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ZUSAMMENFASSUNG

Die Entstehung allergischer Reaktionen lässt sich in zwei Phasen einteilen: die Sensibilisierungs- und die Effektorphase. Bei Erstkontakt des Immunsystems mit einem Allergen fehlen noch wichtige Komponenten, die für eine Abwehrreaktion notwendig sind. Liegt allerdings eine Fehlfunktion des Immunsystems vor, kann das Allergen als schädlich eingestuft werden. Daraufhin werden Immunglobulin E (IgE) Antikörper und allergenspezifische T-Zellen gebildet und der Grundstock für eine Immunreaktion, die auf basophilen Granulozyten und Mastzellen basiert, wird gelegt. Bei neuerlichem Kontakt mit demselben Allergen kommt es im Rahmen der Effektorphase zu allergischen Symptomen.

Das Ziel dieser Arbeit ist, Methoden zu etablieren, mit deren Hilfe die verschiedenen Stadien von Allergien untersucht werden können.
Im ersten Teil wurden Methoden entwickelt, um die Aufnahme von aufgereinigten Allergenen und ihren Abbau durch dendritische Zellen (DCs) zu darzustellen. Dadurch soll die Frage geklärt werden, warum manche Proteine trotz hoher Sequenzhomologie ein unterschiedliches Potenzial besitzen, allergische Reaktionen hervorzurufen. Die in dieser Arbeit verwendeten Proteine sind zum einen das 2S Albumin aus Sonnenblumenkernen und zum anderen das dazu homologe Protein aus der Paranuss. DCs konnten auf Deckgläsern kultiviert werden, die Aufnahme und die Rezeptorbindung von fluoreszenzmarkierten Proteinen wurden sichtbar gemacht und die Vesikel, die diese markierten Proteine enthielten konnten zu verschiedenen Zeitpunkten an verschiedenen Orten lokalisiert werden. Schlussendlich konnte auch die Aufnahme beider 2S Albumine, die untersucht werden sollten, durch DCs bestätigt werden. Die Rolle der Oberflächenrezeptoren bei der Aufnahme in die Zellen bleibt noch eindeutig zu klären.

Im zweiten Teil dieser Arbeit sollten die Seren eines Patientenkollektivs hinsichtlich ihres IgE-Bindungsprofils und somit auch ihres Sensibilisierungsstatus bestimmt werden, wofür ein neuartiger Allergenchip verwendet wurde. Die Resultate wurden daraufhin mit den Ergebnissen der konventionellen diagnostischen Methoden verglichen. Die zentrale Frage des Projektes ist, ob der Allergenchip in der Diagnose von Nahrungsmittelanaphylaxien eingesetzt werden kann. Deshalb wurden die Patientenseren mit Hilfe des Allergenchips, enzymgekoppelten Immunadsorptionstests (ELISA) und Immunblots auf IgE getestet und die Resultate mit den Krankengeschichten abgeglichen, die auf aufwändigen *in vitro* und *in vivo*

Tests basieren. Anzustreben ist es, die für die Diagnose einer Allergie erforderlichen Tests patientenspezifischer zu gestalten. Der Allergenchip ist eine vielversprechende Methode, die Diagnose von Nahrungsmittelanaphylaxien zu erleichtern. Für seine standardmäßige Anwendung ist es allerdings erforderlich, die Sensitivität weiter zu erhöhen, da IgE-Antikörper gegen einige Allergene fälschlicherweise nicht mit dem Chip identifiziert werden konnten.

1 ABSTRACT

The development of an allergic reaction is based on two phases: sensitization and effector phase. The first time the immune system of a person encounters an allergen it is not prepared for a defensive reaction. However, due to a malfunction of the immune system the allergen is recognized as harmful. As a consequence, Immunoglobulin E (IgE) antibodies, allergen-specific T cells and an immune response involving mast cells and basophils are established. The next contact with the allergen leads to an effector phase resulting in allergic symptoms.

The aim of this work is to establish different methods for the observation of the different stages of the allergic disease.
In the first part methods were set up to follow the route of the uptake and processing of purified allergens by dendritic cells (DCs). Using these methods it should later on be possible to identify the reasons why some proteins have a different allergenic potential although they have high sequence similarities. The proteins used in this case were 2S albumins from sunflower seeds and Brazil nut. It was possible to grow DCs on coverslips, to detect the uptake and the binding to corresponding surface receptors of labelled proteins by immunofluorescence microscopy, to detect the location of the vesicles at different time points and to show the uptake of both proteins of interest by DCs. The involvement of receptors in the uptake still has to be identified.

In the second part of this work the IgE-binding pattern and thus the sensitization status found in the sera of a patients' cohort was determined by a novel allergen microarray. The results were compared to the outcomes of conventional diagnostic methods. The underlying question of this project was if the allergen chip could be a useful tool in the diagnosis of food anaphylaxis. Therefore, allergen microarray tests, immunoblots and Enzyme-linked Immunosorbent Assays (ELISAs) were done and compared to case histories obtained by conventional diagnostic methods consisting of extensive in vitro and in vivo testing which is time-consuming and expensive. The goal for the future will be to make the testing more patient-friendly and more economic. The microarray is a good candidate to facilitate the diagnosis of food anaphylaxis. However, to establish the microarray as an important component in testing for anaphylaxis, the sensitivity of the method needs to be increased as there were several eliciting allergens that were not detected by the chip.

2 LIST OF ABBREVIATIONS

AID	Activation-induced deaminase
AKP	Alkaline phosphatase
APC	Antigen-presenting cell
APT	Atopy patch test
BP	Birch pollen
BSA	Bovine serum albumin
CCD	Cross-reactive carbohydrate determinant
cDC	classic dendritic cell
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CRD	Component-resolved diagnostics
CS	Coverslip
CTL	Cytotoxic T lymphocyte
DBPCFC	Double-blind placebo-controlled food challenge
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
FA	Food allergy
FCS	Fetal calf serum
FD	Fluorescein isothiocyanate-coupled dextran
FDEIA	Food-dependent exercise-induced anaphylaxis
FITC-Tf	Fluorescein isothiocyanate-coupled transferrin
GB	Gold buffer
GM-CSF	Granulocyte macrophage colony-stimulating factor
IgE	Immunoglobulin E
IL	Interleukin
ISAC	Immuno solid-phase allergen chip
ISU	ISAC standardized unit
ITAM	Immunoreceptor tyrosine-based activation motif
MHCI/II	Major histocompatibility complex I/II
MIA	Microarray image analysis
NC	Nitrocellulose
NHS	Normal human serum

NK	Natural killer cell
nsLTP	non-specific lipid transfer protein
O/N	Over night
OFC	Oral food challenge
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
pDC	plasmacytoid dendritic cell
PR-10	Pathogenesis-related protein family 10
PRR	Pattern recognition receptor
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIP	Standard isotonic percoll
SPT	Skin prick test
TBST	Tris buffered saline with Tween-20
Th1/2	T helper cell type 1/2
TLR	Toll-like receptor

3 INTRODUCTION

3.1 The allergic reaction

The European Academy of Allergology and Clinical Immunology (EAACI) defined allergy as a *hypersensitivity reaction initiated by immunologic mechanisms* [1]. According to the Gell and Coombs classification, allergic reactions can be grouped into 4 different classes [2].

Type I allergic reactions are immediate-type reactions mediated by cross-linked IgE antibodies on the surface of mast cells and basophils resulting in mediator release. This induces the local or systemic allergic reactions that affect respiratory tracts, the gastrointestinal tract, skin, or blood circulation. Mostly the symptoms occur within minutes after the contact, but rarely late-onset reactions are reported [2-4].

Type II antibody-cytotoxic reactions are mainly IgG- or IgM-mediated. Symptoms are caused either by antibody-dependent activation of macrophages, eosinophils and neutrophils, or by activation of complement-mediated cell lysis. While the onset of the reaction after exposure is shortly delayed, clinical symptoms become apparent only days later [2-4].

Type III reactions are immune complex-mediated. Soluble IgG and IgM antibodies bind antigen and precipitate in vessels or tissues, thus causing complement activation and subsequent recruitment of inflammatory cells. Clinical manifestations such as serum sickness develop 9 – 11 days after contact [2-4].

Type IV reactions, or delayed-type hypersensitivity, are predominantly T cell-mediated. Contact of the allergen with sensitized cytotoxic T lymphocytes (CTL) or T-helper cells leads to the activation of T cells and subsequent tissue damage or inflammation. Symptoms occur within 2 – 14 days [2-4].

In this work the focus will be placed on IgE-mediated immediate-type allergic reactions.
Due to various factors that are not very well studied but seem to comprise environmental influences like infections as well as genetic predisposition, sensitization occurs in previously non-allergic individuals. In this process, the equilibrium between the actions of regulatory T cells (Treg) and the induction of T helper cells type 2 (Th2)-mediated responses is erroneous. In healthy individuals, Tregs are responsible for the induction of tolerance to harmless pro-

teins that are in frequent contact with the body under non-inflammatory steady-state conditions, especially in the gastrointestinal tract and in the respiratory system in addition to physical barriers like the mucosa or epithelial cells [5].

