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DEVELOPMENT OF A GLYCOCLUSTER ADJUVANT MIMICKING NATURAL YEAST β-(1,2)-STRUCTURES

Kaarina Mäkinen (née Ranta)

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ABSTRACT

Kaarina Mäkinen (née Ranta)
Development of a glycocluster adjuvant mimicking natural yeast β-(1,2)-structures

Department of Pulmonary Diseases and Clinical Allergology, University of Turku
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Allergy is characterized by T helper (Th) 2-type immune response after encounter with an allergen leading to subsequent immunoglobulin (Ig) E-mediated hypersensitivity reaction and further allergic inflammation. Allergen-specific immunotherapy (SIT) balances the Th2-biased immunity towards Th1 and T regulatory responses. Adjuvants are used in allergen preparations to intensify and modify SIT. β-(1,2)-oligomannoside constituents present in *Candida albicans* (*C. albicans*) cell wall possess Th1-type immunostimulatory properties. The aim of this thesis was to develop a β-(1,2)-linked carbohydrate compound with known structure and anti-allergic properties to be applied as an adjuvant in SIT. First the immunostimulatory properties of various fungal extracts were studied. *C. albicans* appeared to be the most promising Th1-inducing extract, which led to the synthesis of various mono- or divalent oligomannosides designed on the basis of *C. albicans*. These carbohydrates did not induce strong cytokine production in human peripheral blood mononuclear cell (PBMC) cultures. In contrast to earlier reports using native oligosaccharides from *C. albicans*, synthetic β-(1,2)-linked mannotetraose did not induce any tumor necrosis factor production in murine macrophages. Next, similarities with synthesized divalent mannosides and the antigenic epitopes of β-(1,2)-linked *C. albicans* mannan were investigated. Two divalent compounds inhibited specific IgG antibodies binding to below 3 kDa hydrolyzed mannan down to the level of 30–50% showing similar antigenicity to *C. albicans.* Immunomodulatory properties of synthesized carbohydrate assemblies ranging from mono- to pentavalent were evaluated. A trivalent acetylated dimannose (TADM) induced interleukin-10 (IL-10) and interferon-γ responses. TADM also suppressed birch pollen induced IL-4 and IL-5 responses in allergen (Bet v) stimulated PBMCs of birch pollen allergic subjects. This suppression was stronger with TADM than with other used adjuvants, immunostimulatory oligonucleotides and monophosphoryl lipid A. In a murine model of asthma, the allergen induced inflammatory responses could also be suppressed by TADM on cytokine and antibody levels.

Keywords: adjuvant, murine model, specific immunotherapy, triacedimannose

TIIVISTELMÄ

Kaarina Mäkinen (née Ranta)
Sienien β-(1,2)-sokerirakenteita muistuttavan adjuvantin kehittäminen

Turun yliopisto, keuhkosairausoppi ja kliininen allergologia
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Allergeeni aiheuttaa allergisen ihmisen elimistössä tyypin 2 auttaja-T-soluvälitteisen (Th2) puolustusvasteen, josta seuraa immunoglobuliini E (IgE) -välitteinen yliherkkyysreaktio ja allerginen tulehdus. Siedätyshoidolla pyritään Th2-soluvasteiden sijaan aiheuttamaan tyypin 1 auttaja- (Th1) ja säätelijä-T-soluvaste (Treg). *Candida albicans* -hiivan (*C. albicans*) β-(1,2)-sidoksia sisältävien mannoosisokerien on havaittu vaimentavan allergista immuunivastetta. Tämän tutkimuksen tavoitteena oli kehittää β-(1,2)-sidoksia sisältävä allergista immuunivastetta tehokkaasti vaimentava mannoosisokeri, jota voitaisiin käyttää siedätyshoidossa allergeenin kanssa tehosteena eli adjuvanttina. Tutkimuksessa seulottiin useita eri sieniä, niiden uutteita sekä mannaaneja uudella menetelmällä. *C. albicans* ja sen mannaanit osoittautuivat tehokkaimmiksi Th1-immuunivasteen aktivoijiksi. Tämän vuoksi *C. albicans* -hiivan rakenteen pohjalta kehitettiin ja tutkittiin kemiallisesti tuotettuja mono- ja divalentteja oligomannosideja. Vastoin aiempia tutkimustuloksia β-(1,2)-sidoksia sisältävä, neljästä mannoosisokerista koostuva rakenne ei saanut hiiren makrofageja tuottamaan TNF-sytokiinia. Seuraavaksi tutkittiin divalenttien mannosidien ja β-(1,2)-sidoksia sisältävien *C. albicans* -hiivan mannaanien antigeenisten epitooppien yhtäläisyyksiä. Kaksi divalenttia sokerirakennetta estivät IgG-vasta-aineiden sitoutumisen alle 3 kDa:n kokoiseen mannaaniosaan 50–70-prosenttisesti. Tulos osoittaa, että tutkitut divalentit sokerirakenteet ovat samankaltaisia *C. albicans* -hiivan antigeenisten rakenteiden kanssa. Seuraavaksi tutkittiin kemiallisesti tuotettujen sokerirakenteiden immuunivasteita muokkaavia ominaisuuksia. Trivalentti asetyloitu mannobioosi (TADM) aktivoi Treg- ja Th1-tyyppistä immuunivastetta *in vitro*. TADM vaimensi koivuallergeenin aiheuttamien sytokiinien (IL-4, IL-5) tuoton koivuallergisilta henkilöiltä eristetyissä soluissa *in vitro*. TADM vaimensi koivuallergeenin aiheuttamat sytokiinivasteet siitepölykauden aikana eristetyissä allergisten henkilöiden soluviljelmissä tehokkaammin kuin tunnetut adjuvantit, CpG-oligonukleotidit ja monofosforyloitu lipidi A. Vastaavia tuloksia saatiin myös *in vivo*, kun TADM vaimensi allergeenin aiheuttamaa välittäjä- ja vasta-aineiden tuottoa hiirimallissa.

Avainsanat: adjuvantti, hiirimalli, siedätyshoito, trivalentti asetyloitu mannobioosi

CONTENTS

ABBREVIATIONS

A. bisporus	*Agaricus bisporus*
alum	aluminum hydroxide
APC	antigen presenting cell
BAL	bronchoalveolar lavage
BCR	B cell antigen receptor
Bet v	Betula verrucosa
BSA	bovine serum albumin
C. albicans	*Candida albicans*
CD	cluster of differentiation
CpG ODN	unmethylated oligodeoxynucleotide sequences containing cytosine-phosphate-guanosine dinucleotides
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DNA	deoxyribonucleic acid
DP	degree of polymerization
ELISA	enzyme-linked immunosorbent assay
FcRI	Fc receptor I
HBSS	Hanks' balanced salt solution
H&E	hematoxylin and eosin
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MPL	monophosphoryl lipid A

MR	macrophage mannose receptor
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor-κB
NMR	nuclear magnetic resonance
O.D.	optical density
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PAS	periodic acid–Schiff
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
P. involutus	*Paxillus involutus*
PRR	pattern recognition receptor
PSAc	bacterial polysaccharide A
RPMI	Roswell Park Memorial Institute medium
RT-PCR	real-time polymerase chain reaction
S. chartarum	*Stachybotrys chartarum*
SCIT	subcutaneous immunotherapy
SEM	the standard error of the mean
SIT	specific immunotherapy
SLIT	sublingual immunotherapy
TADM	trivalent acetylated dimannose
TCR	T cell receptor
TGF-β	transforming growth factor β
Th	T helper
TLC	thin layer chromatography
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	T regulatory

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals (**I-V**).

I Ranta K, Nieminen K, Saariaho T, Kortekangas-Savolainen O, Kumpula EK, Kosonen J, Pasanen AL, Savolainen J. Evaluation of fungal extracts to determine immunomodulatory properties. J Investig Allergol Clin Immunol. 2013;23:226–33.

II Ranta K, Nieminen K, Ekholm FS, Poláková M, Roslund MU, Saloranta T, Leino R, Savolainen J. Evaluation of immunostimulatory activities of synthetic mannose-containing structures mimicking the β-(1,2)-linked cell wall mannans of *Candida albicans*. Clin Vaccine Immunol. 2012;19(11):1889–93.

III Mukherjee C, Ranta K, Savolainen J, Leino R. Synthesis and immunological screening of β-linked mono- and divalent mannosides. Eur J Org Chem. 2012;2957–68.

IV Mukherjee C, Mäkinen K, Savolainen J, Leino R. Chemistry and Biology of Oligovalent β-(1,2)-Linked Oligomannosides: New insights into carbohydrate-based adjuvants in immunotherapy. Chemistry Eur J. 2013;19:7961–74.

V Mäkinen K, Mukherjee C, Leino M, Panchadhayee R, Lehto M, Wolff H, Alenius H, Leino R, Savolainen J. A novel mannoside-glycocluster adjuvant: compared *in vitro* to CpG ODN and MPL and tested *in vivo* in mouse asthma model. Submitted.

1 INTRODUCTION

In allergic subjects, an allergen encounter leads to a T helper (Th) 2-type immune response followed by a subsequent immunoglobulin (Ig) E-mediated hypersensitivity reaction and further allergic inflammation (James L and Durham S 2008). An effective treatment for allergic diseases is allergen-specific immunotherapy (SIT) which balances the Th2-biased immunity towards Th1 and T regulatory (Treg) responses (James L and Durham S 2008). Since the prevalence of allergic diseases is increasing, also the requirements for SIT are high, some of them being optimal potency with minimal disadvantages. Immunotherapy is laborious and adverse effects occur frequently. Therefore, intensive research has focused on adjuvants conjugated to allergen preparations to intensify and modify the immune response and to shorten the injection regimens.

Insights into cellular events and mechanisms behind allergen-specific immune responses and further tolerance provide the means to design and develop powerful adjuvants for allergy preparations to be applied in SIT. Early exposure to microbes has been associated with the lower prevalence of asthma among children with an agricultural background (Ege MJ et al 2011). In general, immune deviation from Th2 to Th1 and Treg responses is considered to be the key factor behind this protective capacity (Umetsu DT et al 2002). In light of these data, many structures of microbial origin conjugated to allergen preparations have been studied in order to find a potent adjuvant for SIT. Such microbial structures are bacterial lipopolysaccharide (LPS), unmethylated oligodeoxynucleotide sequences containing cytosine-phosphate-guanosine dinucleotides (CpG ODN) and monophosphoryl lipid A (MPL) (Bohle B et al 1999, Gerhold K et al 2002, Puggioni F et al 2005, Moingeon P et al 2011). In a murine model of allergy, bacterial deoxyribonucleic acid (DNA) containing CpG ODN skewed the immunity towards Th1 (Hemmi H et al 2000). Accordingly, CpG ODN conjugated to a ragweed-pollen antigen administered in six preseasonal injections effectively reduced allergic symptoms in a clinical study (Creticos PS et al 2006). Another well-characterized adjuvant is MPL, a non-toxic modified LPS (Ulrich JT and Myers KR 1995). MPL promotes Th1-type immune responses and, when conjugated to an allergen, reduces allergic symptoms after just four preseasonal injections (Drachenberg KJ et al 2001, Puggioni F et al 2005).

Also cell wall components of yeasts and diverse carbohydrate structures possibly induce Th1 responses (Mencacci A et al 1994, Savolainen J et al 1999). However, as former studies have been conducted with natural cell wall extracts, the observed responses cannot be associated with a single structure in these heterogeneous mixtures (Masuoka J 2004). Therefore the aim of this thesis was to screen various fungal structures for their immunostimulatory capacities, identify the specific

structure behind the cellular responses, chemically synthesize it and, most importantly, characterize its immunostimulatory properties. The goal of this study was to identify a structure potentially applicable as a novel adjuvant during SIT.

2 LITERATURE REVIEW

2.1 Atopic allergies, pathogenesis and allergic inflammation

Allergic diseases are a major health concern worldwide and their prevalence is continuously increasing globally (Hansen TE et al 2013). In allergy the immune responses to harmless environmental allergens are aggressive and unnecessary. The tendency to produce IgE in response to common environmental allergens is called atopy. Common allergens include dander, dust, mold and pollen. The clinical manifestations of allergy include food allergy, conjunctivitis, rhinitis, asthma, atopic dermatitis and anaphylaxis (Kucuksezer U et al 2013). According to the hygiene hypothesis, the increased prevalence of allergic disorders is a result of missing microbial exposure (Strachan DP 1989). During development of the immune system in childhood, the lack of microbial antigens due to clean environment and the use of antibiotics is associated with allergy (Droste JH et al 2000). It is stated, that the exposure to microbes induces Th1-type responses and thereby protects against atopy and allergic asthma (Braun-Fahrländer C et al 1999). An extended version of the hygiene hypothesis, called the biodiversity hypothesis, is defined by the variability among living organisms. Loss of the macrodiversity leads to reduced interactions between environmental and human microbiotas. This is associated with alterations of the microbiota and immune dysfunction leading to impaired tolerance mechanisms (Haahtela T et al 2013).

Tolerogenic environment for harmless antigens is lost in allergy. For example, the gastrointestinal flora of atopic subjects has been shown to be different from that of healthy counterparts (Björkstén B et al 2001). Many other factors are also suggested to have an effect on the pathogenesis of allergy, such as western lifestyle, dietary factors, infections and sibling number (Prokopakis E et al 2013). In addition to this, allergy is known to have a clear genetic predisposition (Meyers DA 2010). Indeed, several genes and genetic regions have been linked to asthma, although their clinical relevance is still unknown (Akhabir L and Sandford AJ 2011).

The pathogenesis of allergy includes the following steps. First a subject becomes exposed to an allergen provoking an immune response towards the unfamiliar allergen. After repeated exposures to the allergen, some subjects become sensitized and develop specific memory T and B lymphocytes. However, in healthy subjects continuous allergen exposure would lead to peripheral tolerance without any sensitization. Some sensitized subjects develop allergic inflammation when exposed to the same allergens, leading to classical symptoms of allergy and tissue injury. (Kay AB 2001.)

Cells of the innate immunity, including antigen presenting cells (APCs), are essential mediators of the innate immune response, circulating freely in the body, until after allergen exposure they become activated. Diverse pathogen-associated molecular patterns (PAMPs) on the surface of allergens are recognized by APCs, especially dendritic cells (DCs), with pattern recognition receptors (PRRs). Interactions between any structures in nature are mediated by receptors and their ligands. The term affinity describes the binding strength between an antigen and an antigen-binding site. The intermolecular forces involved in the binding process include hydrogen bonding, electrostatic interactions, van der Waals interactions and dipole attraction. Avidity refers to the strength of multivalent ligand binding. (Astronomo RD and Burton DR 2010.)

The uptake of allergens by APCs happens through macropinocytosis, phagocytosis or receptor-mediated endocytosis. Specific PRR activation initiates signaling cascades in APC leading to production of transcription factors, such as nuclear factor-κB (NF-κB). Transcription factors stimulate further gene expression and APC begins to produce chemokines and cytokines directing the immune responses. Activated APCs with processed allergens on their cell membrane mature according to the type of allergen on their way to the lymphoid organs, including thymus, bone marrow, lymph nodes, spleen, tonsils and mucosa-associated lymphoid tissue. After this maturation, they express high levels of co-stimulatory and adhesion molecules on their membranes.

T lymphocytes are important players in the acquired immunity, both cell-mediated and humoral immune reactions. They mature in the thymus from progenitor T cells originated in the bone marrow (Figure 1). During this maturation T cells divide into different lineages by expressing different molecules on their membranes. They develop into cluster of differentiation 4 (CD4) positive Th or CD8 positive cytotoxic T cells. Thereafter the so-called naive Th cells with unique T cell receptors (TCRs) enter the circulation and start to travel in the human body.

