between Mala s 11 and hMnSOD is not likely to be due to LPS contamination, since the LPS levels in the preparations were equally very low (2.7 pg/ml for rMala s 11 and 15 pg/ml for rhMnSOD) and in control experiments these LPS levels did not affect the MDDC phenotype or the release of cytokines. However, the possible adjuvant effect of additional contaminants such as CpG DNA can not be entirely ruled out.

4.3 MUTATIONAL ANALYSIS OF AMINO ACID RESIDUES INVOLVED IN IgE-MEDIATED CROSS-REACTIVITY BETWEEN MALA S 11 AND HUMAN MNSOD (PAPER IV)

IgE mediated cross-reactivity between allergens of high sequence homology is a well established phenomenon [30, 56]. Such a cross-reactivity has also been reported between allergens and human endogenous proteins [74, 75]. This can be exemplified by MnSOD from *A. fumigatus*, Asp f 6, which cross-reacts with human MnSOD [140]. A comparison of the crystal structures of Asp f 6 and hMnSOD revealed patches of identical amino acid residues that are displayed to the surface of both enzymes and which could therefore constitute cross-reactive IgE-binding epitopes [71]. Since IgE antibodies to hMnSOD along with positive SPT and APT have been observed in AE patients sensitized to *M. sympodialis* [141], we hypothesized that such a cross-reactivity also occurs between Mala s 11 and hMnSOD. In addition, we aimed to identify amino acids involved in such cross-reactivity.

4.3.1 IgE-mediated cross-reactivity between Mala s 11 and hMnSOD

In order to investigate a possible cross-reactivity between Mala s 11 and hMnSOD we first screened plasma samples from AE patients sensitized to *M. sympodialis* for specific IgE to both rMala s 11 and rhMnSOD. The ability of rMala s 11 and rhMnSOD to inhibit IgE binding to each other was then studied using inhibition ELISAs. In contrast to the negative control BSA, rMala s 11 and rhMnSOD exhibited comparable inhibition of IgE-binding to rMala s 11 for the five plasma samples that were tested (Figure 5a). IgE binding to solid-phase coated rhMnSOD was also inhibited by rMala s 11 added in fluid phase (Figure 5b). These results demonstrate that Mala s 11 and hMnSOD share common IgE binding epitopes and indicates that hMnSOD may maintain activation of the immune response in the absence of Mala s 11. MnSODs from various species have been demonstrated to cross-react at B- and T-cell levels [140]. We thus demonstrate that Mala s 11 should be added to the group of cross-reacting MnSODs, providing a plausible explanation for the autoreactivity observed in a subgroup of AE patients.

IgE-mediated autoreactivity to proteins other than Mala s 11 has previously been suggested to have implications in AE [85, 86, 172, 173]. However, an autoimmune response to MnSOD might enhance inflammatory skin processes. This may be due to the fact that MnSOD expression is up-regulated by factors such as mechanical trauma [174] and hence the scratching associated with AE [81] might cause an increased release of endogenous MnSOD. Higher levels of MnSOD actually occur in lesional skin areas of AE patients when compared to unaffected skin as well as the skin of healthy individuals [141].

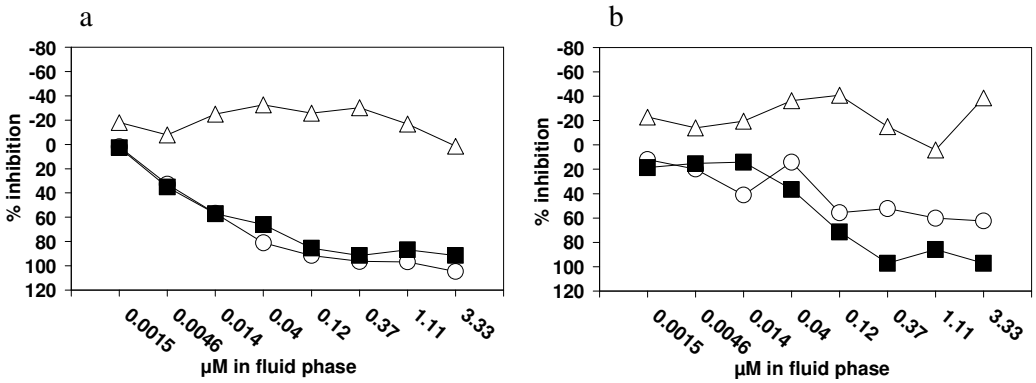

Figure 5. Inhibition of IgE-binding to rMnSODs in the solid phase. Plasma from an AE patient sensitised to rMala s 11 and rhMnSOD was pre-incubated with increasing amounts of rMala s 11 (○), rhMnSOD (■) or BSA (Δ). Pre-incubated plasma was transferred to plates pre-coated with rMala s 11 (**a**) or rhMnSOD (**b**) and IgE binding was analysed by ELISA. Representative results from one out of five assays performed are depicted.