3.2 Type I allergic reactions: The sensitization phase

After the first contact with the respective allergen, professional antigen-presenting cells (APCs) internalize and digest it, generating small peptides that can be presented by major histocompatibility complex type II (MHC II) to the receptor of naïve CD4+ T cells. Additional interleukin-4 (IL-4) secretion of the APC drives differentiation of the T cell towards the Th2 and subsequent clonal expansion. Interaction between Th2 and naïve B cells as well as IL-4 and IL-13 secretion by the Th2 cells then leads to class-switch of the B cell resulting in the production of allergen-specific antibodies of the IgE type. Additionally, IgE+ memory B cells specific for the sensitizing allergen are produced [6].

3.2.1 Dendritic cells

In 1868, Paul Langerhans discovered a novel cell type that he thought was some sort of nerve cell [7]. However, the term ***dendritic cell*** was first mentioned only in 1973 when Steinman and Cohn discovered a heterogenous, stellate cell type among the splenic cells they were investigating [8].

Today it is known that DCs form an important link between the innate and the adaptive immunity. They comprise various subtypes that can be classified according to their developmental stage, their life history, the host state, or the DC activation status [9, 10].

They are important professional APCs that are able to take up antigens, to process them, and to present them to T cells [11]. The subsequent activation of antigen-specific T cells is an important trigger for the adaptive immune response.

3.2.1.1 Dendritic cell development

DCs derive from $CD34^+$ hematopoietic progenitor cells in the bone marrow [12, 13] branching off afterwards into many various subtypes which can be seen in Figure 1. In a first decision, multipotent progenitors commit either to the myeloid or to the lymphoid lineage and develop into common myeloid (CMP) or common lymphoid progenitors (CLP). Both lineages can give rise to plasmacytoid DCs (pDCs) if they express the receptor for the cytokine Flt3

ligand (Flt3L) on their surface, Flt3 [11, 14-16]. These cells form a DC subtype that is involved in viral immunity [17, 18] and maintenance of tolerance [18, 19].
Both CMPs and CLPs can also give rise to conventional DCs (cDCs). In addition, CLPs bear the potential to develop into B cells, T cells, or natural killer (NK) cells while CMPs can differentiate to megakaryocytes, erythrocytes, granulocytes, or monocytes. The latter, in turn, act as progenitors for macrophages and cDCs (Figure 1).

DCs that reside in the periphery have been shown to penetrate the tight junctions of gut epithelia in order to sample antigens, just like M cells [20] indicating their role in food allergy.

In immature DCs, MHCII molecules are located within the lysosomal compartments of the cells rather than on their surfaces ready for the delivery of antigens to their intracellular compartment by excessive endocytosis. After encountering pathogenic structures, Toll-like receptor (TLR) signalling leads to a short phase of enhanced endocytosis [21] followed by a dramatic physiologic alteration including a massive down-regulation of the endocytic activity of the DC and maturation. These events prepare the DCs for their new task, to present digested peptides via MHCII molecules, that are now located on the surface, to T cell receptors and thus, to induce T cell maturation [22, 23].

Additionally to their long known immunogenic function, DCs are also able to induce tolerance [24, 25].

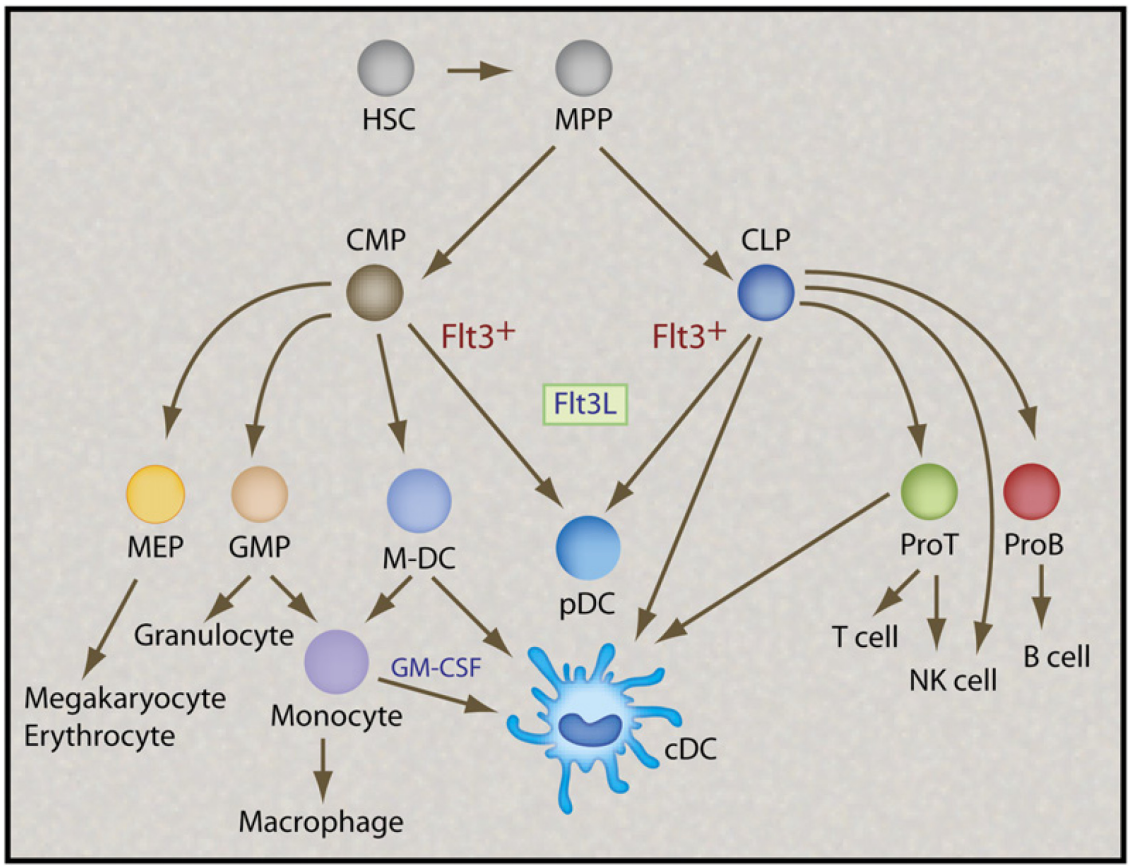

Figure 1 Schematic development of murine DCs, taken from Wu et al., 2007 [14].

3.2.1.2 Dendritic cell subsets

The classification of DCs can be based on various aspects one of which is their developmental stage (pre-DCs or developed DCs). Pre-DCs will differentiate into DCs upon stimulation by signals specific for infection or inflammation or also by default [11].

Another criterion for the classification of DCs is their life history (migratory, resident, or circulating DCs). **Migratory DCs** capture pathogen or self-antigen in the periphery and consequently migrate through the lymphatic vessels towards the draining lymph node [26]. At the same time the DCs already undergo maturational steps [9-11].

In contrast, **resident DCs** stay in lymphoid tissues in an MHCII immature state. They can be divided into $CD8^+$ cells that are located in T cell areas of spleen and lymph nodes and $CD8^-$ DCs that can be found in marginal areas [9, 11].

As a third class, **circulating DCs** are found in the blood circulation in low numbers [9].

As another DC type follicular DCs (FDCs) were described. They differ in some major characteristics from pDCs and cDCs as they reside in B cell follicles of secondary lymphoid organs and are responsible for the maintenance of immunological memory and are even discussed to be able to stimulate B cells without the necessity of Th cells [27, 28].

3.2.1.3 Endocytosis

Endocytosis is performed by virtually every cell type in order to take up molecules that cannot cross the plasma membrane because of their size or polarity. However, there are various ways of endocytosis for particles of various sizes. Basically, there are four major types of endocytosis: phagocytosis, macropinocytosis, and clathrin- and caveolae-mediated endocytosis.

Phagocytosis is performed by professional phagocytic cells, such as DCs. The term describes the uptake of large particles or even entire cells in a receptor- and actin-mediated manner [23, 29]. The involved receptors comprise Fc receptors, complement receptors, scavenger receptors, integrins, lectins, and toll-like receptors (TLRs) which are activated by different ligands and activate different signal pathways. Thus, the first stage of endocytosis, the receptor binding, already has a great impact on the subsequent development of the cell, see also Figure 2 [30]. Receptor binding leads to actin-mediated engulfment of the particle. Involved receptors include pattern recognition receptors (PRRs) like C-type lectin receptors, NOD-like receptors, RIG-I-like receptors and TLRs [31] as well as complement receptors, and Fc receptors loaded with adequate antibodies [7, 23]. All of these receptors often have various functions besides the induction of antigen presentation, see Figure 2.