In lymphoid organs, matured APCs with major histocompatibility complex (MHC) molecule class II present allergens to specific Th cells with compatible TCRs. After encounter with an allergen in the context of MHCII, Th cell binds the allergen with its TCR and CD4 molecules. This leads to Th cell activation, differentiation and clonal expansion. Th cell activation requires also co-stimulatory signals, which must be delivered by the APC. These co-stimulatory signals are mediated by binding of CD28 on T cells to CD80 or CD86 on APCs.

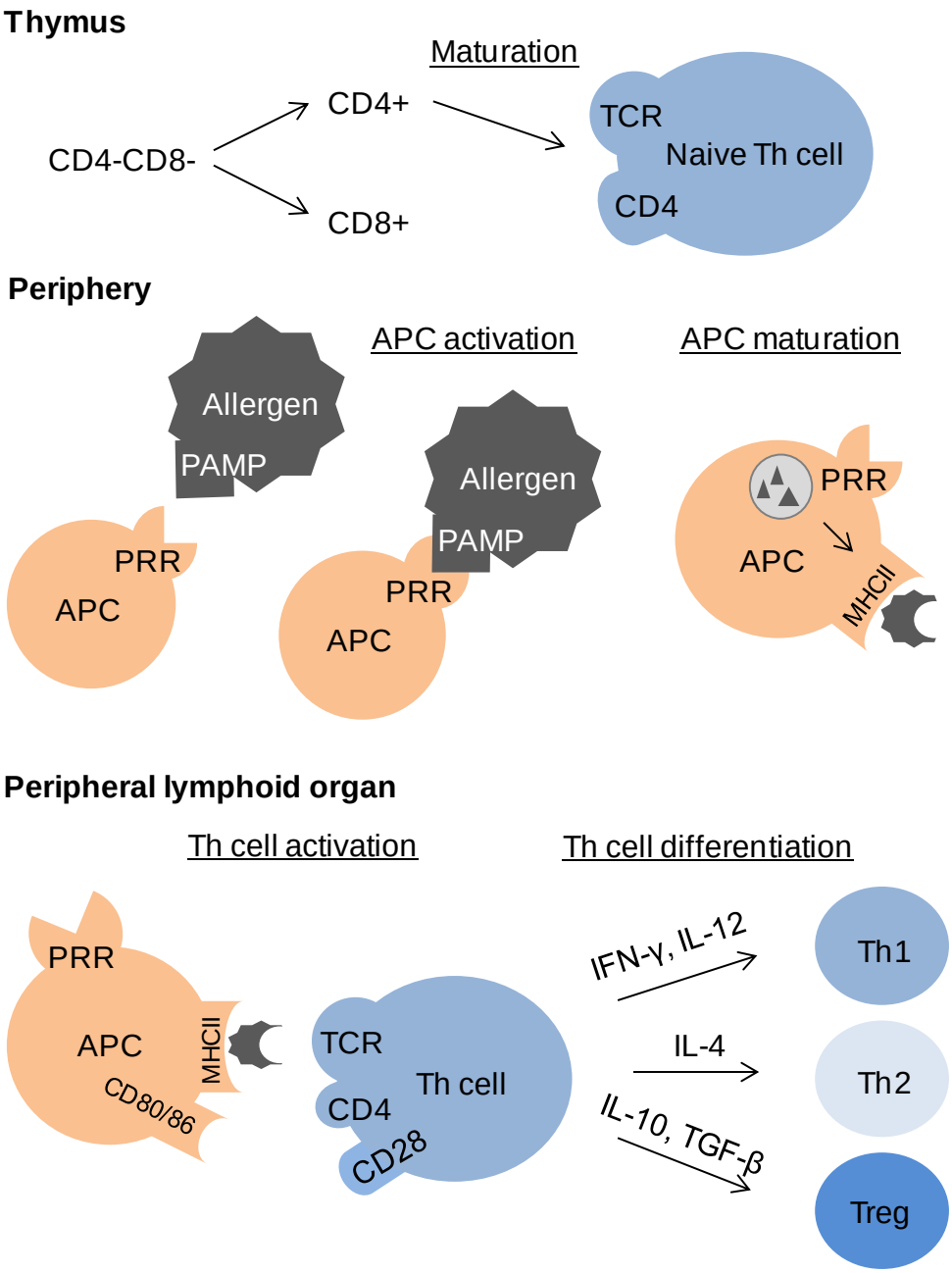

Figure 1. Th lymphocytes mature in the thymus. Allergens are recognized according to their PAMPs by various PRRs on the surface of the APCs. In the lymphoid organs, APCs present allergens on MHCII to the Th cells with specific TCRs. APC, antigen presenting cell; CD, cluster of differentiation; IFN-γ, interferon-γ; IL, interleukin; MHCII, major histocompatibility complex molecule class II; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; TCR, T cell receptor; TGF-β, transforming growth factor β; Th, T helper; Treg, T regulatory.

The differentiation of Th cells into different classes of T effector cells (Th1, Th2, Th17, Treg) depends on the type and amount of allergen as well as on the cytokines produced by DCs (Rautajoki KJ et al 2008). The consequences of Th differentiation are profound, since the various subsets of Th cells have different functions. Th1 lymphocytes control cell-mediated immunity by producing cytokines such as interferon-γ (IFN-γ) (Szabo SJ et al 2003). Treg cells producing interleukin (IL)-10 are important in maintaining the balance of the immune system by suppressing both Th1 and Th2 responses (Jutel M et al 2003). They maintain the peripheral tolerance by suppressing and eliminating autoreactive lymphocytes. In contrast, cytokines important in allergic inflammation are IL-4 and IL-5, produced by Th2 cells (Akdis M et al 2011).

In allergy, low amounts of allergens favor the differentiation of naive Th cells to Th2 cells. The impaired function of Treg cells and the predominance of Th2 cells is apparent (Akdis M et al 2004, Ling EM et al 2004). In fact, T cell subsets differ vastly in healthy and allergic individuals. After allergen exposure Treg cells dominate in healthy subjects, while allergic subjects have a predominance of Th2 cells (Akdis M et al 2004).

The effector Th cells drive both the cell-mediated and humoral immunity. Later, any encounter with the specific allergen activates effector Th cells without any co-stimulation. The developed T effector cells activate other cells of the immune system before undergoing apoptosis. However, some T effector cells persist forming the basis of immunological memory. These memory cells respond more rapidly to a second stimulus with the same allergen. (Itano AA and Jenkins MK 2003.)

B lymphocytes are essential players of the humoral immunity. They possess surface Igs, which recognize and bind allergens efficiently. These structures are also known as B cell antigen receptors (BCRs). BCR activation and allergen binding leads to allergen internalization, processing and presentation on MHCII molecules. Effector Th cells sensitized to the same allergen recognize the presented allergens and start to produce cytokines that cause the B cells to proliferate and terminally differentiate into antibody-secreting plasma cells or memory cells. During B cell differentiation isotype switching occurs according to cytokines released by Th cells. IL-4 produced by Th2 cells in association with a co-stimulatory signal (CD40) induces switching to IgG1 and IgE producing plasma cells. On the other hand, Th1 cells participate in isotype switching by producing IFN-γ, which induces switching to IgG2a and IgG3. Transforming growth factor β (TGF-β) induces switching to IgG2b and IgA. Treg cells can potently suppress IgE production and increase IgG4 and IgA production.

Antibody-secreting plasma cells differ from B cells, since during differentiation antigen presentation is lost due to finished expression of MHCII molecules. Memory B cells are specific to the allergen encountered during the primary immune response. These cells survive in the body and respond quickly, when re-encountered with the same allergen.

During sensitization the encounter of differentiated allergen-specific Th2 cells and B cells expressing the specific allergen leads to production of activating signals and rapid proliferation of both B and Th2 cells. Th2 cells induce B cell differentiation to plasma and memory cells and class switching from IgM to IgE antibodies (Figure 2). In fact, atopic subjects are predisposed to produce IgE antibodies against the allergens (Ryanna K et al 2009). Healthy subjects respond to an allergen with Th1-type cellular reactions leading to production of allergen-specific IgG1 and IgG4 antibodies. These antibodies block the interactions with IgE and allergens. (Kay AB 2001.)

During the early-phase response upon re-exposure to an allergen the formerly produced IgE antibodies are bound to Fc receptor I (FcRI) present on mast cells, basophils and eosinophils. IgE antibodies catch the specific allergen leading to the immediate allergic reaction with mast cell degranulation and histamine and leukotriene release. This is followed by smooth muscle activation, itching, mucus production from goblet cells, vasodilatation and edema. Allergen binding to basophils, mast cells and eosinophils also activates the production of IL-4 and CD40 ligand driving the class switching of B cells and IgE production. (Galli SJ et al 2008.)

Late-phase reaction is coordinated by allergen-specific Th2 cells and mediators released by mast cells during the early-phase reaction. Th2 cells and eosinophils are recruited to the sites of allergic inflammation. DCs present specific allergens on MHCII molecules to TCRs on Th2 cells, which leads to Th2 cell activation, proliferation and cytokine production. IL-4 and IL-13 drive IgE production and IL-5 assists in eosinophil development and survival. Eosinophils release cytokines and chemokines that generate an inflammatory response at the sites of allergen exposure. During the late-phase response, cellular changes are observed, including local IgE production and T cell activation. (Galli SJ et al 2008.)

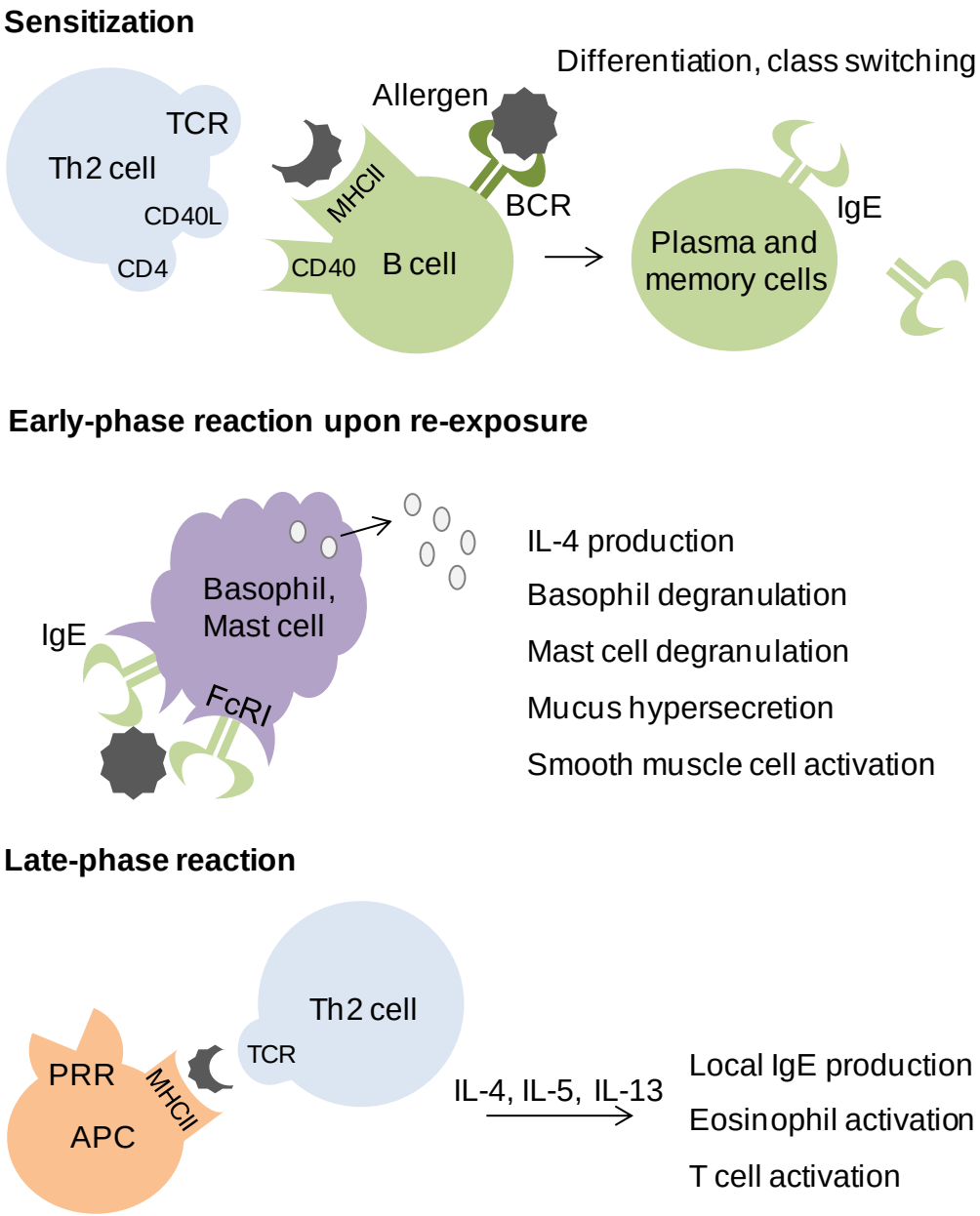

Figure 2. During sensitization, the Th2 cells induce B cells with the specific allergen to differentiate to plasma cells. Th2 cells induce class switching of plasma cells leading to production of allergen-specific immunoglobulin E antibodies. When the IgE antibodies attached to FcRI-receptors present on mast cells and basophils catch allergens, the immediate allergic reaction is mediated with mast cell degranulation and histamine and leukotriene release. Thereafter eosinophils are activated by Th2 cells provoking the late-phase response (modified from Larche M et al 2006). APC, antigen presenting cell; BCR, B cell antigen receptor; CD, cluster of differentiation; CD40L, CD40 ligand; FcRI, Fc receptor I; IgE, immunoglobulin E; IL, interleukin; MHCII, major histocompatibility complex molecule class II; PRR, pattern recognition receptor; TCR, T cell receptor; Th, T helper.

2.2 Specific immunotherapy

An immune system biased towards Th2 mediated immunity can be deviated towards protective Th1- and Treg-type immunity by SIT, which modifies the underlying pathological immune responses present in allergy (Maggi et al 2012). SIT consisting of repeated administrations of increasing doses of specific allergens is a potent treatment for atopic allergic diseases. Animal, insect, house dust mite and pollen allergies are treated with subcutaneous injections (SCIT). Sublingual immunotherapy (SLIT) is used in the treatment of grass pollen-induced allergic rhinitis (Clavel R et al 1998). Food allergies can be treated with specific oral tolerance induction (Calvani M et al 2010). Nowadays SIT is considered to be the only available form of therapy influencing the natural course of allergy. Although SIT was first described in 1911 independently by Freeman and Noon (Noon L 1911, Freeman J 1911), the detailed mechanisms of SIT are still partly unclarified. The pivotal objectives of SIT are achieved by modulating allergen-specific antibody and T cell responses (Akdis CA 2012). Although SIT relieves the immediate and the late-phase inflammatory reactions to allergens, its fundamental goal is to increase tolerance to allergens, which is achieved by three mechanisms: through the induction of Treg cells, through the immune deviation towards Th1 cells and through the induction of anergy of Th2 cells (Figure 3).

The different immune responses of allergic subjects provoked by natural allergen exposure and allergen administration during SIT are largely due to the allergen dose, route and the continuity of the allergen exposure. The side-effects of SIT are mainly a result of the low quality of the used allergen extracts. High allergenicity stems from the crude, non-standardized allergen sources from which the allergen extracts are prepared. The natural allergen exposure of nasal mucous membranes during the pollen season is relatively weak, and predominantly induces Th2 responses in pollen allergic subjects, as described previously. It is known that Th cells presented with low levels of allergen on APCs differentiate preferentially into Th2 cells. Instead, when a high dose of allergen is presented to Th cells, like in SIT, they differentiate into Th1 cells. Therefore, SIT stimulates the differentiation of Th1 cells, which further produce IFN-γ. IFN-γ inhibits Th2 differentiation and also stimulates the B cells to produce IgG antibodies.