4.3.2 Identification of potential cross-reactive IgE binding epitopes

After having demonstrated that Mala s 11 and hMnSOD do in fact share common IgE cross-reacting epitopes, we also aimed to identify the residues involved in this cross-reactivity. Based on primary sequence alignment we mapped the homology between Mala s 11 and MnSODs from *A. fumigatus*, *H. sapiens*, *D. melanogaster* and *S. cerevisiae*. We also created a molecular model of Mala s 11 based on its homology to hMnSOD [175], to identify the conserved residues that are exposed on the surface of Mala s 11 and which could hence constitute cross reacting IgE epitopes. Our model of Mala s 11 exhibits an α/β fold in accordance with known crystal structures of MnSODs [71, 141, 175]. The crystal structures of *A. fumigatus* and *H. sapiens* MnSOD indicate that both enzymes form tetramers. We thus hypothesized that Mala s 11 may also form a tetramer. Mapping of conserved residues in the model of Mala s 11 allowed the identification of four potential cross-reacting regions that included a total of 17 key residues.

Two of the residues in region 1 (K43 and N50) point towards the solvent in both the monomer and the tetramer, whereas accessibility of residues A77 and K79 to the solvent is relatively reduced in the tetramer in comparison to in the monomer. It is therefore likely that residues K43 and N50 are the most important for IgE binding in region 1 and they have also been suggested to be involved in IgE-mediated cross-reactivity between *A. fumigatus* and *H. sapiens* MnSOD [140]. All four residues in region 2 protrude into the solvent both in the monomer and in the tetramer models of Mala s 11, indicating their accessibility for antigen-antibody interactions. In region 3, residues L180, Q181, Y182 and N184 are exposed to the solvent in both the monomer and the tetramer. However, upon the formation of a hypothetical tetramer, our model suggests that the side chain of residue L180 may interact with the side chain of residue L180 in the adjacent monomer, and may thus not be as exposed to the solvent when compared to the four other residues that comprise region 3. Similarly, our molecular model suggests that antibody access to residue Q136 could be sterically hindered in the tetramer. Three residues, P19, P97 and Q98, are accessible to the solvent in region 4

within the tetramerical model of Mala s 11. As for Q136 in region 3, the side chain of residue Y23 is buried within the tetrameric form of Mala s 11.

All of these amino acid residues were altered using site-directed mutagenesis. For each of the four regions two mutated versions were produced. In the first version all residues except A77 were modified to alanine (A versions). In the second version all residues were mutated to aspartate or to residues with opposing polarity (B versions). All mutated versions of Mala s 11 migrated as single bands in agreement with the predicted sizes when analysed by SDS-PAGE. In addition, they were all enzymatically active with CD (circular dichroism) spectra similar to that of wild-type (WT) rMala s 11, indicating a high secondary structure similarity. In order to study the importance of the identified regions for IgE binding, the IgE binding capacity of mutated rMala s 11 proteins was compared to that of WT rMala s 11 in inhibition ELISA assays (Figure 6).