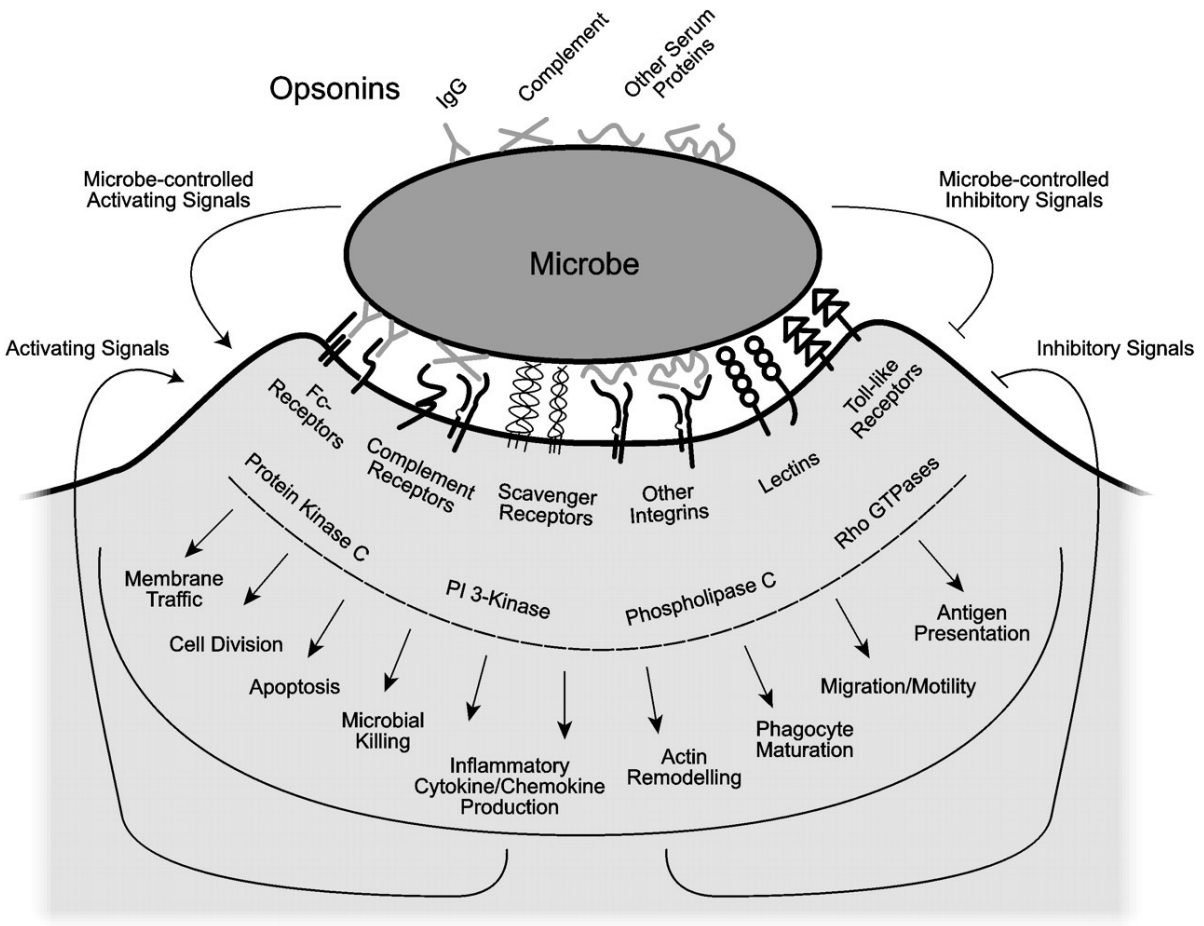

Figure 2 Different signal transduction pathways in phagocytosis upon receptor binding by antigens, taken from Underhill et al., 2002 [30].

The patterns that are recognized by PRRs comprise lipids, lipoproteins, proteins, and nucleic acids that are present on various pathogens [32]. One of the best studied allergen concerning its sensitizing potential is Der p 2. It is a major allergen from the house dust mite that was shown to display great functional homology with a lipid-binding component of the TLR 4 complex [33, 34]. Thus, it is presumed to act as an adjuvant in the activation of the DC with a possible lipopolysaccharide (LPS) cargo attached to the allergen enhancing the activating effect.

Glycoproteins act via a more indirect route with their carbohydrate moieties engaging C-type lectin receptors on the surface of DCs. In particular, fucosylated glucans that occur naturally on microbes but not on mammalian cells [31]. The mannose receptor from the C-type lectin family has been shown to mediate the uptake of major allergens like Der p 1 and Der p 2 from house dust mite, Can f 1 from dog dander, Bla g 2 from cockroach, and Ara h 1 from peanut binding to their carbohydrate residues [35].

Macropinocytosis (*cell drinking*) also is an actin-dependent mechanism that is constitutive in immature DCs and macrophages and is down-regulated in mature DCs [23, 36]. During the process of macropinocytosis, large amounts of extracellular fluids are internalized including dissolved antigens.

Alternative forms of endocytosis are ***clathrin-mediated endocytosis*** and uptake by ***caveolin-containing invaginations (caveolae)*** of soluble receptor-antigen complexes [23, 29]. However, until now there is no indication that these mechanisms play a major role in DCs. Internalized proteins then reach endosomal or lysosomal compartments where they are processed by resident enzymes.

3.2.1.4 Processing and antigen presentation

While MHCI is responsible for the presentation of mostly endogenous proteins in virtually all cell types, MHCII presentation is restricted to professional APCs that take up and process exogenous protein before loading small peptides to MHCII receptors and presenting them to a T cell.
Processing of exogenous antigen happens in endosomes and lysosomes that contain various degrading enzymes e.g. endo- and exopeptidases, reductases and esterases [37]. Since phagosomes of DCs provide rather mild conditions (neutral or alkaline pH) compared to the highly acidic phagosomes of macrophages, the antigens are not degraded completely but can be loaded onto MHCII molecules as peptides of approximately 10 amino acid residues [37]. Upon binding, further processing takes place and the final peptides of 10 – 20 amino acids are presented on the surface to CD4+ T cells. These fragments are big enough for the TCR to recognize antigenic epitopes.

In contrast, endogenous antigen, such as a viral protein, is recognized in the Golgi apparatus and transported into the cytosol where it is degraded by the proteasome complex. Peptides of 8 – 9 amino acids are transported back into the Endoplasmic Reticulum, loaded onto MHCI, and transported to the surface where it is presented to CD8+ cytotoxic T cells [37].

Beside these classic routes of antigen presentation, there is an alternative third way of antigen presentation. Exogenous antigen taken up by the DC can be degraded to large fragments in

phagosomes and then be released to the cytosol. The fragments then follow the classic MHCI pathway and are presented to CD8+ T cells [37, 38]. Thus, the immune system developed a way to kill bacteria or tumor cells, for example.

3.2.2 Immunoglobulin E

Immunoglobulin E is one of the five known antibody classes besides IgG, IgM, IgA, and IgD. A first idea of its existence was observed in 1919 when the recipient of a blood transfusion developed an allergic reaction. In 1921, a passive transfer of a positive skin test was carried out leading to further insights into the development of allergies. However, the term *atopy* was introduced in 1923 by Coca and Cooke describing an *inherited human hyperresponsiveness*. They also hypothesized on the existence of certain *reagins* which they linked to the development of atopic hyperresponsiveness [39].
Half a century passed by until IgE was finally described as a new immunoglobulin class. In fact, the discovery was made by two different research groups via two different approaches.

The group of Kimishige and Teruko Ishizaka from Denver stumbled on IgE mistaking it for an IgA antibody. Later on, they found out they had discovered a novel class of antibody by serum precipitation. They called it γE-globulin, although they were not able to isolate it.

The Bennich and Johansson group from Uppsala found out about the existence of IgND, as they called it, at around the same time using a different approach. They examined the serum of a myeloma patient and found a component they could not identify. Further research led them to the conclusion that they were dealing with a new immunoglobulin isotype. After the finding that this antibody could be linked to allergic asthma they tested their antibody in combination with the antiserum to γE the Denver group had found resulting in blockage of the antiserum by the Uppsala-antibody indicating that both antibodies shared biological activity as well as antigenic determinants [39]. In 1968, IgE was officially declared to be a novel immunoglobulin class [40].

Nowadays it is known that IgE is present in the serum at the lowest concentration compared to all the other known immunoglobulin classes [41]. This circumstance can be attributed partly to the low half–life of free IgE which is only 5 to 10 days. However, once the antibody is bound to its high affinity receptor, FcεRI, its half-life increases dramatically [39].

Like all antibodies, IgE is produced by B cells and either expressed on the cell surface or secreted into circulation where it forms the humoral branch of the immune system.
Two signals inducing isotype switching are a prerequisite for the expression of IgE: Interleukin (IL) 4 or IL-13 provides the first signal binding to its respective receptor leading to transcription of the S□ switch region. For signal 2, CD40 ligand (CD40L) on T cells binds to CD40 on B cells activating DNA switch recombination that, in combination with activation-induced deaminase (AID), is involved in the class switch and somatic hypermutation. Both signals are produced by T cells [41].