High allergen concentrations and regular allergen exposure during SIT activate also Treg cells. Initially, the Treg activation leads to production of anti-inflammatory cytokines, including IL-10 and TGF-β. This results in active suppression of the effector cells, including mast cells and eosinophils (Jutel M et al 2003, Akdis C and Akdis M 2011). The early-phase reaction to allergens is attenuated by diminished amounts of mast cells and the secreted mediators. IL-10 and TGF-β also inhibit IL-4-mediated allergen-specific IgE synthesis in B lymphocytes and increase

protective IgG production (Akdis CA et al 1998). IgG antibodies further inhibit the functions of mast cells and eosinophils and interactions between allergens and IgE antibodies diminishing early-phase responses to allergens. IL-10 is also known to induce tolerogenic DCs producing IL-10 on their own.

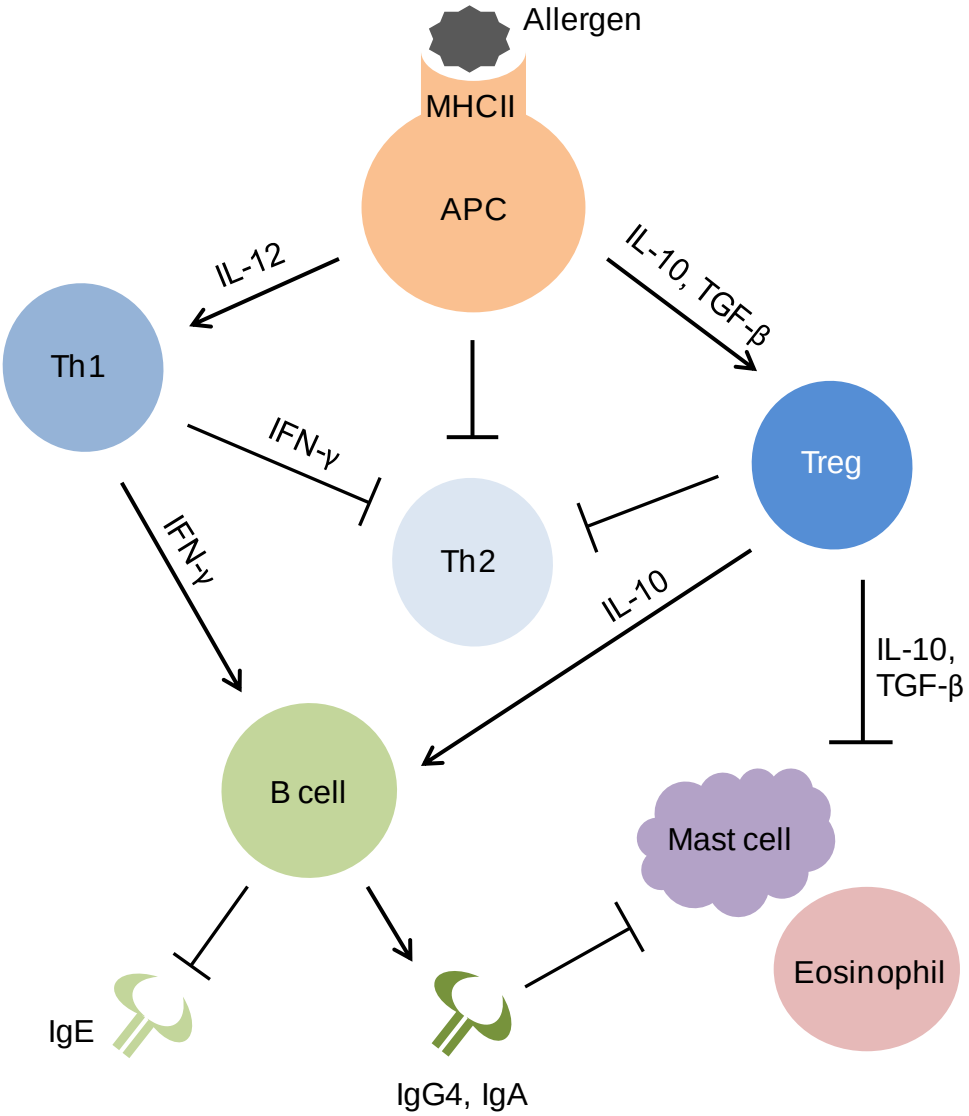

Figure 3. During SIT Treg and Th1 cells are activated. These cells produce cytokines that mediate active suppression of the effector cells, allergen-specific IgE synthesis and Th2 differentiation. Increased IgG production inhibits the functions of the mast cells and eosinophils and interactions between the allergens and the IgE antibodies. This results in diminished effects of the eosinophils, mast cells and basophils (modified from Smarr CB et al 2013). APC, antigen presenting cell; IFN-γ, interferon-γ; Ig, immunoglobulin; IL, interleukin; MHCII, major histocompatibility complex molecule class II; TGF-β, transforming growth factor β; Th, T helper; Treg, T regulatory.

Also the late-phase reaction is suppressed during SIT. The migration of T cells, mast cells, basophils and eosinophils to the inflammation site is diminished. Allergen-specific Th cell anergy is also obtained by production of IL-10. Cell anergy is characterized by suppressed proliferative and cytokine responses against the allergen. This results in down-regulation of activation, priming and survival of allergic inflammatory effector cells leading to peripheral tolerance. Moreover, anergized T cells can be reactivated by distinct cytokines to produce Th1-type cytokines deviating the immune system towards Th1 cells. Overall, SIT induces peripheral tolerance, causing manifestations of allergy to decrease. (Akdis C and Akdis M 2011.)

2.3 The use of adjuvants in immunotherapy

Conventional SIT has several disadvantages, which reduce compliance and attenuate its use. Treatments are lengthy taking up to three years and potentially risky with adverse effects and serious allergic reactions (Senti G et al 2005). Therefore novel approaches to immunotherapy are of considerable interest. Reduced injection regimens and alleviated side effects could be achieved by optimizing the allergen type, dose and route of administration. In order to achieve the optimal effects of immunotherapy, adjuvants are commonly used. These molecules modify the immune response to the allergen and increase the production of antibodies, when combined to a small amount of the allergen (Moingeon P et al 2011). Adjuvants are administered together with allergens to elicit the most potent immune responses (Akdis CA and Akdis M 2011).

Adjuvants enhance the cellular responses of SIT (Figure 4). Adjuvants act on APCs through PRRs, which recognize PAMPs on allergens and adjuvants. Adjuvants target the allergens to tolerogenic APCs, enhance the recognition of allergens and increase allergen uptake by DCs and macrophages. Tolerogenic APCs promote and maintain immunologic tolerance by inducing cell anergy and the differentiation of T suppressor and Treg cells. Tolerogenic APCs express high endocytic and phagocytic properties with low levels of MHCII and co-stimulatory molecules (Ezzelarab M and Thomson AW 2011). Adjuvants also provide necessary signals for activation of APCs (Reed SG et al 2013). They also activate monocytes and modify Th differentiation. The adjuvant-allergen complex is first processed by an APC and then the processed allergen is presented to Th cells. This is followed by Th cell differentiation and pro- or anti-inflammatory immune responses according to the properties of adjuvants. The following immune responses during SIT can be closely modulated by selecting the adjuvant carefully. The optimal adjuvant would favor Th1 and Treg pathways while simultaneously suppressing Th2 responses. (Moingeon P et al 2011.)

Aluminum hydroxide (alum) has been used as an adjuvant in immunotherapy since 1938 (Sledge RF 1938). It has been shown to downregulate Th2 responses and enhance the contact between an APC and an allergen (Mckee AS et al 2007). It also enhances allergen uptake by DCs. In fact, it has been suggested that alum might store allergens and slowly release them to immune cells prolonging the antigen presentation (Harrison WT 1935). Paradoxically, it promotes Th2-type reactions which lead to undesired induction of IgE production (Mckee AS et al 2007). Therefore, other alternatives to immunologic adjuvants in allergy preparations have been explored and proposed.

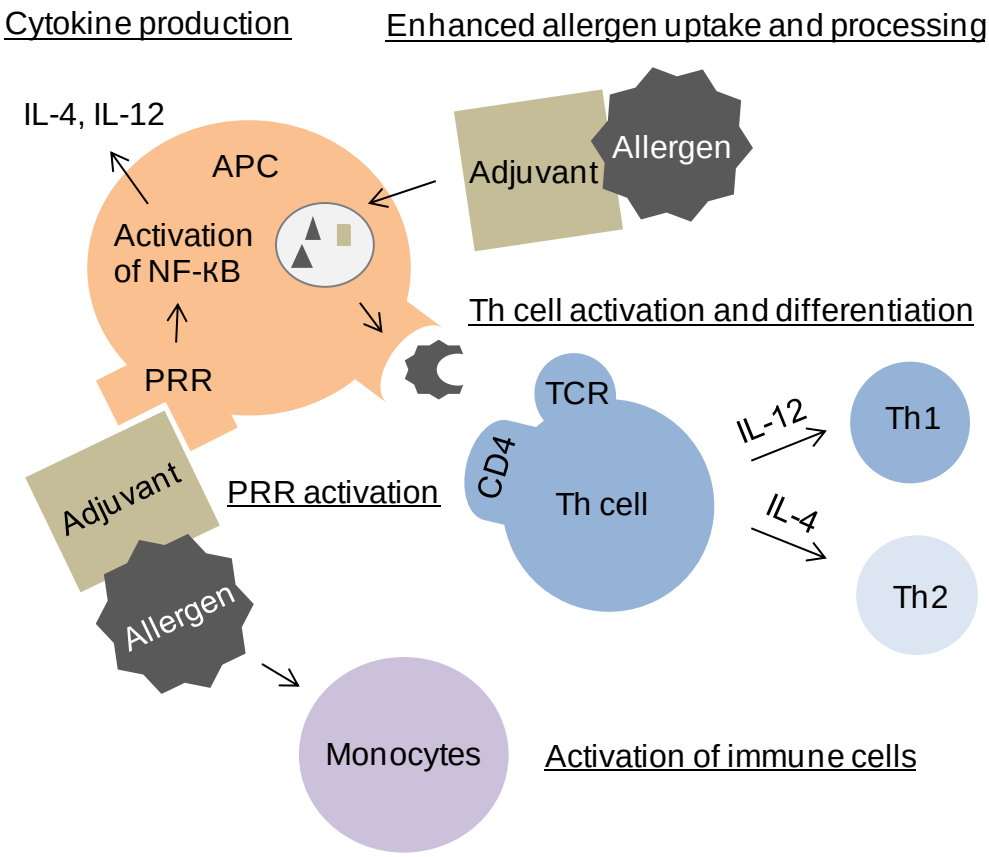

Figure 4. Adjuvants enhance the cellular responses of SIT. Adjuvants target the allergen to tolerogenic APCs, enhance the recognition of allergens, increase allergen uptake by dendritic cells and macrophages and provide necessary signals for activation of APCs. Adjuvants also activate monocytes and modify Th differentiation (modified from Reed S et al 2013). APC, antigen presenting cell; CD, cluster of differentiation; IL, interleukin; NF-κB, nuclear factor-κB; PRR, pattern recognition receptor; TCR, T cell receptor; Th, T helper.

2.4 Immunostimulatory effects of current adjuvants

The increased prevalence of atopic allergies in developed societies has partly been associated with high hygiene and decreased microbial load during childhood diminishing the natural Th1 responses leading to enhanced Th2-type immunity (Yazdanbakhsh M et al 2002). This has initiated a search for microbial structures to be used as adjuvants. These adjuvants would induce a local Th1-type environment and reduce allergen-induced Th2 responses, thereby suppressing allergic inflammation (Moingeon P et al 2011). Indeed, several structures from microbes, including bacterial LPS, CpG ODN and MPL, induce Th1 immune responses and down-regulate allergen-induced Th2 responses (Bohle B et al 1999, Gerhold K et al 2002, Puggioni F et al 2005). In fact, MPL conjugated to allergen was approved for human use in 2009, and has completed the phase III trials. However, none of these molecules have established a firm position as allergy adjuvants in clinical use.

Mycobacteria have been widely studied for their immunostimulatory properties. The favorable effects of tuberculin seem to be related mainly to induction of Treg responses, including IL-10 and TGF-β production, rather than enhanced Th1 responses (Zuany-Amorim C et al 2002). However, among Japanese school children the Th1-type responses to Mycobacterium tuberculosis were inversely associated with atopy (Shirakawa T et al 1997). Despite the promising studies with a murine model (Wang C and Rook G 1998), unsatisfactory results were received in a clinical trial where repeated doses of non-pathogenic Mycobacterium vaccae were administered to a group of established asthmatics (Shirtcliffe PM et al 2004).

Another interesting adjuvant candidate was discovered, when bacterial DNA was observed to possess immunostimulatory activity *in vitro* among microbial components (Tokunaga T et al 1984). Allocated studies indicated oligodeoxynucleotides containing CpG motifs (CpG ODN) to be responsible for these effects (Klinman DM et al 1996). Further studies confirmed that CpG ODN promoted Th1-type immunity with IFN-γ production by natural killer lymphocytes and inhibited Th2 cell activation and cytokine production (Klinman D et al 1996, Hessel EM et al 2005). Also in murine models and clinical studies CpG ODN have shown strong immunostimulatory potency and wide antiallergic activities in the treatment of asthma (Santeliz JV et al 2002), allergic conjunctivitis (Magone MT et al 2000) and allergic rhinitis (Creticos PS et al 2006). A study by Tulic et al clearly showed decreased nasal inflammatory responses, relief of allergic symptoms and induction of immunogenicity with reduced allergenicity during immunotherapy with the bacterial CpG ODN bound covalently to allergens (Tulic MK et al 2004).

Another molecule with favourable immunostimulatory properties is bacterial LPS, which strongly activates macrophages and monocytes to produce IL-6 and tumor

necrosis factor (TNF) (Hirohashi N and Morrison DC 1996). In fact, LPS is a PAMP recognized by PRRs on the surface of macrophages. However, LPS is known for its toxic effects on cells and thus it is not suitable for adjuvant use. Therefore, research has focused on a non-toxic monophosphorylated derivative of lipid A, termed MPL, originally derived from the cell wall of *Salmonella minnesota* (Ulrich JT and Myers KR 1995). MPL reduces allergic inflammation by inducing IFN-γ production from peripheral blood mononuclear cells (PBMCs) and IL-10 and IL-12 production from monocytes (Puggioni F et al 2005) with subsequent suppression of Th2-type response and IgE responses (Drachenberg KJ et al 2001). However, the difficult synthesis of MPL has restricted its use, since MPL consists of fatty acid chains which differ by the length and number of chains.

Another immunopotent carbohydrate of microbial origin is bacterial polysaccharide A (PSAc), expressed on the surface of *Bacteroides gracilis*, an anaerobe colonizing the gut and the lower gastrointestinal tract of mammals (Tzianabos AO et al 1992, Könönen E et al 1992). The capsular polysaccharides of this bacterium are zwitterionic featuring both positively and negatively charged moieties in each repeating unit. PSAc is recognized and taken up by the intestinal APCs, which process it and present it to T cells on MHCII molecules (Cobb BA et al 2004). PSAc is known to drive Th cell proliferation and differentiation towards Th1-type cytokine production (Mazmanian SK et al 2005, Vercelli D 2006). It also stimulates macrophages to produce TNF (Gibson FC et al 1996).