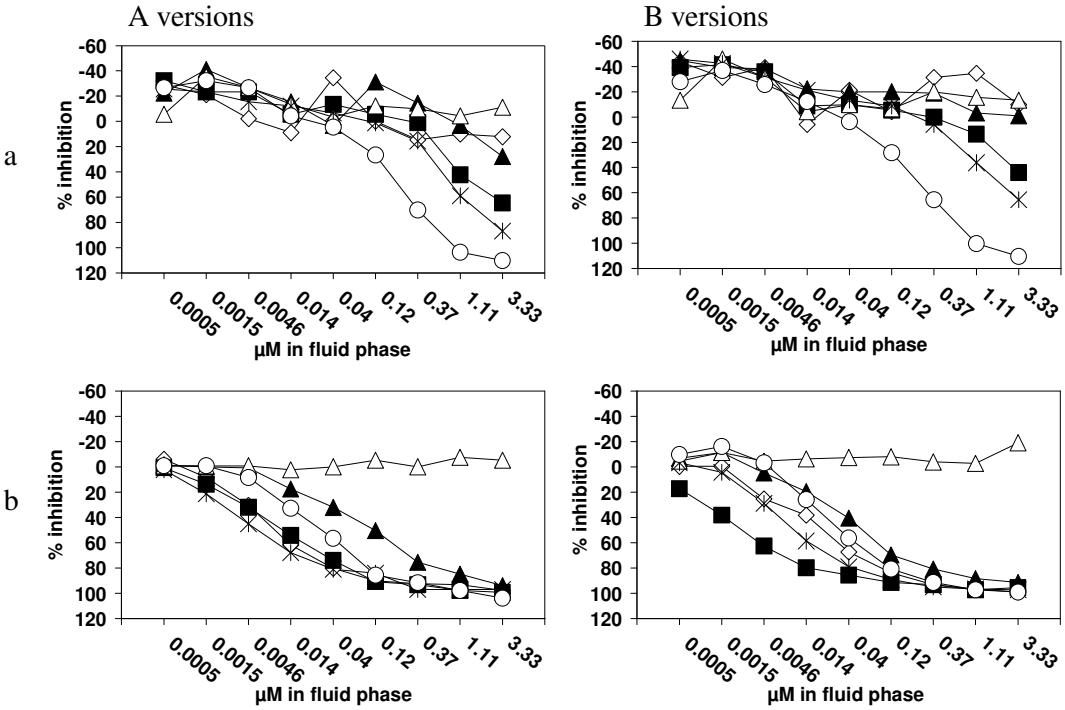

Figure 6. Inhibition of IgE binding to WT rMala s 11 in solid phase. In each assay plasma from an AE patient sensitised to rMala s 11 and rhMnSOD was pre-incubated with increasing amounts of WT rMala s 11 (○) or the A or B versions of rMala s 11 mutated in region 1 (ж), region 2 (▲), region 3 (■), region 4 (◊) or BSA (Δ) as control. Pre-incubated plasma was transferred to plates pre-coated with rMala s 11 and IgE-binding was analysed with ELISA. a) Plasma sample in which all mutated versions showed lower IgE binding in comparison to WT rMala s 11 (○). b) One representative out of four assays performed where only mutations in regions 2 (▲) led to lower IgE binding in comparison to WT rMala s 11 (○).

Five ELISA assays, each using a different patient sample, were performed using plasma from AE patients sensitised to both rMala s 11 and rhMnSOD. rMala s 11 inhibited IgE binding to itself in a dose-dependent manner in all assays, whereas the negative control protein BSA did not inhibit IgE binding to rMala s 11. In one assay lower IgE-binding was observed for all mutated rMala s 11 versions compared to the

binding capacity of WT rMala s 11 (Figure 6a). In the additional four assays only the rMala s 11 versions where mutations were introduced in region 2, resulted in lower IgE-binding when compared to WT rMala s 11 (Figure 6b). Thus our results indicate that region 2, comprising residues E29, P30, E122 and K125, is of importance for IgE binding to Mala s 11. Our results indicate that additional residues involved in IgE binding to Mala s 11 remain to be mapped since the mutations in region 2 only lowered IgE binding to a certain extent.

The results presented herein reveal IgE-mediated cross-reactivity between Mala s 11 and human endogenous MnSOD, which could provides an explanation at the molecular level for the autoreactivity to hMnSOD observed in AE patients. We also identified residues possibly involved in such autoreactivity.

5 CONCLUSIONS

I. In this study we established the crystallization conditions for Mala s 1. Crystals formed in 25 % PEG 8K, 0.2 M $(NH_4)_2SO_4$. They belonged to space group $P2_12_12$, with unit-cell parameters a = 44.4, b = 163.7, c = 50.6 Å and diffracted to 1.35 Å resolution. The crystal structure of Mala s 1 will provide us with important insights into both the structure and the function of Mala s 1.