3.2.3 IgE receptors

IgE exerts its function upon binding to receptors that are specific for their Fc domain which is constant for any antibody of the E class no matter what antigen is recognized by the variable epitope-binding domain. To date, two distinct types of IgE receptors are known. One of them is the high-affinity receptor **FcεRI** which is located on basophils and mast cells as well as on antigen-presenting cells [41]. It can be made up of one α, one β and two γ domains forming a tetrameric receptor that is expressed on mast cells and basophils [41, 42]. Trimeric receptors lacking the β subunit are found predominantly on APCs and eosinophils inducing endocytosis upon receptor cross-linking [42]. The half-life of unbound FcεRI on the cell surface was shown to be significantly shorter in comparison to elongated presence in case of FcεRI-IgE complexes. In absence of IgE, FcεRI is removed from the cell surface more rapidly by endocytosis [43, 44]. The receptors then either escape the endosome by recycling to the membrane, a process in which IL-3 is likely to play an important role, or they undergo lysosomal degradation [45, 46]. If an allergen binds to two sIgE-FcεRI complexes on the surface of an effector cell, immunoreceptor tyrosine-based activation motif (ITAM)-dependent cell signalling takes place leading to mediator release from the cell and thus, elicits an immediate allergic reaction is triggered.

In addition to the high-affinity receptor FcεRI there is also the so-called low-affinity receptor **FcεRII**, or **CD23**. It is a type 2 transmembrane protein of about 45 kDa with C lectin-like features [47]. It is a monomeric protein that is expressed by B and T cells as well as by monocytic cells, among others [48-51]. As indicated by the term *low-affinity receptor,* CD23 displays a much lower affinity to IgE. Another fundamental difference to the high-affinity recep-

tor is its function upon activation. While the FcεRI pathway leads to mediator release, IgE binding to CD23 causes a halt of IgE production by B cells. Like FcεRI, CD23 can also be cleaved from the cell surface which turns it into a soluble receptor that exerts cytokine-like functions [48].

3.3 Type I allergic reactions: The effector phase

Upon secondary exposure to the allergen source, the immune system of the sensitized individual is already prepared for a new encounter with the allergen. Allergen-specific IgE (sIgE) antibodies are secreted to the circulation by corresponding activated B cells and consequently bind via their constant Fc region to respective high-affinity receptors (FcεRI) on the surface of mast cells and basophils. Cross-linking of adjacent receptors by a multivalent allergen triggers a signal cascade in the mast cell resulting in degranulation. During this process pre-formed mediators are released within seconds to minutes after allergen contact. Mediators comprise vasoactive amines, lipid mediators, chemokines, and cytokines [41, 52, 53].
However, in the late phase at a few hours to even days after the allergen contact, symptoms occur that are caused by the various cytokines secreted by Th2 cells or prostaglandins produced by mast cells.

3.3.1 Effector cells

The effector cells involved in the allergic reaction are mast cells, basophils and eosinophils. In sensitized individuals, allergen-specific IgE antibodies are bound to their corresponding FcεRI expressed on the surface of mast cells and basophils. The next exposure to the allergen leads to cross-linking of the IgE molecules and activation of the effector cells.
Mast cells can be found in vascularised tissues, especially in those that are in direct contact with the environment [54]. They derive from CD34+ hematopoietic stem cells and express IgG and complement receptors, TLRs and FcεRI [41]. They contain cytoplasmic granules bearing pre-formed mediators like heparin, histamine and proteases that, upon activation, are released and cause tissue remodelling in order to facilitate the recruitment of immune cells to the site of pathogenic infections [55]. Once activated, mast cells are also able to newly synthesize lipid mediators.

Basophils also express FcεRI and secrete Th2 cytokines as well as histamine from intracellular granules. In contrast to mast cells, they are mainly circulating cells and play a role in the late phase of the allergic reaction. They synthesize large amounts of the cytokines IL-4 and IL-13 which suggests that they have an important role in the amplification of the production of IgE [41].

Eosinophils predominantly reside in the bone marrow and the gastrointestinal tract. Their major role of is thought to be the secretion of cytotoxic granule proteins in order to fight parasitic infections. They are also able to activate mast cells, T cells and epithelial cells [41, 56].

3.3.2 Symptoms

Allergic diseases are dependent on many environmental, genetic and individual factors which makes them a complex disease with diverse manifestations. Symptoms can occur within minutes to several hours.

Food allergies predominantly affect the main site of contact, the gastrointestinal tract starting with the rather mild manifestation of the oral allergy syndrome (OAS). The symptoms of mild edema and pruritus are confined to the oral cavity and are linked to the sensitization to pollen allergens that causes cross-reactivity to a homologous food allergen. The responsible allergens are mainly labile proteins that are degraded in the hostile environment of the stomach [57, 58].

Other symptoms are urticaria or angioedema upon ingestion or direct skin contact, atopic dermatitis and eosinophilic gastroenteritis [57, 58].

In contrast, allergic rhinitis occurs upon contact to airborne allergens like pollens or animal dander. The histamine release from mast cells causes the major symptoms of rhinitis like sneezing, rhinorrhea, nasal pruritus and congestion of the nose [59].

Allergic asthma is a chronic inflammatory airway disease involving shortness of breath, chest tightness, a rapid heart rate and wheezing [60].

The most severe manifestation of an allergic reaction is an anaphylactic episode. Anaphylaxis was defined by the EAACI as a severe, life-threatening, generalized or systemic hypersensitivity reaction [1].

The estimated lifetime prevalence of anaphylaxis is 0.05 – 2% with a high probability of being underdiagnosed [61]. This can occur when experiencing the first anaphylactic episode,

due to a hidden trigger, or due to mild or transient symptoms. Besides airborne allergens, drugs and insect venoms, food allergens are an important elicitor of anaphylactic episodes. Once they are confirmed as a trigger, food allergens are comparatively easy to avoid. Hence, the identification of the triggering allergen is of utmost importance in anaphylactic patients. However, diagnostic tests like provocation tests bear a great risk of inducing another anaphylactic episode and, thus, have to be carried out in appropriately equipped health care facilities and by specially trained professionals [62].

A special form of anaphylaxis is exercise-induced anaphylaxis (EIA). Here, patients experience an anaphylactic episode after physical exercise, often in combination with certain foods or other co-factors. A very common form of food-dependent exercise-induced anaphylaxis (FDEIA) is elicited by the consumption of wheat products and subsequent exercise.
The underlying principle could be the increased permeability of the gastrointestinal tract caused by exercise and/or drugs like, for example, aspirin. A study by Matsuo and co-workers [63] confirms this hypothesis showing an increased level of gliadin in six patients suffering from wheat-dependent exercise-induced anaphylaxis as well as in a healthy control group. The authors suggest that increased gastrointestinal permeability caused by exercise and/or aspirin results in enhanced absorption of allergens and, thus, could be the reason why in some individuals anaphylaxis occurs only in combination with these co-factors.
Another cause of FDEIA could be the exercise-induced activation of tissue transglutaminase in the intestine which, in combination with the gastric proteases pepsin and trypsin, leads to the formation of complexes of ω-5 gliadin. These complexes increase IgE binding in vitro and in vivo [64].

3.4 Diagnosis of food allergy

The diagnosis of food allergy is established by a combination of clinical history, physical examination, and laboratory studies.
According to the expert panel of the National Institute of Allergy and Infectious Diseases of the US, the methods to identify the eliciting food comprise *in vitro* determination of circulating allergen-specific Immunoglobulin E (IgE), *in vivo* skin prick tests (SPT), and oral food challenges (OFC) as well as atopy patch test (APT) in the case of contact food allergy [65].

3.4.1 Determination of specific IgE antibodies

To date, there are several assays to measure sIgE one of which is the CAP system fluorescent enzyme immunoassay (CAP-FEIA). This method evolved from the total serum IgE radioimmunosorbent test (RAST), a first-generation IgE antibody assay. The underlying mechanism is the covalent binding of the suspected allergen to a hydrophilic carrier polymer and the subsequent exposition to the serum of a patient. Bound sIgE can be detected by a labelled antibody.

3.4.2 Skin prick tests

The SPT is a useful test to screen for the presence of sIgE in vivo and thus, for the determination of the sensitization status of the patient if there are any suspected allergies. An allergen extract of the allergic reaction is applied intracutaneously to the skin of the upper forearm or the back by a metal lancet. Histamine dihydrochloride serves as a positive control [66] while a saline solution is applied as a negative control [67]. If there are mast cells loaded with IgE specific to a protein from the tested allergen extracts the respective allergen crosslinks the sIgE releasing a signal for the mast cells to perform mast cell degranulation. This causes a typical *wheal and flare* reaction within minutes after the injection on pricked skin. However, although the test has good sensitivity it shows a high frequency of false-positive outcomes [68-70].

3.4.3 Food challenges

Oral food challenges are utilized for both IgE- and non-IgE-mediated allergies [68] and can be single-blinded, double-blinded, or open. The double-blind placebo-controlled food challenge (DBPCFC) is considered the gold standard in the diagnosis of food allergy, but since it is time-consuming and expensive, open or single-blinded challenges are often preferred.

3.5 Allergen families and cross-reactivity

Many species share highly conserved proteins that display, to a certain degree, amino acid sequence similarity and shared conformation. These similarities can lead to cross-reactivities

if an individual gets sensitized to a protein from one source and is afterwards exposed to a homologous protein from a different source. In this case an allergic reaction can take place without previous sensitization to the second allergen.

An important group of plant-derived allergens are ***seed storage proteins***. They comprise proteins from the prolamin and cupin superfamilies [71]. Cupins can be further divided into vicilins, that are trimeric 7S globulins, and the hexameric 11S legumins [72]. 2S albumins belong to the prolamin superfamily.