2.5 Immunostimulatory fungal components

Microbes, including fungi, are nowadays widely demonstrated as Th1-inducing structures. Conventionally, the immunostimulatory structures are considered to be proteins, but nowadays also various carbohydrate structures are known to actively influence the innate immune system (Lindequist U et al 2005). Polysaccharide and protein extracts from a variety of fungi have been demonstrated to modulate cytokine production *in vitro* in human PBMC cultures possibly leading to indirect immunomodulation of the T cell activation (Jeurink P et al 2008). Potent Th1 responses have been reported after cell stimulations with glucose homopolymers, termed β-glucans, extracted from mushrooms such as *Grifola frondosa*, *Lentinus edodes* and *Sclerotinia sclerotiorum* (Suzuki Y et al 2001, Kodama N et al 2002, Murata Y et al 2002). Also protein-bound polysaccharides extracted from mushrooms, called polysaccharide peptide and polysaccharide krestin, which consist of monosaccharides with α-(1,4)- and β-(1,3)-glucosidic linkages, have been shown to induce lymphoproliferation, activation of T cells and production of IFN-γ (Tzianabos AO 2000). In addition, some yeasts are capable of inducing IFN-γ production in whole blood cultures of healthy individuals (Heintel T et al 2003). The mannan and mannoproteins of *Cryptococcus neoformans* induce a Th1

response (Pietrella D et al 2001). In addition, glycoprotein and mannan extracts from *Candida albicans* (*C. albicans)* are immunostimulatory (Mencacci A et al 1994).

2.6 *C. albicans* cell wall carbohydrates in immunostimulation

2.6.1 Cell wall of *C. albicans*

C. albicans is a member of the normal human microflora but also an opportunistic pathogen leading to systemic candidiasis mainly during immunosuppression. The rigid cell wall of *C. albicans* is comprised of proteins and other components, including chitin, β-glucans and mannans (Figure 5) (Mora-Montes HM et al 2009). The term mannan can be used about a variety of molecules consisting of mannosides, proteins and lipids. The core of the cell wall contains β-(1,3)-glucan moieties which are covalently attached to branched β-(1,6)-glucans and β-(1,4)-linked *N*-acetylglucosamine polymers, called chitin (Chaffin WL et al 1998). The cell wall proteins are highly glycosylated through *N*- (amide bond) or *O*-linkages (ester bond) by branched polymers of mannose and are therefore referred to as mannoproteins. Some of these *N*- and *O*-linked mannans are modified with side chains containing mainly α-linked, but also β-(1,2)-linked oligomannosides. These structures are referred to as phosphomannans because of the α-linked phosphodiester bonds between the side chains and mannans (Shibata K et al 1995, Trinel P et al 1997). The hydrolysis of the β-linked chains yields β-(1,2)-D-mannotriose and β-(1,2)-D-mannobiose that accounts for about 1% of the amount of the mannan. β-(1,2)-mannosides are primarily found in the acid-stable and acid-labile components of mannoproteins (Trinel PA et al 1997). Another structure comprising of β-(1,2)-linked mannose glycans is phospholipomannan. In this linear structure, β-(1,2)-mannosides are linked to the phytoceramide derivatives of *myo*-inositol phosphate (Trinel PA et al 1993).

2.6.2 Immune response against *C. albicans* carbohydrates

The recognition of carbohydrate structures is essential for defending the body against foreign material. Traditionally, carbohydrates have not been considered active inducers of immune responses. In fact, structures consisting solely of carbohydrate components typically elicit a poor immune response due to the lack of Th-dependent activation and thus weak class switching of B-cell-mediated immunity. However, nowadays certain carbohydrates are considered highly antigenic. The main immune cells involved in the recognition of *C. albicans* are DCs, monocytes, neutrophils and macrophages. Mannans in the cell wall of *C. albicans* possess diverse immunostimulatory functions from the activation of specific Th subsets (Mencacci A et al 1994, Ashman RB and Papadimitriou JM 1995) to the induction of DC maturation and TNF production (Pietrella D et al

2006). *Candida*-specific antibodies are often found in human sera, presumably due to colonization by *C. albicans* (Martinez JP et al 1998).

2.6.3 Pattern recognition receptors involved in *C. albicans* recognition

Recognition of the different PAMPs in the *C. albicans* cell wall by APCs, mainly DCs, is mediated by a diverse range of PRRs (Poulain D and Jouault T 2004) (Figure 5). Four major classes of PRRs have been identified and they are classified on the basis of their function to two different groups; signaling PRRs include Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors, whereas endocytic PRRs include C-type lectin receptors and retinoic acid-inducible gene-I-like receptors. C-type lectin receptors recognize polysaccharide structures. This class features Dectin-1 recognizing β-glucans and the macrophage mannose receptor (MR) and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) both recognizing *N*-linked mannans and *N*-acetylglucosamines (Poulain D and Jouault T 2004, Garcia-Vallejo J et al 2009).

Various TLRs are involved in the recognition of fungal components. The α-linked mannosides are recognized by the TLR4, MR and DC-SIGN (Netea MG et al 2008). TLR2 recognizes phospholipomannan on its own or in coordination with Dectin-1 inducing the production of proinflammatory cytokines, such as TNF (Jouault T et al 2003, Poulain D and Jouault T 2004). Activation of TLR2 also induces IL-10 production, generating Treg cells with immunosuppressive potential (Netea MG et al 2004). The sole activation of Dectin-1 induces IL-2 and IL-10 production (Netea MG et al 2008). TLR4 recognizes *O*-linked mannans (Netea MG et al 2006). Mycobacteria interact with DCs via TLR2 and TLR4 resulting in strong Th1-type responses. TLR9 detects fungal DNA, such as CpG ODN motifs (Johansen P et al 2001). The MR recognizes mannose units on the pathogens and facilitates their phagocytosis and assists in antigen presentation and recognition as well as in proper leukocyte response.

Galectin-3 is a 30 kDa receptor expressed on macrophages, DCs and epithelial cells (Kohatsu L et al 2006). Galectin-3 has the ability to amplify TLR2-mediated cellular effects, especially in macrophages (Jouault T et al 2006). Galectin-3 also regulates inflammatory and allergic responses (Rabinovich GA et al 2002). It downregulates the IL-5 gene expression in eosinophils and T cell lines (del Pozo V et al 2002). Furthermore, the instillation of a plasmid DNA encoding galectin-3 diminished asthmatic reactions, including eosinophil inflammation and T cell proliferation in a rat model (del Pozo V et al 2002). Enhanced inflammatory cytokine production was observed in response to LPS in galectin-3 knockout mice (Li Y et al 2008). Galectin-3 enhances cytokine production (TNF) in macrophages exposed to fungi in collaboration with Dectin-1 (Esteban A et al 2011).

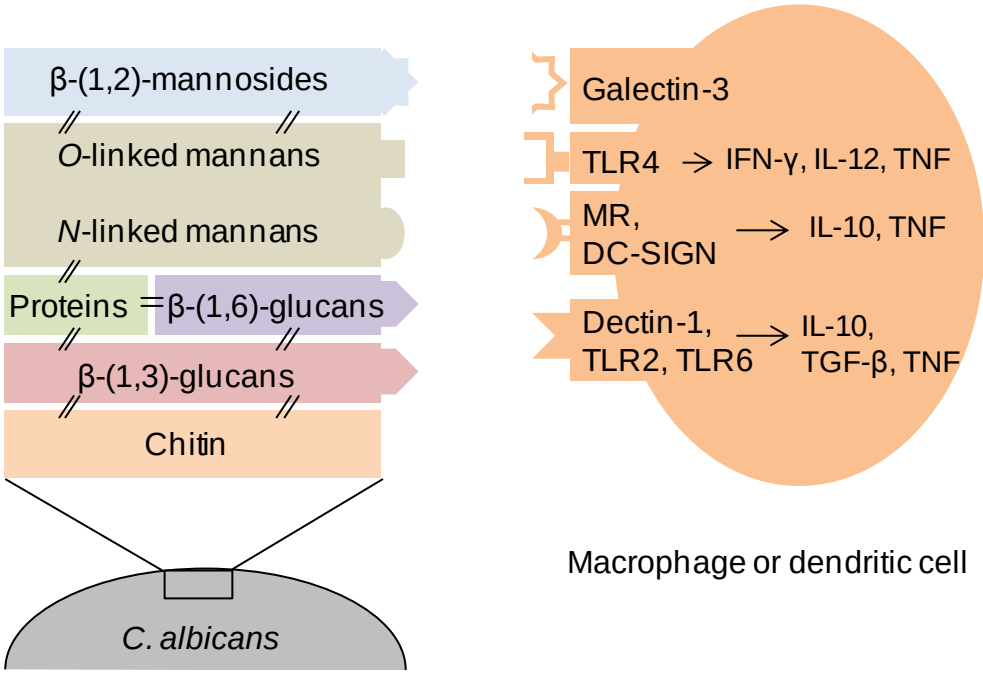

Figure 5. The rigid cell wall of *C. albicans* is comprised of protein and carbohydrate components. The cell wall proteins are highly glycosylated through *N*- or *O*-linkages. PRRs are important in recognition of these different PAMPs. TLR2 recognizes phospholipomannan and TLR4 *O*-linked mannans. Dectin-1 recognizes β-glucans and MR and DC-SIGN both recognize *N*-linked mannans and *N*-acetylglucosamines. The α-linked mannosides are recognized by the TLR4, MR and DC-SIGN. β-(1,2)-linked oligomannosides are recognized by galectin-3 (modified from Perez-Garcia LA et al 2011). *C. albicans*, *Candida albicans*; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; IFN-γ, interferon-γ: IL, interleukin; MR, mannose receptor; TGF-β, transforming growth factor β: TLR, Toll-like receptor; TNF, tumor necrosis factor.

2.6.4 β-(1,2)-linked oligomannosides of *C. albicans*

β-(1,2)-linked oligomannosides present in the *C. albicans* cell wall are recognized by galectin-3. β-(1,2)-oligomannosides possess immunostimulatory properties as evidenced by the induction of TNF production in murine macrophages (Jouault T et al 1995). TNF is a key factor in inflammation inducing cell differentiation, proliferation and migration (Bradley JR 2008). On the other hand, the functions of the murine macrophages were suppressed in response to a second stimulus of β-(1,2)-linked oligomannosides, which refers to desensitization of macrophages leading to inhibition of LPS-induced TNF release and nitric oxide production in cell cultures (Jouault T et al 2000). In another murine model, monoclonal antibodies specific to β-(1,2)-linked oligomannosides have been shown to protect against candidiasis (Han Y and Cutler JE 1995, Han Y et al 1997). These antibodies are specific to β-(1,2)-linked mannotriose, also present in *C. albicans* phosphomannan (Han Y et al 1997). It has also been demonstrated that the

induction of IgG antibodies is achieved with epitopes consisting of albumin-coupled di- and trisaccharides resembling β-(1,2)-mannosides in the *C. albicans* cell wall. Inhibitory activity did not increase with size (Nitz M et al 2002).

2.7 Structural aspects of immunostimulatory carbohydrates

Glycoscience is a relatively young field of research, which may partly be due to the structural complexity of carbohydrates and the difficulties in determining their composition. Polysaccharides are comprised of branched oligosaccharides, which in turn contain repeated moieties of monosaccharides. Monosaccharides are joined together with glycosidic linkages. Monosaccharides and oligosaccharides mediate interactions between cells, molecules and different organisms. Carbohydrates have been conventionally classified as classic T-cell independent antigens which do not mediate cellular immune responses but rather elicit humoral immunity with weak-affinity IgM antibodies. This mechanism involves activating BCRs leading to stimulation of antigen-specific B cells. However, nowadays carbohydrates are known to modulate both humoral and cellular immune systems.

Various polysaccharide structures alter immune responses by inducing cytokine production (Tzianabos AO 2000). Therefore carbohydrates possess beneficial adjuvant properties. In addition to the carbohydrate structures extracted from the *C. albicans* cell wall, also other carbohydrates possess immunostimulatory properties. Oligosaccharides derived from human milk direct naive umbilical cord blood derived Th cells towards Th1 differentiation (Eiwegger T et al 2004). Also carbohydrate based particles joined to an allergen induce strong IgG responses and relieve allergic inflammation (Grönlund H et al 2002).

The recognition of and binding to carbohydrates by immune cells depend on a variety of carbohydrate-related factors. These include the stereochemistry and the degree of polymerization (DP), chain length, flexibility, glycosidic linkage and multivalency of the carbohydrate (Nitz M et al 2002, Masuoka J 2004, Astronomo RD and Burton DR 2010). These factors altogether contribute to the three-dimensional secondary structure of the carbohydrate. Also molecular weight, degree of branching, the number of functional groups and helices have an effect on the biological activities of β-glucans (Chatterjee S et al 2011). Another important characteristic of an immunostimulatory carbohydrate is solubility, which can be increased by phosphorylation, sulfation or amination, as evidenced with β-(1,3)-glucans (Tzianabos AO 2000).

Clustered carbohydrate epitopes are common in many natural glycoproteins (Lee R and Lee Y 2000). Multivalent structures possess several moieties of various carbohydrate structures linked to each other. Therefore they can simultaneously

bind to multiple receptors (Mammen M et al 1998). It is actually postulated that multivalent receptors interacting with multivalent targets are needed in molecular recognition (Dwek RA 1996). This mechanism is proposed to have evolved for the discrimination of self and nonself in the immune system (Dwek RA 1996). Molecular recognition in biological systems usually relies on multivalent ligand-receptor interactions with increased avidity (Mammen M et al 1998, Kiessling LL et al 2000). Many lectins possess low single-site-binding affinities and thus by increasing the number of bond interactions the overall strenght of the interaction can be enhanced and high avidity gained leading to biologically relevant events *in vivo*. The binding capacity to receptors can be varied by altering the linker or the carbohydrate density of the multivalent molecule (Lee R and Lee Y 2000). However, multivalent interactions may also lead to nonspecific aggregation of various molecules and cell types since specificity may be lost due to possible cross-reacting structures.

3 AIMS OF THE STUDY

The goal of this study was to characterize immunostimulatory mannosides potent for suppressing allergic inflammation in the prevention and treatment of atopic allergy. The detailed aims of this thesis were:

1. to screen fungal extracts from different sources for their immunostimulatory properties (**I**).

2. to analyze various synthetic mannosides mimicking the β-(1,2)-linked mannosides of *C. albicans* for their immunostimulatory properties *in vitro* (**II**, **III, IV**).

3. to analyze the inhibitory effects of synthetic β-(1,2)-linked mannosides on specific IgG binding to low molecular weight hydrolyzed *C. albicans* mannan (**III**).

4. to study the effects of a synthetic immunostimulatory glycocluster adjuvant (trivalent acetylated dimannose, triacedimannose, TADM) on birch allergen induced cytokine responses of PBMCs in allergic subjects (**IV, V**) and to compare the effects with other known adjuvants, CpG ODN and MPL *in vitro* (**V**).

5. to evaluate the effects of TADM on allergen-induced allergic inflammation in a murine model of asthma (**V**).

4 MATERIALS AND METHODS

4.1 Fungal antigens (I)

Fungal extracts were isolated from 24 different yeasts, moulds and mushrooms (Table 1 in study **I**). The yeast extracts include two commensal species (*C. albicans, Pityrosporum ovale:* strains CBS and ATCC), three environmental species (*Rhodotorula rubra*, *Cryptococcus albidus* and *Candida utilis*) and one species used in commercial house-hold products (*Saccharomyces cerevisiae*).

The *Aspergillus umbrosus* strain was originally isolated from mouldy hay and cultured to prepare whole antigen extracts as previously described (Kaukonen K et al 1993). The whole extracts of *Aureobasidium pullulans*, *Acremonium furcatum*, *Aspergillus versicolor*, *Chaetomium globosum*, *Phialophora repens*, *Stachybotrys chartarum (S. chartarum)*, *Ulocladium botrytis* and *Alternaria alternata* (kindly provided by Dr. P. Raunio, Department of Environmental Sciences, University of Kuopio, Finland) were originally isolated from water-damaged building materials and cultivated to prepare whole extracts as described earlier (Pasanen AL et al 1999, Raunio P et al 2001).