II. In this study we solved the crystal structure of Mala s 1. Mala s 1 was shown to exhibit a 6-fold β-propeller fold. This is a novel fold among allergens. The putative active site of Mala s 1 overlaps structurally to putative active sites of the potential fungal protein homologues Q4P4P8 and Tri 14, derived from the plant parasites *Ustilago maydis* and *Gibberella zeae*, respectively. This resemblance suggests that Mala s 1 and the parasite proteins may share similar functions. In addition, we demonstrate that Mala s 1 binds to phosphatidylinositol (PtdIns) (3)-phosphate (P), PtdIns(4)P, and PtdIns(5)P, lipids with a possible role in the localization of Mala s 1 to the cell surface.

III. We herein demonstrate that whereas rhMnSOD did not affect the MDDC phenotype in healthy individuals, rMala s 11 up-regulated expression of the maturation marker CD83, the co-stimulatory molecules CD40, CD80 and CD86 and HLA-DR to a similar extent as did LPS. Furthermore, rMala s 11, but not rhMnSOD, induced significantly higher levels of TNF-α, IL-6, IL-8, IL-10 and IL-12p70 in cell culture supernatants after 24 h in comparison to MDDCs cultured in medium alone. Finally, MDDCs pre-incubated with rMala s 11 induced a significantly higher proliferation of allogeneic CD14-depleted peripheral blood monocytes than did MDDCs pre-incubated with rhMnSOD. Our results suggest that Mala s 11 but not hMnSOD affects the immune response of healthy individuals through DC maturation and cytokine release. This indicates that DCs possess the ability to distinguish between Mala s 11 and its human homologue MnSOD.

IV. We herein demonstrate that rMala s 11 is able to inhibit IgE-binding to rhMnSOD and *vice versa*, indicating that these two homologues share common IgE epitopes. In addition, we identified residues possibly involved in such cross-reactivity. These results provide an explanation at a molecular level for the autoreactivity to hMnSOD observed in AE patients sensitized to *M. sympodialis*.

In summary, we have determined a novel three dimensional structure not previously demonstrated among allergens. We demonstrate the ability of DCs to distinguish between proteins with high sequence homology and we provide a structural basis underlying the autoimmune response to hMnSOD in AE based on IgE-mediated cross-reactivity.

6 FUTURE PERSPECTIVES

The research I have performed during my PhD was done with the aim of deepening knowledge of the structure and function of *M. sympodialis* allergens. Indeed, the results presented herein have provided new insights into these areas. However, they have also raised new questions. I will here outline my ideas of how to proceed with the research within this field in order to further understand the host-microbe interactions of *Malassezia* and its implications in AE.

An allergic reaction to *Malassezia* is observed in around 50% of adult AE patients, while this is very rarely observed in patients with allergic diseases or in healthy controls [132]. This suggests a specific link between *Malassezia* and AE and it is therefore my opinion that this interaction is worthy of further study. Based on what is known today it is not clear whether *Malassezia* can contribute to the development of AE or if it only worsens the symptoms in patients already having AE. Given that *Malassezia* is part of the normal skin flora [117] and induces IgG production and PBMC proliferation in healthy individuals [176], this yeast also comes into contact with the immune system in healthy conditions. Currently, efforts are ongoing into understanding what causes the dysfunctional skin barrier observed in AE and what consequences it has on host-microbe interactions. My suggestion would be to examine the role that the dysfunctional skin barrier has in sensitization to *Malassezia*. Likely, this facilitates the penetration of *Malassezia* cells and released allergens into the skin. Further studies comparing the interaction of *M. sympodialis* allergens with immune cells from AE patients and healthy individuals will help us to understand why an allergic sensitization to this yeast is frequently observed among AE patients and is very rare in other allergic conditions [101, 132]. It has been shown that MDDCs can take up whole *Malassezia* yeast cells, leading to maturation and release of inflammatory cytokines [147, 148], and here I show that Mala s 11 alone can induce such a response in MDDCs from healthy individuals. Additional studies involving DCs from AE patients will be necessary to understand the role of DCs in the sensitization to *Malassezia* allergens in AE.