Proteins that frequently give rise to allergenic cross-reactivity reactions are, for example, ***non-specific lipid transfer proteins (nsLTPs)***. These proteins, as indicated by the name, are lipid-binding proteins and belong to the prolamin superfamily [72]. They are very stable proteins of 7 to 9 kDa that can be found in a variety of fruits, vegetables, nuts, and seeds [72]. Their resistance to proteolysis and heat makes them full allergens with the potential to sensitize by ingestion [71].

In addition to nsLTPs, ***2S Albumins*** are another subgroup of the prolamin superfamily.
Proteins from this group are composed of a large chain of 9 kDa and a small chain of 3 kDa that are linked by disulphide bonds [72]. An immature form of the protein in an earlier stage of development was showed to have a molecular mass of 18 kDa as well as an intermediate protein of 15 kDa still bearing an ER secretion signal [73]. Upon SDS-PAGE analysis the subunits are separated and studies showed that the IgE-binding epitope resides on the large chain bearing a helix-loop-helix region [74].
Important allergenic representants of the 2S albumin group are Jug r 1 from walnut, Ses i 2 from sesame, Ara h 2, 6, and 7 from peanut, and Ber e 1 from Brazil nut. In contrast, the 2S Albumin from sunflower, SFA8, does not seem to be of clinical relevance. Thus, in this work we will compare Ber e 1, which is a major allergen, to SFA8.

What is known about the allergenicity of Ber e 1 is that the 9 kDa chain could be shown to be responsible for the IgE binding in 100% of a patients' collective of 11 individuals who had reported anaphylactic reactions [75].

In comparative experiments carried out by Kean and co-workers, the effects caused by exposition of dendritic cells to the 2S albumins from Brazil nut, Ber e 1, and sunflower seed,

SFA8, were assessed [76]. Despite their high structural similarity the two proteins caused crucial differences in terms of cytokine production and cell surface phenotype.
While incubation of DCs with the well known allergen Ber e 1 resulted in IL-4 and IL-5 production which was lacking in incubation with SFA8, whose allergenic capacity is uncertain. Conversely, only SFA8 was able to induce production of IL-12 and TNF-α. Based on these findings the different roles of the two homologous proteins in the development of allergic reactions were confirmed and could be linked to the induction of either Th1 response in the case of SFA8, or Th2 response in the case of Ber e 1 with the latter being a prerequisite for the development of an allergic reaction.

The high allergenic potential of Ber e 1 is partly explained by the finding that it is the only protein from Brazil nut that is rather resistant to acidic pH and pepsin digestion so that it is possible to reach the gastrointestinal tract as an intact protein and can be taken up by DCs [77]. However, experiments in mice revealed that intraperitoneal injections of Ber e 1 induced sIgE production if injected in combination with lipid fractions from Brazil nut [78].

Profilins are ubiquitously expressed in eukaryotic cells and, thus, are also an important candidate for allergenic reactions due to cross-reactivity. They are proteins of 12 – 15 kDa containing highly conserved sequences that are labile to digestion by pepsin which is an enzyme found in the gastrointestinal tract. Thus, the route of sensitization is supposedly restricted to the airway route. Despite their wide cross-reactivity profilins have a rather low clinical relevance giving rise mostly to oral allergy syndrome (OAS) [72, 79].

The members of the ***pathogenesis-related protein family 10 (PR-10)***, in contrast, are not as highly conserved and ubiquitous as profilins [80]. They can be found in pollen from trees of the Fagales order and in plant foods from the Rosaceae, Apiaceae, and Fabaceae [81]. However, they seem to have a greater potency to elicit clinical symptoms [79]. PR-10 molecules, which are of about 17 kDa, are labile to pepsin digestion indicating that the primary route of sensitization is by inhalation [82]. Nonetheless, severe allergic reactions could be ascribed to PR-10 proteins [83].
An important representative of the PR-10 family is the major birch pollen allergen, Bet v 1, with particular relevance in Central and Northern Europe as it is considered the primary sensitizing allergen in the birch-plant food syndrome. Patients sensitized to Bet v 1 often develop

oral symptoms upon the ingestion of plant food containing Bet v 1-related protein. These symptoms occur immediately after contact with the concerning food and are mostly mild and restricted to the oropharynx [84]. However, there were also cases of severe allergic reactions caused by PR-10 proteins.

Today, a variety of allergy syndromes on the basis of allergic cross-reactivity are known. A frequently observed example is the pollen-plant food syndrome [71, 85-87]. This so-called type 2 food allergy results from IgE cross-reactivity between homologous proteins from pollen and plant food.

Other proteins that cause allergic cross-reactivities are, for example, parvalbumins causing allergic reactions upon the ingestion of various types of fish [71, 88]. Similarly, tropomyosins are responsible for cross-reactivities caused by shellfish [89, 90].
In nuts, several proteins are known to be able to cause allergenic cross-reactivity. In particular, these cross-sensitizing proteins can be the non-specific lipid transfer proteins (nsLTPs), storage proteins, profilins, oleosins, or proteins from the pathogenesis-related (PR-10) family [71].

Apart from proteins, also carbohydrate structures can be a source of cross-reactivity. These structures are called ***cross-reactive carbohydrate determinants (CCDs).*** The actual clinical importance of CCDs is subject of debate as there is not much evidence that they actually elicit symptoms in patients. To the contrary, they are often responsible for false positive skin test results [91]. In the case of the CCD-bearing protein bromelain, for example, the low clinical relevance is due to only one single carbohydrate moiety making it unable to cross-link IgE on the surface of mast cells [92, 93]. However, there were also reports assigning a greater role to CCDs describing cases of anaphylaxis due to the administration of a glycosylated antibody that is recognized by cross-reactive sIgE [94].

4 MATERIAL AND METHODS

4.1 Material

Chemicals	Suppliers
4-Nitro blue tetrazodium chloride (NBT) 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) Coomassie Brilliant Blue R-250 (CBB) Dithiothreitol (DTT) Glycine Tris(hydroxymethyl)aminomethane, Ultra Pure	Biomol GmbH, Hamburg, Germany
Blaubrand counting chamber 0.1 mm	Brand, Wertheim, Germany
Bovine serum albumin (BSA) Glycerol ≥ 99.5%, p.a. waterfree Microscope slides Milk powder N,N,N,N-tetramethylethylenediamine (TEMED) Sodium dodecyl sulfate HCl	Carl Roth GmbH + CO. KG, Karlsruhe, Germany
PageRuler Prestained Protein Ladder	Fermentas, St. Leon-Roth, Germany
Ammoniumperoxidisulfate (APS) Dulbecco's Phosphate Buffered Saline (DPBS) Trypan Blue stain 0.4%	Gibco BRL, MD,USA
Iscove's Modified Dulbecco's Media (IMDM) Hank's Buffered Salt Solution (HBSS)	Lonza, Verviers, Belgium
Acetic Acid Acrylamide Bis-acrylamide Bromphenol blue Ethanol Isopropanol KCl KH_2PO_4 Methanol $MgCl_2 \cdot 6H_2O$ NaCl $NaHCO_3$ $Na_2HPO_4 \cdot 2H_2O$ $NaH_2PO_4 \cdot H_2O$ NaN_3 NaOH	Merck, KGA, Darmstadt, Germany
Transferrin from human serum, Alexa Fluor® 488 conjugate	Molecular Probes (Invitrogen), OR, USA
Percoll-Paque PLUS Ficoll	GE Healthcare
ImmunoCAP ISAC Assay Kit IgE	Thermo Fisher Scientific, Vienna
4' 6-Diamidine-2'-phenylindole di-hydrochloride (DAPI)	Roche, Mannheim, Germany
Tween 20 Fluorescein isothiocyanate-Dextran (FITC-Dextran)	Sigma-Aldrich Co., St.Louis, MO, USA
Fluoromount-G	SouthernBiotech, Birmingham, AL, USA
Cover glasses	ThermoScientific, Braunschweig, Germany

4.1.1 Blood samples from healthy donors

The blood samples used for culturing of PBMCs and the generation of DCs were taken from 6 healthy individuals without any known allergy.

4.1.2 Serum samples from anaphylactic patients

Sera used for microarray analysis

For the chip analysis sera from 55 food anaphylactic patients (mean age 42.7 years; range 21-68) were obtained from the Anaphylaxie Register of the Charité Hospital in Berlin. For these sera conventional diagnosis had been carried out in order to identify the eliciting allergen source. For all 55 patients total IgE levels had been determined (mean 310.8 kU/l, range ~2 to >5000 kU/l), 54 patients had undergone skin prick testing (SPT), 52 had been tested for sIgE, and for 29 supervised provocation tests had been performed.

Sera from soy allergic patients

The sera of 20 soy allergic patients (10 male, 10 female ranging from 10-69 years, median age 36) were kindly provided by R. Lang, Paracelsus Medical University Salzburg. They were used for microarray analysis, ELISA and Western blotting. The patients were tested before for a soy drink and a birch pollen extract by skin prick testing resulting in 18 positive outcomes for soy drink and 19 for birch pollen (BP) extract. RAST testing using soybean extract resulted negative in 17 patients while 18 presented sIgE to the soy allergen Gly m 4, 18 had a positive RAST outcome for BP extract and 12 for Bet v 1.