The mushroom extracts were prepared from one cultivated mushroom species (*Agaricus bisporus,* champignon, *A. bisporus*) and seven wild mushroom species (*Cantharellus tubaeformis*, *Paxillus involutus (P. involutus)*, *Kuehneromyces mutabilis*, *Cortinarius triumphans*, *Lactarius rufus*, *Hydnum repandatum*, *Boletus edulis*). *A. bisporus* was acquired from a mushroom farm (Mykora, Kiukainen, Finland). The wild mushrooms were collected from the Turku region in Finland by an experienced mushroom consultant. Special attention was paid to the purity and freshness of the material and mushrooms with a slightest hint of larvae were excluded. Each species was collected in separate paper bags and deep-frozen within three hours. Prior to the extraction, the mushrooms were washed with 10% ethanol and distilled water, and roughly homogenized in 0.125 M NH_4HCO_3 with a hand blender. After centrifugation at +4 ˚C with 14 000 *g* for 30 min, the pelleted fractions were resuspended in 0.125 M NH_4HCO_3, sonicated and shaken for 24 hours at +4 ˚C. Unlike all the other preparations, the *A. bisporus* suspension was ruptured by freezing at -25 ˚C and passing four times trough X-press (AB Biox, Gothenburg, Sweden). After centrifugation (14 000 *g*, 30 min at +4 ˚C), the supernatants were sterile-filtered (0.2 μm Acrodisc PF filter, Pall Gelman Laboratory, Ann Arbor, MI, USA) and stored lyophilized at +4 ˚C.

4.2 Hydrolysis and fractionation of the *C. albicans* mannan (I)

The mannans of *C. albicans*, *S. chartarum* and *P. involutus* were isolated by hot citrate extraction and precipitated with Cetavlon according to a previous study (Nakajima T and Ballou CE 1974).

The hydrolysis of the *C. albicans* mannan (20 mg/ml) was performed under mild acidic conditions with 0.1 N HCl for up to 60 min at +100 ˚C. The neutralization of the *C. albicans* mannan hydrolysis products was performed by adding NaOH. The outcome was analyzed by thin-layer chromatography (TLC) using silica gel-coated aluminium sheets (Merck, Darmstadt, Germany) and *n*-butanol-acetic acid-water (2:1:1 v/v/v) as an eluent. A preliminary rough fractionation of the hydrolysis products by size was performed with Amicon YM100, YM10, YM3 and YM1 ultrafiltration membranes (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. Below 3 kDa fraction was further fractionated by liquid chromatography on Bio-Gel P-2 Fine (1.7 x 131 cm) according to the manufacturer's instructions (Bio-Rad).

4.3 The syntheses of the carbohydrates (II-V)

The Leino research group at Åbo Akademi University provided the synthesized mannosides. The syntheses of the carbohydrate constructs used in this thesis are described in detail in the articles listed in Table 1. The compounds prepared were designed as simple mimicks and analogues of the hydrolyzed oligosaccharide fractions from the *C. albicans* cell wall with the β-(1,2)-linkage serving as a basis for all structural modifications. Crich's methodology for β-mannosylation was successfully utilized in introducing the β-linkages, whereas a click chemistry protocol was utilized in generating the oligovalent derivatives. A convenient protecting group strategy utilizing both *p*-methoxybenzyl and benzylidene groups simultaneously for a simple and cost effective global deprotection step was applied.

Table 1. The nomenclature and syntheses of the carbohydrate constructs.
* Constructs were not water-soluble and therefore could not be tested.

Structure	Number	Results presented in article	Synthesis described in article
β-D-Mannopyranosyl-(1,2)-D-mannopyranose, "β-(1,2)-linked mannobiose"	1	II	Ekholm FS et al 2010
β-D-Mannopyranosyl-(1,2)-β-D-mannopyranosyl-(1,2)-D-mannopyranose, "mannotriose"	2	II	Ekholm FS et al 2010

β-D-Mannopyranosyl-(1,2)-β-D-mannopyranosyl-(1,2)-β-D-mannopyranosyl(1,2)-D-mannopyranose,"mannotetraose"	3	II	Ekholm FS et al 2010
Cyclohexyl β-D-mannopyranosyl-(1,2)-α-D-mannopyranoside, "α-cyclohexyl"	4	II	Ekholm FS et al 2010
Methyl-β-D-mannopyranosyl-(1,2)-α-D-mannopyranoside, "α-methyl mannoglycoside"	5	II	Ekholm FS et al 2010
Man-β(1,2)-Man-β(1,2)-Man-OMe	6	II	Ekholm FS et al 2010
Methyl-β-D-glucopyranosyl-(1,2)-α-D-mannopyranoside, "β-(1,2)-linked oligomannosides containing GlucNAc residues"	7	II	Ekholm FS et al 2010
GlcNAc-β(1,2)-Man-OMe	8	II	Ekholm FS et al 2010
Methyl-β-D-glucopyranosyl-(1,2)-β-D-mannopyranosyl-(1,2)-α-D-mannopyranoside, "β-(1,2)-linked oligomannosides containing glucose"	9	II	Ekholm FS et al 2010
1,4-bis(α-D-mannopyranosyloxy)butane	10	II	Ekholm FS et al 2009
1,4-bis(2-*O*-β-D-mannopyranosyl-(1,2)-α-D-mannopyranosyloxy)butane	11	II	Ekholm FS et al 2009
1,4-bis(cyclohexyl 2-*O*-α-D-mannopyranosyl)butane	12	II	Ekholm FS et al 2009
1,4-bis(2-*O*-D-mannopyranose)butane	13	II	Ekholm FS et al 2009
1,4-bis(2-*O*-β-D-mannopyranosyl-(1,2)-D-mannopyranose)butane	14	II	Ekholm FS et al 2009
1,4-bis(methyl-α-D-mannopyranosyl-(2,1)-2-*O*-β-D-glucopyranosyl)butane	15	II	Ekholm FS et al 2009
1-[*β*-D-Mannopyranosyloxyethyl]-4-hydroxymethyl-1,2,3-triazole, "monovalent monosaccharide with β-linkage"	1	III	III
1-[(*β*-D-Mannopyranosyl)-(1,2)-(*β*-D-mannopyranosyloxyethyl)]-4-hydroxymethyl-1,2,3-triazole, "monovalent disaccharide with two β-linkages"	2	III	III
1-[(*β*-D-Mannopyranosyl)-(1,2)-(*α*-D-mannopyranosyloxyethyl)]-4-hydroxymethyl-1,2,3-triazole, "monovalent disaccharide with one α- and β-linkage"	3	III	III
l-[β-D-mannopyranosyloxyethyl]-4-[β-D-mannopyranosyloxymethyl]-[1,2,3]-triazole, "divalent monosaccharide with β-linkage"	4	III	III

1-[β-D-mannopyranosyl-(1,2)-β-D-mannopyranosyloxyethyl]-4-[β-D-mannopyranosyl-(1,2)-β-D-mannopyranosyloxymethyl]-[1,2,3]-triazole, "divalent disaccharide with two β-linkages"	5	III	III
1-[β-D-mannopyranosyl-(1,2)-α-D-mannopyranosyloxyethyl]-4-[β-D-mannopyranosyl-(1,2)-α-D-mannopyranosyloxymethyl]-[1,2,3]-triazole, "divalent disaccharide with one α- and one β-linkage"	6	III	III
1-[2,3,4,6-Tetra-*O*-acetyl-β-D-mannopyranosyloxyethyl]-4-hydroxymethyl-1,2,3-triazole	18	IV	IV
1-[(2,3,4,6-Tetra-*O*-acetyl-β-D-mannopyranosyl)-(1,2)-(3,4,6-tri-*O*-acetyl-β-D-mannopyranosyloxyethyl)]-4-hydroxymethyl-1,2,3-triazole	19	IV	IV
1-[(2,3,4,6-Tetra-*O*-acetyl-β-D-mannopyranosyl)-(1,2)-(3,4,6-tri-*O*-acetyl-α-D-mannopyranosyloxyethyl)]-4-hydroxymethyl-1,2,3-triazole	20	IV, *	IV
1-[*β*-D-Mannopyranosyloxyethyl]-4-hydroxymethyl-1,2,3-triazole	21	IV	IV
1-[(*β*-D-Mannopyranosyl)-(1,2)-(*β*-D-mannopyranosyloxyethyl)]-4-hydroxymethyl-1,2,3-triazole	22	IV	IV
1-[(*β*-D-Mannopyranosyl)-(1,2)-(*α*-D-mannopyranosyloxyethyl)]-4-hydroxymethyl-1,2,3-triazole	23	IV	IV
1,3-Di[1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-mannopyranosyloxyethyl)-4-(methyl-1-oxy)-1,2,3-triazolyl]propane	24	IV, *	IV
1,3-Di[1-[(2,3,4,6-tetra-*O*-acetyl-*β*-D-mannopyranosyl)-(1,2)-(3,4,6-tri-*O*-acetyl-*β*-D-mannopyranosyloxyethyl)]-4-[methyl-1-oxy]-1,2,3-triazolyl]propane	25	IV, *	IV
1,3-Di[1-[(2,3,4,6-tetra-*O*-acetyl-*β*-D-mannopyranosyl)-(1,2)-(3,4,6-tri-*O*-acetyl-*α*-D-mannopyranosyloxyethyl)]-4-[methyl-1-oxy]-1,2,3-triazolyl]propane	26	IV, *	IV
1,3-Di[1-(*β*-D-mannopyranosyloxyethyl)-4-(methyl-1-oxy)-1,2,3-triazolyl]propane	27	IV	IV
1,3-Di[1-[(*β*-D-mannopyranosyl)-(1,2)-(*β*-D-mannopyranosyloxyethyl)]-4-[methyl-1-oxy]-1,2,3-triazolyl]propane	28	IV	IV
1,3-Di[1-[(*β*-D-mannopyranosyl)-(1,2)-(*α*-D-mannopyranosyloxyethyl)]-4-[methyl-1-oxy]-1,2,3-triazolyl]propane	29	IV	IV
1,2,3-Tris[1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-mannopyranosyloxyethyl)-4-(methyl-1-oxy)-1,2-3-triazolyl]propane	30	IV	IV
1,2,3-Tris[1-[(2,3,4,6-tetra-*O*-acetyl-*β*-D-mannopyranosyl)-(1,2)-(3,4,6-tri-*O*-acetyl-*β*-D-mannopyranosyloxyethyl)]-4-[methyl-1-oxy]-1,2-3-triazolyl]propane	31	IV, *	IV
1,2,3-Tris[1-[(2,3,4,6-tetra-*O*-acetyl-*β*-D-mannopyranosyl)-(1,2)-(3,4,6-tri-*O*-acetyl-*α*-D-mannopyranosyloxyethyl)]-4-[methyl-1-oxy]-1,2-3-triazolyl]propane, "TADM"	32	IV, V	IV
1,2,3-Tris[1-(*β*-D-mannopyranosyloxyethyl)-4-(methyl-1-oxy)-1,2,3-triazolyl]propane	33	IV	IV

1,2,3-Tris[1-(*β*-D-mannopyranosyl)-(1,2)-(*β*-D-mannopyranosyloxyethyl)]-4-[methyl-1-oxy]-1,2,3-triazolyl]propane	34	IV	IV
1,2,3-Tris[1-(*β*-D-mannopyranosyl)-(1,2)-(*α*-D-mannopyranosyloxyethyl)]-4-[methyl-1-oxy]triazolyl]propane	35	IV	IV
1,1,1,1-Tetramethyl[1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-mannopyranosyloxyethyl)-4-(methyl-1-oxy)-1,2-3-triazolyl]methane	36	IV, *	IV
1,1,1,1-Tetramethyl[1-[(2,3,4,6-tetra-*O*-acetyl-*β*-D-mannopyranosyl)-(1,2)-(3,4,6-tri-*O*-acetyl-*β*-D-mannopyranosyloxyethyl)]-4-[methyl-1-oxy]-1,2-3-triazolyl]methane	37	IV, *	IV
1,1,1,1-Tetramethyl[1-[(2,3,4,6-tetra-*O*-acetyl-*β*-D-mannopyranosyl)-(1,2)-(3,4,6-tri-*O*-acetyl-*α*-D-mannopyranosyloxyethyl)]-4-[methyl-1-oxy]-1,2-3-triazolyl]methane	38	IV, *	IV
1,1,1,1-Tetramethyl[1-(*β*-D-mannopyranosyloxyethyl)-4-(methyl-1-oxy)-1,2-3-triazolyl]methane	39	IV	IV
1,1,1,1-Tetramethyl[1-[(*β*-D-mannopyranosyl)-(1,2)-(*β*-D-mannopyranosyloxyethyl)]-4-[methyl-1-oxy]-1,2-3-triazolyl]methane	40	IV	IV
1,1,1,1-Tetramethyl[1-[(*β*-D-mannopyranosyl)-(1,2)-(*α*-D-mannopyranosyloxyethyl)]-4-[methyl-1-oxy]-1,2-3-triazolyl]methane	41	IV	IV
1,2,3,4,6-Penta[1-(2,3,4,6-tetra-*O*-acetyl-*β*-D-mannopyranosyloxyethyl)-4-(methyl-1-oxy)-1,2-3-triazolyl]-β-D-glucopyranose	42	IV, *	IV
1,2,3,4,6-Penta[1-[(2,3,4,6-tetra-*O*-acetyl-*β*-D-mannopyranosyl)-(1,2)-(3,4,6-tri-*O*-acetyl-*β*-D-mannopyranosyloxyethyl)]-4-[methyl-1-oxy]-1,2-3-triazolyl]-β-D-glucopyranose	43	IV, *	IV
1,2,3,4,6-Penta[1-[(2,3,4,6-tetra-*O*-acetyl-*β*-D-mannopyranosyl)-(1,2)-(3,4,6-tri-*O*-acetyl-*α*-D-mannopyranosyloxyethyl)]-4-[methyl-1-oxy]-1,2-3-triazolyl]-β-D-glucopyranose	44	IV, *	IV
1,2,3,4,6-Penta[1-(*β*-D-mannopyranosyloxyethyl)-4-(methyl-1-oxy)-1,2-3-triazolyl]-β-D-glucopyranose	45	IV	IV
1,2,3,4,6-Penta[1-[(*β*-D-mannopyranosyl)-(1,2)-(*β*-D-mannopyranosyloxyethyl)]-4-[methyl-1-oxy]-1,2-3-triazolyl]-β-D-glucopyranose	46	IV	IV
1,2,3,4,6-Penta[1-[(*β*-D-mannopyranosyl)-(1,2)-(*α*-D-mannopyranosyloxyethyl)]-4-[methyl-1-oxy]-1,2-3-triazolyl]-β-D-glucopyranose	47	IV	IV

4.4 Endotoxin testing (I-V)

All compounds were screened for endotoxin contamination with E-Toxate Kit (Sigma-Aldrich, St Louis, MO, USA) by spot checking during the preparation and by double-checking all compounds showing any immunostimulatory activity.

Endotoxin levels in all tested samples (highest used stimulation concentration) were below 0.015 EU/ml.

4.5 Subjects (I-V)

In study **I,** the evaluation of the fungal extracts was done with five atopic subjects (31.6 ± 7.2 years; 3 females, 2 males) and five healthy subjects (34.4 ± 12.0 years; 5 females). All atopic subjects had skin prick test verified pollen allergy and seasonal allergic rhinitis. In addition, two of them had specific IgE to timothy grass pollen (Phleum pratence) and three to birch tree pollen (Betula verrucosa, Bet v). 8 atopic dermatitis patients and 8 healthy subjects were included in cell stimulation studies with the mild acid hydrolyzed mannan of *C. albicans* and its fractions. The 8 atopic dermatitis patients had specific IgE to commensal yeasts, Malassezia furfur and *C. albicans*. The cell stimulation studies with the below 3 kDa fraction of the *C. albicans* mannan were performed with three healthy study subjects without any history of allergic diseases or specific IgE antibodies to common allergens.

The PBMCs used in the stimulation tests in studies **II** and **IV** were retrieved from the blood samples donated by voluntary healthy laboratory personnel (n = 3-5). In study **III** serum samples for competitive enzyme-linked immunosorbent assay (ELISA) studies were collected from vaginal candidiasis patients (n = 5-12) after confirmed elevated levels of IgG antibodies against *C. albicans* mannan. The PBMCs used in birch allergen induced cell cultures in study **IV** were collected from 6 birch pollen allergic subjects.