Autoimmunity based on IgE-mediated cross-reactivity has been suggested to be of importance in AE [74, 75] and we demonstrate that Mala s 11 can be added to the previously presented cross-reactive structures of particular importance in this disease. It would be interesting to examine whether Mala s 11 and hMnSOD also cross-react at the T-cell level. In addition, the use of our mutated versions of Mala s 11, in which the residues involved in IgE-mediated cross-reactivity have been altered, in SPT and APT would clarify the *in vivo* importance of these residues. Here, I also show the ability of MDDCs from healthy individuals to distinguish between Mala s 11 and hMnSOD despite their high degree of homology. Future work on MDDCs or possibly directly isolated DCs from AE patients is needed to elucidate whether the immunological tolerance to hMnSOD is broken at a DC level in patients sensitized to hMnSOD. This would help to clarify if endogenous allergen homologues can induce an immune response leading to a switch to IgE production in B-cells or if the clinical response

observed is only due to IgE-mediated cross-reactivity. In addition to an autoimmune response based on molecular mimicry, a subgroup of AE patients also have a response to autoantigens lacking environmental allergen homologues [85]. This appears to be related to disease severity [85]. It would be of great value to further investigate the immunological responses underlying sensitization to these substances.

As a step in clarifying the immune response to *Malassezia*, additional studies of which particular T lymphocyte subsets that are involved is needed. The studies performed so far have focused on the induction of T_H1 and T_H2-type subsets [150]. T_{regs} have been suggested to have implications in AE [177] and in allergic diseases in general [22]. The newly characterized T lymphocyte subset T_H17 plays a role in protection against fungi [24]. For these reasons it would be of great value to establish the role of these two subsets in the responses to *M. sympodialis* in AE patients and in healthy individuals.

Concerning the allergenic components of *Malassezia*, although several reports deal with this topic, much work remains. Firstly, as indicated by phage display analysis of the IgE-binding repertoire of *M. sympodialis* [138, 178], the list which at present contains 10 allergens is far from complete. In addition to these, allergens from *Malassezia* species other than *M. sympodialis* have to be taken into account. To further understand the allergenicity of *Malassezia* and to improve diagnostic methods, additional allergens have to be sequenced, expressed as recombinant proteins, verified in terms of their relative IgE-binding capacity and T-cell reactivity.

Even when it comes to the allergens already identified and expressed, many questions remain to be answered. Among other things, the function of four of these *Malassezia* allergens is still unknown. One way to find out more about the nature of these allergens is by determining their three dimensional structures. This can provide clues both to the allergenicity of these proteins and to their function in *Malassezia*. In addition they can be added to the panel of *Malassezia* allergens currently used for research purposes. In this thesis I have presented the crystal structure of the major allergen Mala s 1. Although this has provided valuable information about this protein, its exact function remains to be determined. Mala s 1 is of particular interest since it is a major allergen located on the yeast cell surface, rendering it easily accessible to the immune system and is therefore, in my opinion, worth further investigation. One way to study the function of Mala s 1 and other functionally unknown allergens is by generating a knock-out version of *Malassezia* and to study how this affects the properties of the yeast. According to our results, homologues to Mala s 1 likely to have similar functions exist in the plant parasites *Ustilago maydis* and *Gibberella zeae*. Hence, a breakthrough in understanding the function of these homologues will also assist in elucidation of the function of Mala s 1.

In addition to the IgE binding components, other substances *e.g.* lipids or carbohydrates present in the cell wall are likely to influence the immune response, as evident for other allergenic sources [47]. *M. sympodialis* is of particular interest given that is the most commonly isolated *Malassezia* species, at least in studies performed in Sweden, Russia and Canada [117, 118, 127]. Sequencing the whole genome for *M. sympodialis*, as

recently done for *M. globosa* and *M. restricta* [128, 129] would help to further understand the nature of the fungus and its interaction with the host.

In addition to allow for standardized diagnostics, recombinant allergens are used in specific immunotherapy (SIT) [33]. Although a few studies indicate a positive effect of SIT in AE patients [179, 180], its potential use in this patient group needs to be further investigated. None of the studies have been performed using *Malassezia* allergens and their usefulness for this purpose is currently unclear.

It is evident that AE represents a complex skin disease with several factors contributing to the symptoms. It also seems that AE patients can be divided into subgroups based on the presence or absence of *e.g.* elevated total serum IgE, sensitization to *Malassezia*, reaction to autoantigens and co-existing asthma. In addition to this comes the involvement of genes related to skin barrier function [81]. In my opinion, this indicates that further efforts should be made into thorough diagnoses of the individual patients to optimize the understanding of the particular pathogenic mechanisms and thereby the prevention and treatment.

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