Fifteen patients had experienced a generalized allergic episode while 5 presented mild symptoms.

All blood and serum samples were obtained according to ethical committee approvals and after signing written consent. Non-allergic subjects signed consent before donating blood samples.

Purified allergens

The allergens used for IgE ELISAs were purified in the lab according to established protocols. Gly m 4 was kindly provided by Prof. Stefan Vieths from the Paul Ehrlich Insitute Langen, Germany, and Gly m 5 and Gly m 6 were provided by Prof. Clare Mills, University of Manchester, UK, deriving from the Europrevall Allergen Library.

Ber e 1 and SFA-8 each labelled with Oregon Green © 488 and pHrodo™ Red were kindly provided by Assoc. Prof. M. Alcocer, University of Nottingham, UK.

4.2 Methods

4.2.1 Protein extract from soy flour

10x Phosphate Buffered Saline (PBS)
100 mM Na_2HPO_4 x 12 H_2O
1.36 M NaCl
27 mM KCl
18 mM KH_2PO_4

For the extraction of soy proteins, certified organic soy flour (SOBO Naturkost, Köln) from steam-heated soybeans was used. 30 g of soy flour were transferred to a beaker and filled up with 150 ml PBS. The beaker was covered and stirred o/n at 4°C. The next day the liquid was transferred to centrifuge tubes and centrifuged at 18000 rpm for 50. The supernatant was transferred to a dialysis membrane and dialyzed against H_2O.

4.2.2 Bicinchoninic Acid Protein Assay

Determination of protein concentration by the BCA assay uses the principle of the Biuret reaction, the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium. The protein forms a chelate complex with a copper ion which leads to its reduction. In its reduced form the copper ion can then form a chelate with BCA which results in a purple-coloured reaction. The amount of BCA-copper chelate increases in proportion to the amount of protein. The reaction can be monitored by photospectrometry at 562 nm. The standard curve to determine protein concentrations is established by BSA standards in a range from 0 – 250 µg/ml.

Procedure:
Preparation of standards was performed according to Table 1.

Standard	BSA (µl)	Buffer (µl)	Final conc.
A	10	70	250 µg/ml
B	8	72	200 µg/ml
C	12	148	150 µg/ml
D	8	152	100 µg/ml
E	4	156	50 µg/ml
0	0	160	0 µg/ml

Table 1 Preparation of standards for the BCA assay

25 µl of the (diluted) samples were pipetted into a 96-well plate (Greiner bio-one, Frickenhausen, Germany) in duplicates. 200 µl of BCA working solution (reagent A : reagent B = 50:1) were added to each well and the plate was incubated at 37°C for 30 min in the dark. OD_{562} was measured and a standard curve was drawn using the software *SoftMax Pro 4.8*. OD values of sample dilutions that were out of range were excluded when calculating the mean sample concentration.

4.2.3 Cell culture

For the generation of monocyte-derived DCs, monocytic cells are purified from peripheral blood by Ficoll and Percoll gradient centrifugation and monocytic adhesion. Such isolated monocytes can be driven to maturate to DCs by the addition of the cytokines GM-CSF and IL-4 within a week. Mature naïve DCs are incubated with labelled allergens in an appropriate concentration for an appropriate amount of time and depending on the temperature, uptake or binding on the cell surface can be monitored by fluorescence microscopy of fixed cells.

Reagents:

HBSS/2%FCS

200 ml HBSS + 4 ml FCS (inactivated)

IMDM/1%FCS

200 ml IMDM + 2 ml FCS + 500 µl antibiotic (0,86 g Gentamycin sulfate + 10 ml SW – make 500 µl aliquots)

IMDM/10%FCS

500 ml IMDM + 50 ml FCS + 500 µl antibiotic antibiotic (0,86 g Gentamycin sulfate + 10 ml SW – make 500 µl aliquots)

4% Paraformaldehyde Fixative

Add 4 g of Paraformaldehyde to 100 ml PBS

Heat to 65°C, stir while cooling

If necessary, small amounts of NaOH are added for better dissolving

Gravity filter through a fine pore filter

4.2.3.1 Isolation of peripheral blood monocytic cells (PBMCs)

For isolation ~ 80 ml of blood from healthy donor, not sensitized to nuts and seeds were obtained. Blood samples were collected in 10 heparinized 8 ml tubes, the blood tubes were decanted into a sterile cell culture flask (75 cm^2). Each tube was washed with 5 ml ice-cold HBSS and added to the flask. Ice-cold HBSS was filled up to 150 ml. 15 ml of Ficoll were added to each of 6 Falcon tubes and 25 ml of blood suspension were pipetted carefully on top of the Ficoll layer in each tube by holding the tube in a semi-horizontal position. Different cell types were then separated by centrifugation for 30 min at 1000xg at room temperature (RT) without brake. The interphase ring containing PBMCs was collected using a Pasteur pipette. The cell suspension obtained by this process was divided to 3 new tubes. HBSS/2%FCS was added to 40 ml and the suspension was centrifuged again (10 min, 800xg, RT). The supernatants were discarded and 10 ml HBSS/2%FCS were used to resuspend the cells of one tube. This suspension was transferred to the next 2 tubes repeating the resuspension process. After 2 washing steps in which remaining cells were also transferred to the last tube the PBMC suspension was concentrated in 30 ml HBSS/2%FCS.

4.2.3.2 Isolation of monocytes from PBMCs

The 30 ml PBMC suspension was centrifuged (10 min, 600xg, RT), the supernatant was discarded and the cells were washed by resuspension in 30 – 40 ml IMDM/1%FCS and centrifugation (10 min, 600xg, RT). The supernatant was discarded again and the cells were dissolved in 8 ml Standard Isotonic Percoll (SIP) 60% (SIP: 1 part 10xPBS + 9 parts Percoll; SIP 60%: 6 ml SIP + 4 ml IMDM/1% FCS). The suspension was mixed and 2 ml were transferred to

each of 4 tubes (10 ml). Then 4.5 ml of SIP 47.5% (9.5 ml SIP + 10.5 ml IMDM/1%FCS) and 2 ml of SIP 34% (3,4 ml SIP + 6,6 ml IMDM/1%FCS) were carefully pipetted in order to make layers of different densities. The tubes were centrifuged (45 min, 2100xg, RT, no brake) resulting in 2 interphases between the 3 gradient layers. The upper interphase contained monocytes whereas the lower one contained peripheral blood lymphocytes (PBLs). Monocytes were collected with a Pasteur pipette and transferred to a new tube. They were washed once using 30 – 40 ml IMDM/1%FCS, centrifuged (10 min, 600xg, RT) and after discarding the supernatant the monocyte cell pellet was resuspended in 10 ml IMDM/1%FCS.

4.2.3.3 Generation of DCs from PBMCs

The cell suspension was diluted 1:2 in TrypanBlue for staining of dead cells. The mixture was applied to a counting chamber and the number of cells was determined by counting under the light microscope. The cell suspension was centrifuged again (10 min, 600xg, RT) and the pellet was resuspended in the right amount of IMDM/1%FCS to obtain a concentration of $4x10^5$ cells/ml. The cells (1ml per well) were transferred to the desired amount of wells of a 24-well-plate, each containing 1 sterile coverslip (CS). The plate was incubated at 37°C for 45 min in order to let the monocytes attach to the surface. Other contaminating cells would still be floating and thus be washed away (wash 2x with pre-warmed IMDM/1%FCS). Then 1 ml/well of IMDM/10%FCS containing 500 U/ml GM-CSF and 250 U/ml IL-4 was added to the wells and the plate was incubated at 37°C for 3 – 4 days. Afterwards, the medium was changed by removing 500 µl of medium and replacing it with 500 µl of new medium containing double the concentration of cytokines. The plate was placed back in the incubator. After one week in total the majority of the monocytes had become naïve Dendritic cells.

4.2.3.4 Optimal conditions for the stimulation of DCs

In order to find out the optimal conditions for the stimulation of DCs, changes were made in incubation time, surface on which the cells grow, and allergen concentration.

Surface

The cells had to be grown on slides suitable for microscopy. It was known from previous experiments in our lab that DCs grow on plastic surfaces, but we tested if they also grew on CS.

Thus, cells were grown on both, glass CS and plastic slides and density was checked under a light microscope. Cells were also maturated to DCs, stimulated with fluorescence-labelled allergen, and analyzed under a fluorescence microscope in order to detect any qualitative differences between the two types of surfaces.

Concentration

In order to identify the optimal concentration of our OregonGreen- and pHrodo-labelled proteins for immunofluorescence analysis, different dilutions of FITC-Dextran (2 µg – 10 µg) were tested.

Medium

Cells are grown in medium containing 10% FCS which provides proteins that are necessary for their survival. However, some components of the FCS might inhibit the binding of the labelled allergens to receptors on the dendritic cells. Therefore we used serum-free medium for our experiments.