In study **V**, 26 adult birch allergic subjects with allergic rhinoconjunctivitis (22 females and 4 males) were included (37.7 ± 2.2 years; mean birch-specific IgE (Immunocap, Thermo Fisher Scientific Phadia, Uppsala, Sweden) 25.2 kU/l ± 5.3 kU/l).

4.6 hPBMC (I, II, IV and V)

In all of the studies discussed in this thesis, the PBMCs were isolated from heparinized blood samples donated by study subjects by Ficoll-Paque density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The cells were washed twice with Hanks' balanced salt solution (HBSS) buffered with $NaHCO_3$ (pH 7.4) and resuspended in Roswell Park Memorial Institute -based medium (RPMI) (Invitrogen, Paisley, Scotland, UK or Carlsbad, CA, USA) supplemented with a 5% autologous serum, 2-2.5 mM L-glutamine (Fluka Chemie AG, Buchs, Switzerland or Sigma-Aldrich Co., St. Louis, MO) and 100 mg/ml gentamycin (Biological Industries Ltd., Kibbutz Beit Haemek,

Israel). The cells were thereafter stimulated with various concentrations of different compounds on 48- or 96-well flat-bottomed cell culture Costar plates (Corning Inc., Corning, NY, USA) in duplicate at +37 ˚C in humidified atmosphere at a density of 10^6/ml. The medium alone served as an unstimulated control. After stimulation, the PBMCs were harvested in 0.5 ml of TRIzol Reagent (Invitrogen) and stored at -70 ˚C.

4.7 PBMC stimulations in the presence of birch pollen (IV, V)

In this thesis a formerly developed *in vitro* method for testing the suppression of the Th2-type cytokine response induced by birch pollen was used (Savolainen J et al 2000). After PBMC isolation from heparinized blood samples the PBMCs (10^6/ml) were applied on 48-well flat-bottomed cell culture plates (Costar, Corning Inc.) and incubated in the presence of a birch allergen (50 μg/ml, Bet v, Aquagen, ALK-Abelló A/S, Hørsholm, Denmark) and various concentrations of different compounds or medium. All incubations were performed at +37 ºC in a humidified atmosphere with 5% CO_2. All stimulations were done in duplicate. The medium alone served as an unstimulated control. After stimulation, the cells and the supernatants of the cultures were collected and stored at -70 ºC.

4.8 Adjuvants (V)

In study **V,** synthetic MPL and an active CpG ODN (tlrl-2006) and a control CpG ODN (tlrl-2006c) were purchased from Invivogen (San Diego, CA, USA). The active CpG ODN had a sequence of 5´-TCG TCG TTT TGT CGT TTT GTC GTT-3´, identical to the one used in a previous study (Marshall JD et al 2001).

In study **V**, the cells obtained during the pollen season (n = 26) were co-cultured in the presence of a birch allergen (50 μg/ml, Bet v, Aquagen, ALK-Abelló A/S, Hørsholm, Denmark) and stimulated with TADM (20, 200 μg/ml), MPL (10 μg/ml), active or control CpG ODN (5 μg/ml). The used concentrations of MPL and CpG ODN were based on the manufacturer´s instructions (Invivogen).

4.9 Mouse cell lineage J774.2 (II)

Mouse cell lineage J774.2 was bought from Sigma-Aldrich (Lot 06/C/015, Germany). The handling and culturing of the cells were done according to the manufacturer´s instructions. The cells were cultured for 24 hours and then stimulated with different concentrations of the synthetic β-(1,2)-linked mannotetraose.

4.10 Cytokine expression (I-V)

In studies **I** and **II,** the potency of various compounds to stimulate IFN-γ messenger ribonucleic acid (mRNA) expression was studied by isolating RNA and by using TaqMan reverse transcription polymerase chain reaction. Briefly, RNA was isolated according to TRIzol instructions and the extracted RNA was stored in 75% ethanol at -20 ˚C. The reverse transcriptase reaction was performed with First-Strand cDNA Synthesis Kit (Amersham Biosciences AB) using oligo (dT) primers. cDNA was stored at -70 ˚C. The relative quantitation of the IFN-γ mRNA expression was performed with ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using a housekeeping gene β-actin as an endogenous reference transcript. The cDNA-specific primer and the dual-labeled probe sequences for β-actin and IFN-γ as well as the PCR reactions were as described earlier (Laaksonen K et al 2003). The data analysis was performed according to the manufacturer's instructions (User Bulletin #2, P/N 4303849, Applied Biosystems) using a comparative Ct ($2^{-\Delta\Delta Ct}$) method, where β-actin served as an endogenous reference gene and an unstimulated cell culture as a calibrator. The resulting $2^{-\Delta\Delta Ct}$ value was used to indicate the fold change in cytokine expression relative to the unstimulated cultures.

High-sensitivity human cytokine Lincoplex kits (LINCO Research, St. Charles, MO, USA) were used to measure cytokine production in studies **IV** (IL-4, IL-5, IL-10, IFN-γ, TNF) and **V** (IL-4, IL-5, IL-10, IL-13, IFN-γ, TNF) and partly in study **II** (IL-4, IL-10, TNF and partly IFN-γ). The Lincoplex assays were performed in accordance with the manufacturer's protocol by employing Luminex technology. This technology of applying fluorescently labeled beads and a flow cytometer (Luminex 100) enables a wide range of cytokines to be measured simultaneously from one sample (Vignali DA 2000).

In study **II,** the amount of TNF produced after stimulating the mouse cell lineage J774.2 with β-(1,2)-linked mannotetraose was measured with high-sensitivity mouse TNF cytokine Lincoplex kit (LINCO Research, St. Charles, MO, USA).

4.11 Lymphoproliferation (II)

In study **II,** lymphoproliferation in the PBMCs was determined with ^{3}H-labeled thymidine (Amersham Biosciences AB) in three replicate cultures (10^6 cells/ml) incubated for 6 days in 96-well flat-bottomed Costar plates (Corning Inc.). Lymphoproliferation, expressed as an index in relation to unstimulated cultures, was calculated from the mean of three replicate cultures.

4.12 Competitive ELISA inhibition (III)

In study **III,** polystyrene 96-well ELISA plates were coated with hydrolyzed below 3 kDa *C. albicans* mannan (50 µl 0.1 mg/ml in phosphate buffered saline (PBS)) and incubated at +4 °C overnight. The plates were then washed three times with 0.05% PBS-tween. Blocking was done by using 2% BSA-PBS (bovine serum albumin fraction V, ICN Biomedicals Inc., Aurora, Ohio, 810034; Tween 20 Fluka, Sigma-Aldrich Co. 93773) for 2 hours at room temperature after which the plates were washed three times. The serum samples were collected from vaginal candidiasis patients (50 µl diluted 1:100 in 0.5% BSA-0.05% PBS-tween) and incubated at +37 °C for 2 hours with serial tenfold dilutions of the studied carbohydrates (0.0001-1 mg/ml) in a total volume of 100 µl. Thereafter the serum dilutions were applied to the coated ELISA plates and blotted and incubated at +4 °C overnight. After washing three times, 100 µl of alkaline phosphatase conjugated antihuman IgG (Dako, Denmark, DO 336) (1:1000 in 0.5% BSA-PBS-tween) was applied to the plates which were incubated for 2 hours at +37 °C. After washing three times, the colour reaction was developed using 100 µl para-nitrophenylphosphate-di-Na-salt (1 mg/ml in diethanolamine, Reagena, Finland, 180288) as a substrate and terminated by adding of 1 M NaOH (AKZO Nobel, the Netherlands) after which the absorbance (optical density, O.D. 405 nm) was read using an automated microplate reader. The inhibition in ELISA was expressed using a formula: (O.D. inhibition – O.D. PBS)/(O.D. no inhibition – O.D. PBS)%. The mean values of the duplicate determinations were used.

4.13 Murine model of asthma (V)

6-8 weeks old female BALB/c mice (NOVA-SCB AB, Sollentuna, Sweden) (n = 8 per group) were maintained on ovalbumin (OVA)-free diets and water *ad libitum.* The mice were anesthetized (isoflurane, Abbott Laboratories Ltd, Queensborough, UK) and their backs were shaved and tape-stripped (Tegaderm 3M Health Care, St. Paul, MN, USA) three times before the injections to induce a skin injury. Weekly intradermal sensitizations were made with 50 µg of OVA (Sigma-Aldrich) in 100 µl, or with PBS (control group) for 4 weeks. TADM (10 or 50 µg) was intradermally injected together with OVA or PBS. On days 28, 29 and 30 all mice were anesthetized and intranasally challenged with OVA (50 µg in 50 µl of PBS) After 24 hours, the mice were killed with isoflurane, and samples (blood, bronchoalveolar lavage (BAL) fluids and lung biopsies) were taken for subsequent analysis (Figure 1 in study **V**).

4.14 BAL cells and the lung histology of the mice (V)

BAL inflammatory cells as well as hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) stained lung biopsies were obtained and prepared as described earlier (Lehto M et al 2005). Finally, the cells were counted under light microscopy (Leica DM 4000B, Wetzlar, Germany).

4.15 RT-PCR analysis of mouse lung tissue (V)

The lung biopsies were quick-frozen and kept at -70 °C for later RNA isolation. The samples were homogenized with Lysing Matrix D tubes (MP Biomedicals, Solon, Ohio; USA) and TRIsure reagent (Bioline, London, UK) in a FastPrep FP120 machine (BIO 101, Thermo Fisher Scientific, Waltham, Mass, USA). Total RNA extraction was performed according to the manufacturer's instructions (TRIsure). Complementary DNA synthesis and real-time polymerase chain reaction (RT-PCR) with 7500 Fast Real-time PCR System and SDS Software v.1.40 (Applied Biosystems, Foster City, CA, USA) were made as previously described (Lehto M et al 2010).

4.16 Mouse serum antibodies (V)

OVA-specific IgE (capture ELISA) and IgG2a (straight ELISA) were analyzed with the ELISA as described earlier with small modifications (Lehto M et al 2003). Coating concentrations were 2 µg/ml rat anti-mouse IgE for OVA-specific IgE and 2 µg/ml OVA for OVA-specific IgG2a. The serial dilutions of the sera were 1/20, 1/60 and 1/120 for OVA-specific IgE and 1/200, 1/600, 1/1800 and 1/5400 for OVA-specific IgG2a. The bound OVA-specific IgE was detected with biotin-conjugated OVA (diluted 1/1000), which was prepared with NHS-LC-Biotin kit (Pierce, Thermo Scientific, Rockford, IL, USA). The monoclonal antibodies and streptavidin-horseradish peroxidase were obtained from BD (San Diego, CA, USA) and the substrate solution from KPL, Inc. (Gaithesburg, MD, USA).

4.17 Ethics committee

All blood samples were taken from the study subjects after informed consent. The study was approved by the ethics committee of Turku University Central Hospital, Finland. All animal experiments were approved by the Social and Health Care Department of the State Provincial Office of Southern Finland.

4.18 Statistical analysis

The Wilcoxon signed-rank test was used to assess the statistical significance of the difference in cytokine expression and lymphoproliferation between the stimulated and the unstimulated cell cultures. In all tests, $P < 0.05$ was used to designate a statistically significant difference. Single mice groups were compared by the nonparametric Mann-Whitney *U*-test using GraphPad Prism software (v.5, GraphPad Software Inc., La Jolla, CA, USA). The data are expressed as mean ± the standard error of the mean (SEM).

5 RESULTS

5.1 Immunostimulatory properties of the fungal extracts (I)

The induction of IFN-γ by the different fungal extracts was studied in PBMC cultures from a total of ten study subjects (Tables 2 and 3 in study **I**). For the statistical analysis, the data of atopic and healthy subjects were merged, as no significant differences were observed. The yeast whole extracts generally induced a strong, dose-dependent enhancement in the IFN-γ mRNA expression. Stimulation with the *C. albicans* extract induced the strongest IFN-γ mRNA expression. In general, the mould and mushroom extracts were weaker inducers of IFN-γ production than the yeast extracts. The *S. chartarum* mould extracts and the *P. involutus* mushroom extracts induced IFN-γ secretion strongly. Most mould and some mushroom extracts resulted in downregulation of the IFN-γ mRNA expression. This downregulation with the higher 200 μg/ml dose was associated with diminished β-actin expression, indicating cytotoxicity of these extracts.

The major components in the fungal cell wall are water-soluble carbohydrates, including mannan. Therefore different fungal mannans were prepared next and their IFN-γ induction capacities were studied in the PBMCs. The mannans of *C. albicans* (yeast), *Agaricus bisporus* (mushroom) and *Aspergillus umbrosus* (mould) induced comparable or augmented IFN-γ expression as compared to the whole extracts (Table 3 in study **I**). When tested in a subset of the study subjects, also the *S. chartarum* and *P. involutus* mannans enhanced IFN-γ expression in the PBMCs at a level similar to the whole extracts (the data not shown).

5.2 Immunostimulatory properties of the *C. albicans* mannan (I)

To further characterize the immunostimulatory components of the fungal mannans the most potent *C. albicans* mannan was chosen for further studies. Hydrolyzed mannan was fractionated to approximately over 100 kDa, 10–100 kDa, 1–10 kDa and below 3 kDa fractions. The prolonged hydrolysis of the *C. albicans* mannan increased the amount of oligosaccharides and monosaccharides in the hydrolysates (Figure 1A in study **I**), but no reduction in the lymphoproliferation of the PBMCs was observed (Figure 1B in study **I**). When analyzed in a subset of the study subjects, no suppression in the IFN-γ secretion was observed after prolonged hydrolysis (the data not shown). All gained fractions induced relatively more IFN-γ secretion in the PBMCs of three study subjects (2.54 ± 2.08 μg/ml; 1.06 ± 0.78 μg/ml; 1.22 ± 0.91 μg/ml; 3.76 ± 1.63 μg/ml) than the intact mannan (0.83 ± 0.54 μg/ml) at corresponding concentrations.

As the below 3 kDa fraction induced the strongest IFN-γ secretion, the immunostimulatory components of this fraction were analyzed. They were further fractionated by size with Biogel P2 liquid chromatography. The highest concentration of carbohydrates was in the fractions 11–13 and when analyzed with liquid chromatography these fractions consisted of di- and monosaccharides (Figure 2B in study **I**). The strongest inducer of TNF production was the pooled fraction 1 (Figure 2A in study **I**). However, no detailed carbohydrate structures could be assessed.

5.3 Immunostimulatory properties of the synthetic mannosides mimicking the β-(1,2)-linked cell wall mannans of *C. albicans* (II, IV)

To explore the carbohydrate structures inducing immunostimulatory responses, we decided to synthesize known mannose-based structures suggested to be in these fractions and test their immunostimulatory capacities in the PBMCs of healthy subjects. Mannobiose (**1**), mannotriose (**2**) and mannotetraose (**3**) induced a moderate dose-dependent IFN-γ response in the PBMCs of healthy subjects (Figure 1C in study **II**). Neither non-reducing cyclohexyl (**4**) and methyl (**5** and **6**) mannosides nor glucose and *N*-acetylglucosamine containing analogues (**7**–**9**) with modifications to the non-reducing end of the oligosaccharide compound did induce any measurable IFN-γ production in the PBMCs (Figures 2B and 3B in study **II**).

None of the divalent compounds (**10-15**) in study **II** induced any measurable IL-4, IL-10 or IFN-γ responses. With a single compound, 1,4-bis(α-D-mannopyranosyloxy)butane (**10**), a dose-dependent stimulation of TNF production was observed (Figure 4B in study **II**).

In study **IV** only 19 out of the 30 synthesized carbohydrate structures (**18-47**) could be tested. The others did not dissolve in water. Very strong induction of IL-10 was observed after PBMC stimulation with TADM (compound **32**, Figure 6). TADM also induced moderate IFN-γ and TNF production. The production of IL-4 and IL-5 was not induced by TADM. Most of the other water soluble mannosides did not have any effect on the cytokine productions of the PBMCs (the data shown for compounds **30**, **32** (TADM) and **35** in Table 1 in study **IV**).

5.4 Effects of the β-(1,2)-linked mannotetraose on the cytokine production in a mouse macrophage cell line J774.2 (II)

The synthetic β-(1,2)-linked mannotetraose (**3**) did not induce any detectable TNF production in a mouse macrophage cell line J774.2 (Figure 1B in study **II**). Under the same experimental conditions, the mild acid hydrolyzed *C. albicans* mannan induced TNF production.

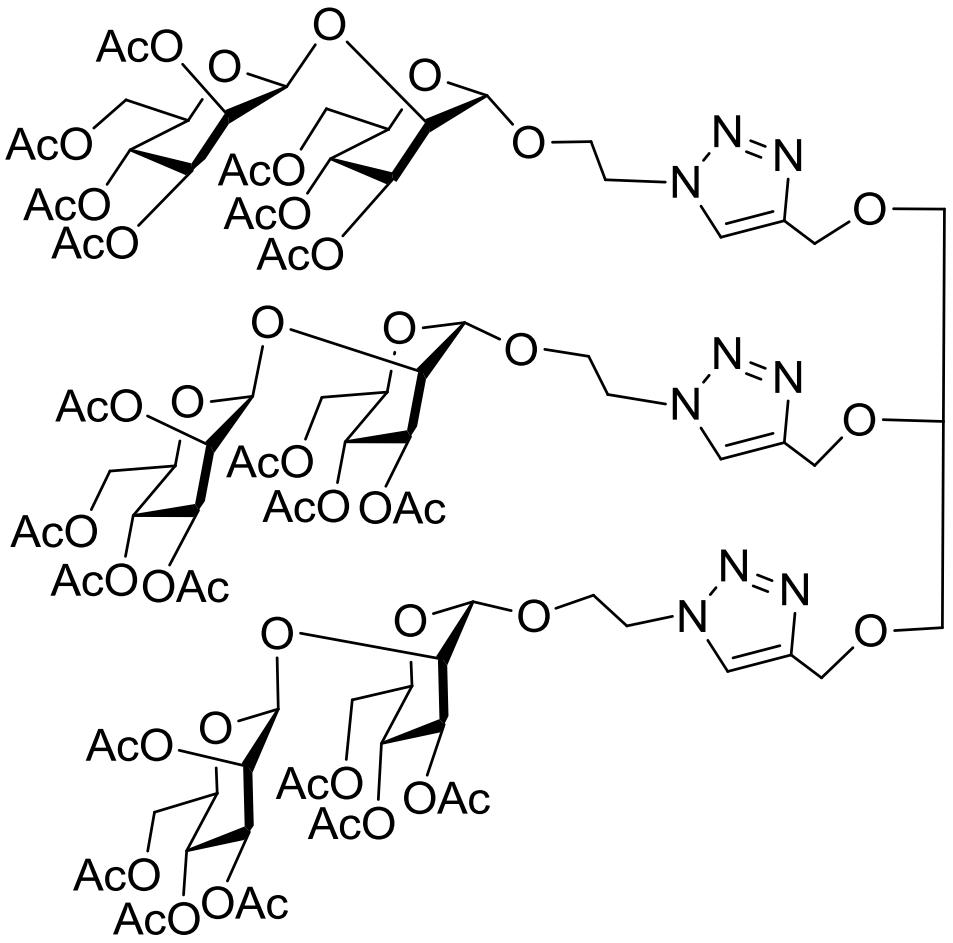

Figure 6. The chemical structure of trivalent acetylated dimannose (TADM).

5.5 The inhibitory effects of synthetic β-(1,2)-linked mannosides on below 3 kDa hydrolyzed mannan of *C. albicans* binding to specific IgG (III)

To characterize the humoral responses to carbohydrates, three different divalent mannosides having β-linkages together with shorter and specific chain length along with their corresponding monovalent counterparts were studied in a competitive inhibition ELISA. The aim of this study was to determine the inhibition of specific human IgG binding to low molecular weight hydrolyzed *C. albicans* mannan by these synthesized mannosides. The binding of these antibodies to below 3 kDa hydrolyzed *C. albicans* Cetavlon mannan was inhibited by divalent mannobiose compounds **5** and **6** down to 50% and 30%, respectively (Figure 4 in study **III**). The inhibition pattern was similar to the corresponding monovalent controls (**2** and **3**) but lesser with mannoses (**1** and **4**).

5.6 Effects of acetylated trivalent mannosides on the birch allergen induced cytokine responses of PBMCs in the allergic subjects (IV)

The effect of TADM (compound **32** in study **IV**) on the birch pollen induced cytokine productions were studied in the allergen (Bet v) stimulated PBMC cultures of birch allergic rhinitis patients. The birch pollen allergen induced strong IL-4 and IL-5 productions of the PBMCs in the allergic subjects. These responses were suppressed by TADM, which also enhanced the IFN-γ production (Figure 4 in study **IV**). Also the allergen induced IL-10 response was suppressed by TADM. The corresponding acetylated trivalent monosaccharide based mannose assembly **30** did not have any effect on the allergen-induced productions of IL-4, IL-5, IL-10 and IFN-γ (Figure 4 in study **IV**).

5.7 Effects of TADM, MPL and CpG ODN on the birch-induced cytokine responses of PBMCs in the allergic subjects during the pollen season (V)

The effects of TADM, MPL and CpG ODN on the allergen (Bet v) induced cytokine responses in the PBMC cultures of 26 birch allergic rhinitis patients were studied next (Figure 2 in study **V)**. Stimulation with birch induced significant responses of Th2 cytokines IL-4 ($P < 0.0001$), IL-5 ($P < 0.0001$) and IL-13 ($P = 0.00012$). Only TADM suppressed the birch-induced production of all three studied Th2 cytokines (IL-4 ($P = 0.0019$), IL-5 ($P = 0.00042$) and IL-13 ($P = 0.034$)), whereas CpG ODN suppressed only IL-5 ($P = 0.019$) and MPL only IL-13 production ($P = 0.0085$). CpG ODN increased the birch-induced IL-4 ($P = 0.0026$) and IFN-γ ($P = 0.00062$) productions. TNF production was enhanced by TADM ($P = 0.012$) and IL-10 production by MPL ($P = 0.012$) and CpG ODN ($P = 0.0046$).

5.8 Airway inflammation, lung expression of IFN-γ mRNA in the OVA treated mice and the OVA-specific antibodies in mice sera (V)

In the mice lungs the OVA treatment induced a mild mRNA expression of IFN-γ, while the expression of other Th1-type cytokines decreased (Figure 3A in study **V**) and all studied Th2-type cytokines significantly increased (Figure 3B in study **V**). Proinflammatory cytokine (TNF) mRNA levels remained at the baseline level, whereas regulatory cytokine (IL-10) mRNA expression significantly increased in the mice lungs after the OVA exposure (Figure 3C in study **V**). TADM significantly increased the mRNA expression of the main Th1-type cytokine (IFN-γ) *(*$P = 0.0398$) (Figure 3A in study **V**). In addition, there was a trend toward decreased IL-4 mRNA expression in TADM mice (Figure 3B in study **V**). Otherwise, the lung expression of different cytokine mRNAs was similar in the PBS and TADM mice.

OVA specific IgE and IgG2a antibodies were found after the OVA treatments in mice sera (Figure 4 in study **V**). TADM significantly decreased the OVA-specific IgE levels in the OVA mice *(P* = 0.0002) (Figure 4A in study **V**), whereas the OVA-specific IgG2a levels increased (Figure 4B in study **V**). There were no differences between the control groups (PBS vs. TADM).

The OVA exposure and challenge induced inflammation in the murine airways. The number of inflammatory cells increased significantly in the H&E stained lung samples as well as the amount of cells producing mucus (PAS cells) according to PAS staining (the results not shown). TADM had no effect on the infiltration of the inflammatory cells to the lungs, although the number of PAS cells decreased slightly (about 10%) with a lower TADM concentration in the OVA-treated mice (the results not shown). The OVA treatment also significantly changed the cell distributions in BAL fluids (Figure 5 in study **V**), decreasing the macrophage cell counts and increasing the amount of the lymphocytes and granulocytes, especially eosinophils. In general, the TADM treatment could not influence the cell types found in the BAL fluids, albeit the number of macrophages declined in the OVA-sensitized and -challenged mice. In addition, eosinophil counts were lower in the TADM control group when compared to the PBS controls (Figure 5 in study **V**).

6 DISCUSSION

6.1 Induction of the IFN-γ production and lymphoproliferation in the PBMCs by the fungal extracts and mannans (I)

According to the hygiene and biodiversity hypotheses, the rich variability among living organisms from all sources protects against allergic diseases by establishing tolerance and immune deviation from Th2 to Th1 response and Treg cell activation (Haahtela T et al 2013, Prokopakis E et al 2013). Also fungal preparations are presented as Th1-inducing compounds (Suzuki Y et al 2001). Because these preparations vary by composition and the actual structures responsible for the detected antiallergic activities are still unclear, we first screened the immunostimulatory properties of 24 fungal extracts, including mushrooms, moulds and yeasts in study **I**. For this study the methods for preparing extracts from a wide variety of fungal species were developed. The strongest IFN-γ response was obtained after stimulation with the *C. albicans* extract. In general, the mould and mushroom extracts were weaker inducers of IFN-γ than the yeast extracts. Our results were in accordance with the previous data suggesting *C. albicans* as an immunopotent organism (Ashman RB and Papadimitriou JM 1995).

Most of the Th1-inducing compounds of the fungal cells have earlier been suggested to origin from their cell wall components, including mannan as the main component of the cell wall of all yeasts (Savolainen J et al 1999, Pietrella D et al 2001). Therefore the fungal mannans, *C. albicans* (yeast), *Agaricus bisporus* (mushroom) and *Aspergillus umbrosus* (mould) were studied next. Accordingly, the Cetavlon-precipitated mannans of various fungi stimulated cytokine production similarly or more than the whole extracts, the most potent of them being the *C. albicans* mannan. This is in line with previous studies showing the *C. albicans* mannan as a potent Th1-inducing molecule (Savolainen J et al 1999).

In study **I** we used PBMCs from healthy subjects to screen the immunostimulatory capacities of fungal extracts. The observed IFN-γ production indicates the fungal extracts to act as recall antigens. In fact, the MHC-restricted antigen recognition mechanisms are likely to be involved due to earlier priming of the immune system by a natural exposure to the antigens present in the studied moulds, yeasts and mushrooms. In line with our results, it has previously been shown that a number of yeast genera are capable of eliciting extensive MHCI-restricted yeast-specific memory T cell responses with IFN-γ production in the whole blood cultures of healthy individuals (Heintel T et al 2003). This is done by presenting processed yeasts as antigens on APCs with MHCI to CD8+ T cells. Furthermore, it has been shown that the innate effector cells are capable of recognizing the fungal cell wall molecules directly through a variety of other PRRs, including MR, β-glucan and

complement receptors (Romani L et al 2002). In addition, the fungal antigens are capable of inducing TNF production in a MHC-independent manner through interactions with TLRs, particularly TLR2 and TLR4 (Jouault T et al 2003). Also immunological cross-reactivity between the common carbohydrate constituents of the fungi, namely mannose and mannan, from different environmental sources may explain the observed results.

6.2 Hydrolysis products of *C. albicans* mannan induce IFN-γ and TNF production in PBMCs (I)

To distinguish the key components behind the induction of IFN-γ by the *C. albicans* mannan composed of mannosides, proteins and lipids, it was next hydrolyzed. The *C. albicans* mannan -derived mild acid hydrolysis products below 3 kDa enhanced the production of IFN-γ secretion similarly as the intact *C. albicans* mannan. This indicates the key components to be of low molecular weight. Next the low molecular weight hydrolysis products of the *C. albicans* mannan were fractionated by size with liquid chromatography in a Biogel P2 column. As microbial immunostimulatory molecules are shown to induce especially good TNF responses, TNF was chosen as the indicator cytokine (Okemoto K et al 2006). A pooled fraction 1 containing oligosaccharides of 12-16 mannoses induced TNF production. Oligosaccharides with at least 4 mannose units have been previously demonstrated to induce TNF production (Jouault T et al 1995). Previous nuclear magnetic resonance (NMR) analyzes have shown the presence of β-(1,2)-linkages in the fractions corresponding to our active fractions (Trinel PA et al 1997). β-(1,2)-linked oligomannosides are known to mediate the attachment of *C. albicans* to murine macrophages and stimulate early and transient TNF production (Jouault T et al 1995, Fradin C et al 1996). The results obtained in this study suggest that the immunological activities detected are, at least partly, due to the presence of β-(1,2)-linked oligomannosides.

However, in addition to the carbohydrates the cell wall extracts evaluated may have contained also other biomolecules including glycoproteins. Compounds derived from natural sources are often heterogeneous and difficult to purify into single chemical entities making batch-to-batch reproducibility problematic or even impossible. Impurities may lead to serious side effects and chemical entities derived from natural sources may also have limited stability in storage. It is possible that the observed immunostimulatory properties do not derive solely from the carbohydrate structures. In these fractions very low amounts of active molecules may induce cytokine production and yet be undetectable by NMR or mass spectrometry. In previous studies designed to evaluate the structure-function relationship of potentially immunostimulatory molecules, the tested molecules have been cell wall preparations consisting of lipids, polysaccharides, proteins and

glycoproteins (Masuoka J 2004). Therefore another approach, in contrast to the fractionated cell wall extracts, to determining the structures behind the immunostimulatory activities of the *C. albicans* mannan had to be employed. The chemical synthesis of the naturally occurring substances, or their close analogues, served as the substitute for isolation of chemically pure natural compounds. Thus the structures developed on the basis of the *C. albicans* carbohydrate structures were applied in the following studies.

6.3 Cytokine production induced by the synthetic mannosides mimicking the β-(1,2)-linked mannosides of *C. albicans* (II, IV)

Cellular interactions can be studied in detail by chemically synthesizing carbohydrates with known reactive groups and scaffold structures. Structure-related factors contributing to the cellular responses include the individual sugar units, the stereochemistry, the DP, chain length, flexibility, nature of the glycosidic linkages and multivalency of the carbohydrate as discussed earlier (Masuoka J 2004, Astronomo RD and Burton DR 2010). These structural details concerning the interactions between oligomannosides and the immune cells have remained largely unknown. To study this, 51 different synthetic molecules based on the β-(1,2)-linked cell wall mannosides and their analogues of *C. albicans* were used in this thesis. Variations of the before-mentioned parameters were used when constructing the compounds for biological screening. Chemical synthesis allows researchers to influence the linker, the number and design of carbohydrates and produce better defined, standardized and contaminant free molecules (Polizzotti BD et al 2007). The production can be closely controlled leading to products with defined amounts of the active compound, which is not possible when using natural extracts (Polizzotti BD et al 2007).

With the first studied monovalent structures in study **II**, the main Th1-type cytokine, IFN-γ, was selected as an indicator for induction of Th1 responses. β-(1,2)-mannobiose (**1**), triose (**2**) and tetraose (**3**) all showed moderate inductions of the IFN-γ expression. These structures correspond to 1% of the mannan of *C. albicans*. Our results were surprising, since the DP has earlier been reported to correlate with the immunomodulatory activity of the mannoside (Jouault T et al 1995). With our small size compounds, no correlations between the immunomodulatory activity and the chain length of the oligomannoside could be demonstrated.