4.2.3.5 Stimulation of DCs with fluorescence labelled allergen

For stimulation, DCs were washed 2x with phosphate-buffered saline (PBS) and incubated with different concentrations of labelled allergen for various amounts of time (5 min-60 min) at 37°C. After incubation the cells were put on ice immediately in order to stop endocytosis.

4.2.3.6 Receptor binding on the surface of DCs by fluorescence labelled allergen

In order to investigate if endocytosis detected at incubation at 37°C is receptor-mediated, binding experiments were carried out. All steps were performed at 4°C to prevent endocytosis. If there was receptor-mediated endocytosis, proteins would bind to receptors on the cell surface and could be detected after washing steps.

DCs were washed 2x with PBS and incubated with serum-free IMDM at 37°C for 15 min. This step removed serum components that were possibly bound to the surface receptors and, thus, were impairing the binding of the labelled proteins.

Cells were washed with PBS again and put on ice for 30 min in order to stop endocytosis. Then labelled protein in appropriate concentration diluted in ice-cold serum-free IMDM was added and incubated for 60 min.

4.2.3.7 Preparation for microscopy

From then on all steps were performed under non-sterile conditions. Cells were washed 2x with ice-cold PBS, then fixated with 1 ml 4% paraformaldehyde in PBS (PFA/PBS) per well for 30 min. Then PBS (RT) was added to the wells and the plate was shaken for 5 min. The next washing step was done with PBS + DAPI (1 mg/ml, 1:10 000) and shaking for 10 min for nuclear staining. The solution was removed and the plate was washed 2x with PBS on the shaker for 5 min each time.

For mounting, a small drop of Fluoromount G was put on a glass slide, a CS was taken out of the well, rinsed shortly in MilliQ water and the liquid was removed with a paper towel. The CS was put on the drop of mounting medium with the cells facing down, taking care that there were no bubbles between the slide and the CS. The slides were dried o/n at RT.

4.2.3.8 Microscopy

Pictures were acquired using an epifluorescence microscope and TissueFAXS software. Squares of 10x10 field of views (FOVs) were selected for each CS and pictures were acquired in the required channels. The pictures were exported and multi-coloured images were created by merging.

4.2.3.9 Controls

In the first few experiments Dextran coupled to FITC (FITC-Dextran) was used as a positive control for endocytic uptake and to make first steps in the handling of DCs without wasting protein.

Since FITC-Dextran is not taken up into the cells by receptor-mediated endocytosis we then switched to FITC-coupled Transferrin (FITC-Tf) as a positive control that reportedly is taken up by DCs in a receptor-mediated manner [95].

4.2.4 Immunological methods

4.2.4.1 IgE Enzyme Linked Immunosorbent Assay

ELISA is a method to detect specific IgE to purified allergens or components in allergen extracts. If specific IgE to the examined allergen is present in the patient's serum, it binds to the corresponding epitopes on the protein that had been immobilized on an inert surface. After washing only these specific IgE antibodies remain bound and are detected by a secondary antibody coupled to an enzyme (Alkaline phosphatase, AKP). After adding a substrate solu-

tion a colour reaction occurs changing the optical density (OD) of the solution. This change can be measured in an ELISA Reader comparing the OD values of the investigated sera to a negative control. Therefore normal human sera (NHS) from 3 different non-allergic people are used for every protein or extract that is coated.

Reagents:

50 mM Sodium carbonate buffer

50 mM $NaHCO_3$

adjust pH to 9.6 with NaOH

10x Tris-buffered saline (TBS)

0.5 M Tris pure

1.5 M NaCl

0.5% NaN_3

adjust pH to 7.4 with HCl

1x TBS

Dilute 1:10 with H_2O

Procedure

100 µl of protein were coated onto microtiter plates (MaxiSorp Immuno Plate, Nalge Nunc International, Roskilde, Denmark) in a concentration of 1-10 µg/ml for purified allergens and 10 – 100 µg for protein extracts. The proteins were diluted in ELISA Coating Buffer (50 mM Sodium Phosphate Buffer, pH 9.6). After overnight incubation at 4°C, the plate was washed three times with Tris-buffered saline containing 0.5% Tween-20 (TBST) and potential free binding sites were blocked with 200 µl of TBST + 3% BSA for 2 hours at room temperature. The plate was washed three times with TBST and incubated overnight with serum (in duplicates; 100 µl per well; diluted 1:10 or 1:5 in TBST + 0.5% BSA) at 4°C. After washing the plate three times with TBST, 100 µl of anti-human IgE AKP (Alkaline phosphatase conjugated mouse anti-human IgE monoclonal antibody; 1:1000 in TBST + 0.5% BSA) were added and the plate was incubated at room temperature in the dark for 60 minutes. The wells were washed three times with TBST and 100 µl of AKP substrate solution (Sigma fast p-Nitrophenylphosphate Tablet Sets; dissolve one tablet set in 5 ml H_2O) were added. The plate was incubated in the dark and at 3 – 5 time points (10 – 180 min) the absorbance was meas-

ured at 405 nm. The mean values of controls plus 3x standard deviation were regarded as baseline. Values above them were defined as positive.

4.2.4.2 Inhibition IgE ELISA

This method is used to assess cross-reactivity between 2 purified allergens or protein extracts. ELISA was performed as described above, the serum of interest without any added inhibitor served as a negative control. In order to confirm the specificity of the inhibition another assay using an unspecific inhibitor that had not shown any cross-reactions to the observed protein was made. On day 1, diluted sera were incubated with the inhibitor (20 – 100 µg/ml) in order to block IgE that was specific to the proteins coated on the plate. After washing and blocking the plate the sera containing the inhibitor were applied to the wells and all following steps were executed as described above. The obtained OD_{405} values of the uninhibited serum were considered as 0% inhibition and based on these values percentage of inhibition caused by the inhibiting agent was calculated.

4.2.4.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

This procedure is used to separate proteins in a mixture according to their molecular weight (MW) or to determine the MW of a specific protein of interest. Therefore it is necessary to linearize the proteins because different foldings influence their migration speed. Additionally, electric charges would also inhibit a strictly size-based migration pattern of the proteins. Thus, SDS is added to the protein denaturing it and adding negative charges proportionally to the length of the protein. DTT further denaturizes the protein by reducing disulfide bonds. Bromophenol blue is added to the sample to monitor the progress of the electrophoresis visually. In the electric field proteins migrate according to their molecular mass resulting in their separation on the gel. To stain the proteins and thus make them visible Coomassie Brilliant Blue is used. Since it also stains the gel, a de-staining solution is used to remove the excess of colour leaving only stained protein bands on the gel.

Reagents:

<u>Lower Buffer</u>

1.5 M Tris pure

0.4% w/v SDS

adjust pH to 8.8 with HCl

Upper Buffer

0.5 M Tris pure

0.4% SDS

adjust pH to 6.8

Reagent C

29.2% w/v Acrylamide

0.8% w/v Bis-acrylamide

filtrate through folded filter

4x Sample Buffer (SB)

200 mM Tris pure

300 mM DTT

adjust pH to 6.8 with HCl

4% w/v SDS

40% v/v Glycerine

Bromophenol blue

10x Electrophoresis Buffer

250 mM Tris pure

192 mM Glycine

1% w/v SDS

Separating gel

Analytical gel	**12%**	**15%**		**Preparative gel**	**12%**	**15%**
Reagent C	2.0 ml	2.5 ml		**Reagent C**	3.6 ml	4.5 ml
Lower Buffer	1.25 ml	1.25 ml		**Lower Buffer**	2.25 ml	2.25 ml
H_2O	1.75 ml	1.25 ml		**H_2O**	3.15 ml	2.25 ml
Temed	2.5 µl	2.5 µl		**Temed**	2.5 µl	2.5 µl
10% APS	25 µl	25 µl		**10% APS**	25 µl	25 µl

Table 2 Chemicals used for the preparation of analytical and preparative separating gels.

Stacking gel (4.5%)

Analytical gel			Preparative gel	
Reagent C	300 µl		**Reagent C**	450 µl
Upper Buffer	500 µl		**Upper Buffer**	750 µl
H_2O	1.2 ml		**H_2O**	1.8 ml
Temed	1 µl		**Temed**	1.5 µl
10% APS	20 µl		**10% APS**	30 µl

Table 3 Chemicals used for the preparation of analytical and preparative stacking gels.

Procedure

At first, the separating gel was prepared according to the concentrations given in Table 2. After adding APS and TEMED the solution was mixed well and immediately poured into a gel chamber leaving enough space for the stacking gel (concentrations see Table 3. During the polymerization process the gel was covered in a layer of isopropanol. After completed polymerization isopropanol was replaced by the stacking gel and a comb was inserted immediately. Once the gel was fully polymerized it was ready to use or could be stored at 4°C wrapped in moist paper towels and a saran wrap.

Before loading onto the gel the sample and 4x sample buffer were pipetted into an Eppendorf tube and heated at 95°C for 5 min for denaturation. Possibly evaporated liquid was spun down in an Eppendorf centrifuge. For determination of the MW of the sample a protein ladder was loaded onto the gel.