To investigate the significance of the individual sugar units and the stereochemistry of the second carbon molecule, we screened corresponding structures with locked anomeric configurations, mannobioside with cyclohexyl or methyl aglycon (**4** and **5**), mannotrioside with methyl aglycon (**6**), and the glucose and *N*-

acetylglucosamine containing analogues (**7**-**9**) with modifications to the non-reducing end of the oligosaccharide compound. The modified monovalent structures did not induce any measurable IFN-γ production, suggesting that the immunostimulatory activity seems to relate to mannose as the core carbohydrate structure.

Oligo- and multivalency of the synthesized β-(1,2)-linked mannan constructs were evaluated next. To characterize the responses related to multivalency, mannosides ranged from monovalent to pentavalent. In contrast to simple monovalent oligosaccharides, the multivalent carbohydrate assemblies may simultaneously interact with multiple receptors, potentially increasing the binding affinities (Kiessling L et al 2006) leading to enhanced cellular responses. As the simplest compounds in oligovalent mannoside structures, a series of divalent mono- and disaccharide based constructs were first screened for induction of cytokines. None of the divalent compounds (**10**-**15**) induced any measurable IL-4, IL-10 or IFN-γ responses. Divalent molecules appeared not to be complex enough for optimal induction, as only one divalent disaccharide (compound **10**), stimulated TNF production dose-dependently.

We continued by testing truly multivalent β-(1,2)-oligomannosides and their analogues in study **IV**. A series of oligovalent carbohydrate assemblies (ranging from mono- to pentavalent) derived from three structurally different β-linked or β-(1,2)-linked mannosides, were chemically synthesized and evaluated in order to investigate their immunostimulatory properties. Only 19 out of the 30 carbohydrate structures (**18**-**47**) were water-soluble and could be tested by *in vitro* PBMC model. The results showed that most of the mannosides did not induce cytokine productions in the PBMCs. However, a trivalent acetylated dimannose (compound **32**, TADM) showed very strong induction of IFN-γ, IL-10 and TNF without inducing IL-4 and IL-5 production. Its fully deprotected hydroxylated congener (compound **35**) did not induce any cytokine responses. It is known, that micro-organisms evoke cells of the innate immunity to produce cytokines leading to antigen-specific Th1-type responses, which is in accordance with our results (Roman M et al 1997).

TADM consists of three acetylated mannobioses with β-(1,2)-linkages between the two mannoses. Most of the acetylated mannosides with increasing valency did not dissolve in the cell culture media. TADM was at the limits of solubility but dissolved in the medium at the maximum concentration of approximately 0.4 mg/ml. The acetylation of carbohydrates hinders intermolecular hydrogen bonding (Simões J et al 2009). This leads to molecule´s enhanced capacity to pass through cell membranes and across hydrophobic barriers resulting in stimulation of the target cells (Witschi MA and Gervay-Hague J 2010). A survey into literature

revealed that one compound, called acemannan, a partially acetylated β-(1,4)-mannan heteropolysaccharide extracted from Aloe vera, shows immunostimulatory activity in *in vitro* and *in vivo* studies (Reynolds T and Dweck AC 1999).

In addition to acetylation, also the length of the carbohydrate part in TADM was partly responsible for the cytokine responses since similar inductions were not detected with the other acetylated mannosides tested. Thus, it appears that both oligovalent three dimensional structure and acetylation of the mannoside are required for immunostimulatory activity.

6.4 β-(1,2)-linked mannotetraose does not stimulate TNF production in a mouse macrophage cell line J774.2 (II)

The focus of this thesis has been on chemically synthesized molecules. The comparison between synthesized products and naturally occurring structures is difficult or even impossible because natural products are poorly characterized. However, Poulain and co-workers demonstrated in 1995 that oligosaccharide fractions prepared by acidic hydrolysis and the subsequent fractionation of the *C. albicans* cell wall oligosaccharides containing β-(1,2)-linked mannotetraose induced TNF production in the mouse macrophage J774 cell line (Jouault T et al 1995). We repeated the experiment under the same experimental conditions but replaced the hydrolyzed *C. albicans* fraction with a synthetic β-(1,2)-linked mannotetraose (compound **3** in study **II).** Synthetic β-(1,2)-linked mannotetraose did not stimulate TNF production in the mouse macrophages in our experiment.

The contradictory results may partially be explained by the fact that in the reference study the macrophages were purified from the peritoneal exudate cells of 20–24-week-old BALB/c mice, whereas we used immortalized macrophages from a mouse cell lineage. The J774 mouse macrophage cells are heterogenous and dependent on several factors in their expression of the macrophage mannose receptor (Fiani ML et al 1998). It is thus possible that the expression of the functional MR was low or absent in our experiments leading to weak TNF production. However, macrophage activation by β-(1,2)-linked mannoside structures is not only dependent on MR but also on various other PRRs (Poulain D and Jouault T 2004) and galectin-3 (Kohatsu L et al 2006). Since acid hydrolyzed mannan, containing β-(1,2)-oligomannosides, induced TNF production in the J774 cells, the negative results with the synthetic mannotetraose cannot be solely explained by the lack of MR expression.

Another possible explanation for the negative results with mannotetraose is its chemical structure. In the earlier studies, the oligomannoside fractions evaluated may have been cell wall glycoproteins comprising of both carbohydrate and

protein. However, only well-defined, chemically synthesized mannosides without a protein component were used in our study and therefore the responses detected can only be due to interactions between the pure small carbohydrate residues and the macrophages. As such these interactions appear inadequate to induce TNF production. The negative result of our study might be explained by earlier observations that β-(1,2)-linked oligomannosides with a DP of eight or more significantly improve TNF secretion (Jouault T et al 1995).

6.5 Inhibition of binding of IgG to *C. albicans* mannan by the synthetic β-(1,2)-linked *C. albicans* derived mannans (III)

According to earlier studies, the β-(1,2)-linked *C. albicans* derived mannans are immunogenic and elicit specific antibodies, which have been shown to protect against *C. albicans* in animal models of systemic and vaginal candidiasis (De Bernardis F et al 1997, Caesar-TonThat T and Cutler J 1997). It is also known that molecular recognition is prone to multivalent ligand–receptor interactions (Kiessling L et al 2006). Therefore, in study **III** inhibition ELISA experiments were done with synthesized mono- and multivalent mannosides and sera from vaginal candidiasis patients were used. The divalent mannosides **5** and **6** inhibited the binding of IgG antibodies present in the sera of candidiasis patients to below 3 kDa hydrolyzed *C. albicans* Cetavlon mannan down to 50% and 30%. A similar inhibition pattern was observed by their monovalent counterparts **2** and **3**. However, relatively lower inhibition was noted with the monovalent mannoside **1** and its divalent counterpart **4**.

The results indicate that the divalent compounds **5** and **6** possess some antigenic elements for *C. albicans* mannan recognition by the IgG antibodies. Antibodies recognize the β-mannans in the cell wall of *C. albicans* on the basis of properties and chain lengths of these antigenic structures (Han Y et al 1997). Bundle and co-workers have previously shown that short β-(1,2)-homo-oligosaccharides from disaccharides up to hexasaccharides inhibit the binding of β-(1,2)-mannan specific monoclonal antibodies (Nitz M et al 2002). The maximum inhibitory activity was observed for di- and trisaccharides, diminishing significantly for the corresponding tetra-, penta-, and hexasaccharides. Consistently with these previous studies, also in our study the β-linked monosaccharide compound **1** and its divalent analogue **4** showed low inhibitory activity. However, the inhibition of IgG binding by the disaccharides (compounds **2**, **3**, **5**, **6**) was much stronger. In fact, the divalent mannoside **5**, with two β-linkages, showed higher inhibitory activity than its corresponding α-linked (at the reducing terminus) counterpart **6**. Previously a tetravalent β-(1,2)-mannan disaccharide cluster, when linked to a protein, has been shown to produce a good antibody response against *C. albicans* (Wu X et al 2007).

Divergent results were got in our study, since no differences in the inhibition of the IgG binding were observed between the divalent and the monovalent compounds.

6.6 Suppression of the birch allergen induced Th2-type cytokine responses in the PBMCs of allergic subjects by TADM and other adjuvants (MPL and CpG ODN) (IV, V)

Stimulation with birch pollen induces strong Th2-type cytokine production (IL-4, IL-5) in allergic subjects (Smole U et al 2010). Also induction of IL-10 production after allergen exposure in allergic subjects has been reported (Thunberg S et al 2010). These strong IL-4 and IL-5 cytokine responses along with IL-10 production were suppressed by TADM in our pilot study with 6 allergic subjects (study **IV**). The PBMC populations from allergic and healthy subjects differ from each other as they consist of different proportions of the allergen-specific Th2 and Treg cells. In allergic subjects, especially during the pollen season, the balance is skewed towards the Th2 cells and in healthy ones towards the Treg cells (Akdis M et al 2004, Ling EM et al 2004). The blood samples for study **IV** were taken from the subjects during high birch pollen season, a period during which the allergen-specific Treg cells are hardly observed. As it is known that also the Th2 cells may produce IL-10 (del Prete G et al 1993), the Th2 cells rather than the Treg cells are the probable source of the produced IL-10 in our study. According to our results, stimulation with TADM led to diminished IL-10 production, apparently because of diminished Th2 cell activity.

In a larger study with 26 birch allergic subjects TADM was compared and benchmarked against the established adjuvants, CpG ODN and MPL (study **V**). Similar results with the pilot study were got, when the birch allergen enhanced the productions of IL-4, IL-5 and IL-13 in the PBMCs of the allergic subjects. During the pollen season, repeated contacts with an allergen enhance the Th2-type responses, as discussed previously (Wosinska-Becler K et al 2004).

With the concentrations applied here, TADM was the only adjuvant that suppressed all birch-induced Th2-type cytokine productions during the pollen season. The applied concentrations of the earlier established adjuvants (CpG ODN 5 μg/mL and MPL 10 μg/mL) were selected based on the data provided by the manufacturers and a previous study (Marshall JD et al 2001). MPL suppressed IL-13 production and CpG ODN only IL-5 production. During SIT repeated administrations of the allergen extracts result in suppression of the Th2-type responses (IL-4, IL-5 and IL-13), together with enhancement of the Treg- (IL-10) and Th1-type responses (IFN-γ) (Smarr CB et al 2013). Consistently, similar suppression of the Th2-type immune responses were observed also with TADM.

All three studied adjuvants also induced proinflammatory cytokine, TNF, production. It is produced by macrophages and the Th1 cells (Beutler B and Cerami A 1989, Mosmann TR and Sad S 1996). Macrophages have a pivotal role in allergic inflammation since they can either enhance or suppress immune responses depending on the cytokine environment (Peters-Golden M 2004). According to literature, MPL stimulates TNF, IL-10 and IFN-γ production by ligating to TLR4, which leads to NF-κB transcriptional activity (Vitoriano-Souza J et al 2012). Also the β-(1,2)-linked oligomannosides significantly improve TNF secretion and these structures are recognized by galectin-3 (Jouault T et al 1995, Kohatsu L et al 2006). The simultaneous induction of TNF and the suppression of the Th2-type cytokines observed in this study with TADM might be due to similar activation cascades shifting the immune responses towards Th1- and Treg-cytokines.

6.7 TADM decreases the specific IgE levels and increases the IFN-γ mRNA responses in an OVA-sensitized murine model of asthma (V)

In order to analyze the immunomodulatory effects of TADM also *in vivo*, the mice were intradermally sensitized and intranasally exposed to OVA to create an experimental allergic asthma model (Haapakoski R et al 2013). The mice were first sensitized by repeated exposures to the OVA allergen. This leads to OVA-specific Th2-type immune responses. Then the mice were challenged with the OVA-allergen to elicit allergic inflammation. Thereafter blood samples, BAL fluids and lung biopsies were taken for analysis.

The cytokine profiles of the lung tissues reflect the main immune responses of the mice. The OVA-treated mice developed strong Th2-type responses (IL-4, IL-5, and IL-13) in the lungs with predominantly eosinophilic cell infiltration. In the mice sera, increased amounts of the OVA-specific Th1-type (IgG2a) and Th2-type (IgE) antibodies were measured after allergen challenge. In accordance with our assumptions, the OVA-specific IgE levels decreased significantly with TADM. In addition, an increase in the OVA-specific IgG2 levels were observed. The key elements of SIT are the decreased allergen-specific IgE levels and the increased levels of IgG (Gleich GJ et al 1982). Allergen-specific IgGs prevent IgE-mediated histamine release, interactions between the allergens and IgE and allergen presentation (Mothes N et al 2003, Wachholz PA et al 2003). These functions inhibit early-phase responses to allergen challenge (Francis JN et al 2008). Indeed, both CpG ODN stimulation and specific immunotherapy with MPL have lead to reduced levels of the allergen-specific IgE (Bohle B et al 1999, Drachenberg K et al 2001).

Although strong alterations were observed in the humoral and cytokine environment after stimulation with TADM in the presence of OVA, no considerable effect on the cells in BAL could be seen, except for a slightly decreased number of macrophages. This is partly explained by the unusually strong OVA-induced eosinophilia, which could not be suppressed efficiently by TADM.

Stimulation with a high concentration of TADM significantly increased IFN-γ secretion and decreased IL-4 mRNA expression in the lung biopsies of BALB/c mice. IFN-γ has previously been demonstrated to inhibit Th2 development and Th2-mediated allergic inflammation (Nakagome K et al 2009). In addition, the Th1 cells and the produced IFN-γ inhibit IgE production, but stimulate B cell class switching to IgG isotypes (Finkelman FD et al 1990). Our results showing decreased IgE levels after allergen exposure and stimulation with TADM are in line with the observed diminished IL-4 production, since IL-4 is needed in IgE production (Del Prete G et al 1988). During SIT tolerance to allergens is increased through the immune deviation from Th2 towards Th1 and Treg cells. Similar immune deviation, at least partly, was also observed with TADM in a murine model of asthma.

7 SUMMARY AND CONCLUSIONS

Novel possibilities to modulate allergic immune responses were investigated by employing synthetic carbohydrate constructs as immunomodulators. The screened fungal preparations induced Th1-type cytokine production. Especially low molecular weight (below 3 kDa) hydrolysis products of the *C. albicans* mannan were strong inducers of TNF. According to literature, oligosaccharides with β-(1,2)-linkages are found in fractions corresponding to our active products. Synthetic compounds mimicking partly the β-(1,2)-linked mannosides originally found in the *C. albicans* cell wall were chemically produced to identify the detailed structures behind the observed stimulatory activity. Some of these synthetic oligosaccharides did induce IFN-γ production in PBMCs of healthy study subjects. Also the antigenic properties of some synthetic mannosides resembling the β-(1,2)-linked mannans present in the *C. albicans* cell wall were studied by ELISA inhibition and were shown to be epitopes of IgG. The biological activity of the carbohydrates is affected by spatial structure, DP and valency. Even slight variations in the structure of the oligosaccharide may lead to diminished cytokine responses. Therefore, oligovalent carbohydrate assemblies (ranging from mono- to pentavalent) were synthesized and their immunostimulatory properties evaluated. Most of the mannosides did not induce any cytokine production in the PBMCs. However, a trivalent acetylated dimannose, TADM, showed very strong induction of IFN-γ, IL-10 and TNF without inducing IL-4 and IL-5 production in the healthy subjects. TADM also suppressed the birch allergen induced Th2-type cytokine responses in the allergic subjects. In fact, TADM suppressed the Th2-type immune responses in the human PBMCs more efficiently than the two other known adjuvants, MPL and CpG ODN. TADM was immunomodulatory also *in vivo* and decreased the specific IgE levels and increased the IgG2 levels and the IFN-γ mRNA responses in an OVA-sensitized murine model of asthma. It is possible to improve the outcome of SIT by modifying the natural course of allergy with novel adjuvants. This results in enhanced efficacy, cost effectiveness, shortened protocol and adjusted side effect profile of SIT. Also patient compliance improves. TADM may be used to suppress allergic inflammation in the prevention and treatment of atopic allergies. Still, studies are needed to further evaluate the properties of TADM as an adjuvant in SIT.

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