4.2.4.4 Immunoblot

This method is used to detect IgE binding to proteins bound to a nitrocellulose (NC) membrane. The proteins are separated by SDS-PAGE before being transferred to the NC membrane, which provides information on their molecular weights. Protein-free spaces on the membrane are blocked in order to prevent unspecific antibody binding and serum is added. During incubation antigen-specific IgE is able to bind to the membrane-bound antigens. This binding is visualized by a secondary antibody coupled to AKP highlighting the marked bands in reaction with a detection solution.

Reagents:

10x Transfer Buffer:

250 mM Tris pure

1.92 M Glycine

1x Transfer Buffer

Dilute 1:2:7 10x Transfer Buffer : methanol : H_2O

Gold Buffer (GB)

42 mM Na_2HPO_4 x 12 H_2O

8 mM NaH_2PO_4 x 2 H_2O

0.5% (w/v) NaN_3

adjust pH to 7.5 with HCl

add 0.5% Tween-20

AP Buffer

100 mM Tris pure

adjust pH to 9.5 with HCl

100 mM NaCl

5 mM $MgCl_2$ x 6 H_2O

Purified proteins or a protein extract were separated by an SDS PAGE as described in section 4.2.4.3. The gel was put into a transfer chamber that holds different layers together (gel and NC membrane surrounded by 2 whatman papers and 1 sponge on each side). Every component was soaked in Transfer Buffer before the chamber was put into a Transphor Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco, CA, USA) and transfer was started (25 mA, 45 – 90 min depending on the thickness of the gel) with the gel being at the side of the cathode while the NC membrane was at the side of the anode. The blot was taken out of the chamber and cut into strips. The strips were blocked with Gold Buffer (GB) + 3% (w/v) milk powder for 2 h. After washing (GB, 3x 10 min) the strips were incubated with serum (1:5 in GB + 0.5% BSA) o/n at 4°C. Serum was removed, NC strips were washed (GB, 3x, 10 min) and incubated with anti-human IgE-AKP antibody (1:750 in GB + 0.5% BSA) for 4 h at RT. The stripes were washed (GB, 3x 10 min; H_2O, 1x 1min; AP Buffer, 1x 1min) and incu-

bated with detection solution (AP Buffer, 0.6% NBT, 0.6% BCIP). Upon detection of violet colour, reaction was stopped with H_2O.

4.2.4.5 Immuno-solid phase allergen chip Assay

The ISAC approach is a semi-quantitative method for screening a patient's serum in order to obtain the IgE-binding pattern to a great number of purified allergens. In the version used in this project, triplets of 103 purified native or recombinant allergens from different allergen sources (food, airborne, and venom allergens as well as a representative for CCDs) are immobilized covalently on a glass slide. In addition to the allergens there are also guiding dots in 3 corners of the reaction field as well as IgE standards and blanks, see Figure 3.

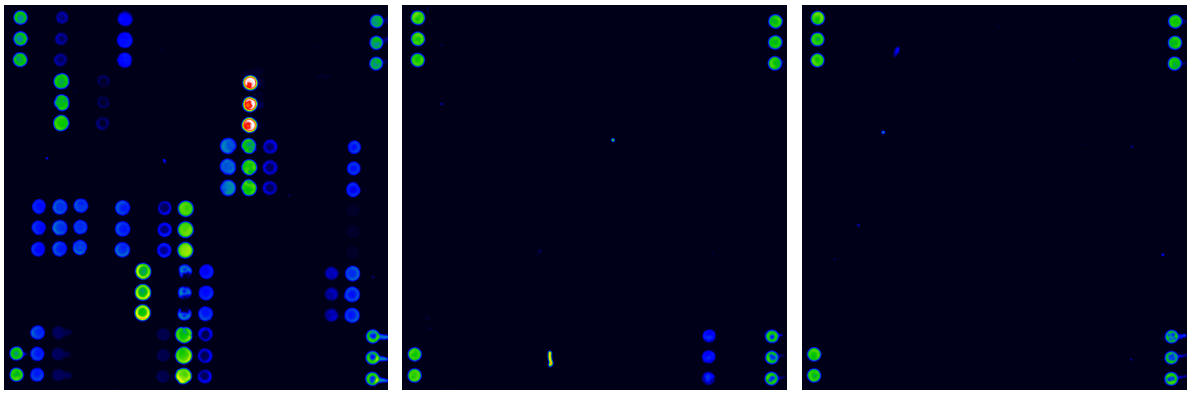

Figure 3 Scan images of patients' sera analyzed by the ISAC microarray.
Vertical triplicates of positive allergens can be seen well. The colour scale ranges from blue (low IgE binding) to white (very high IgE binding).

Procedure:

For the analysis of patients' sera the *ImmunoCAP ISAC Assay Kit IgE* (Thermo Fisher Scientific, Vienna) is used containing ISAC IgE microarrays, an IgE control serum, a fluorescently labelled anti-human IgE detection antibody, and Component A washing buffer.

Before use, the microarray has to be washed in order to remove salt. The slide is put into a glass slide rack and transferred to a washing dish containing 1x concentrated Component A washing buffer (35 ml of 20x concentrated Component A + 665 ml H_2O). The reaction fields have to be covered completely while washing for 1 h using a magnetic stir bar. Then the glass slide rack is transferred to a washing dish containing H_2O and a magnetic stir bar. After 5 min of stirring, the slide is taken out of the slide rack and let dry. After that, 25 µl of undiluted serum are carefully applied to a reaction field making sure the tip of the pipet does not touch it and thus scratches the allergen spots from the glass. The microarray is put into a humidity

chamber for 2 h. During this time the sIgE antibodies in the serum, if present, are allowed to bind specifically to allergens on the chip. After incubation the unbound IgE is removed by washing with fresh Component A for 10 min and subsequently with fresh H_2O for 5 min as described before. The slide is air-dried and remaining IgE is detected by 25 µl of a fluorescence-labelled anti-human IgE antibody. Detection is performed by *Microarray Image Analysis (MIA)* software and provides semi-quantitative information on the amount of specific IgE for distinct allergens based on fluorescence intensity expressed in *ISAC Standardized Units (ISU)* with positive values ranging from 0.3 – 100 ISU as well as *TIFF* images for every reaction field. Based on the ISU values the results were assigned to one of 4 classes, see Table 4.
For establishing the calibration curve, an internal reference serum included in the kit is applied to one reaction field per kit.

ISU Interval	Concentration of IgE antibodies
< 0.3 ISU	below limit of detection or very low
≥0.3 – <1 ISU	low
≥1 - <15 ISU	elevated or high
≥15 ISU	very high

Table 4 Classification of ISAC results according to ISU values.

5 RESULTS

5.1 Dendritic Cells

The aim of this subproject was to analyze the uptake of allergens by dendritic cells, the most important type of antigen-presenting cells. The central question was: Is the uptake of Ber e 1, the 2S albumin from Brazil nut, and SFA from sunflower by DCs different?

Starting from the very beginning, first the experimental setup had to be performed. Thus, changes in concentration of cells and proteins, surface, medium, and incubation time were made in order to find out the optimal conditions for the cells to grow and for the visualization of the labelled proteins.

To investigate if uptake of the allergens by the cells is receptor-mediated, receptor binding assays were carried out at 4°C. At this temperature endocytosis is halted and proteins that are bound by surface receptors of the cells stay bound instead of being taken up by the cells. Subsequent fluorescence microscopy delivers a typical, veiled signal pattern indicating the binding of the stained protein to the surface receptors in contrast to the vesicular staining obtained by endocytosed proteins.

5.1.1 Concentration of cells

In a first step, the conditions had to be determined that provide an optimal environment for the cells to grow. For that purpose, monocytes were isolated from the blood of a birch pollen allergic patient and grown on plastic slides and glass coverslips in a concentration of $4x10^5$ and $1x10^5$ cells per ml. After differentiation to dendritic cells they were checked under the microscope making sure they were growing on both surfaces. Differentiated DCs were incubated with 10 mg/ml FITC-Dextran for 1 h in both plastic and glass coverslip approaches.

Cells	Surface	Protein	Conc.	Incub time	Temp	Medium
$4x10^5$	CS	FD	10 mg/ml	1 h	37°C	IMDM/10%FCS
$1x10^5$	CS	FD	10 mg/ml	1 h	37°C	IMDM/10%FCS
$4x10^5$	PS	FD	10 mg/ml	1 h	37°C	IMDM/10%FCS

Results

The first finding was obtained by light microscopy showing that dendritic cells grow on plastic as well as on glass surfaces. More cells displaying the elongate dendritic shape characteristic for stimulated dendritic cells could be observed on the glass surface than on plastic where the vast majority of the cells presented the typical rounded shape of mature naïve dendritic cells. Fluorescence microscopy showed that FITC-Dextran had been taken up well by the cells, and the concentration was high enough to produce signals that were well detectable (data not shown).

5.1.2 Incubation times

In order to compare the positions of endocytotic vesicles at the beginning of FITC-Dextran-uptake and their route of migration, incubation times were varied. Localization of FITC-Dextran after 5 min and 1 h of incubation were compared.

Cells	Surface	Protein	Conc.	Incub time	Temp	Medium
$4x10^5$	CS	FD	10 mg/ml	1 h	37°C	IMDM/10%FCS
$4x10^5$	CS	FD	10 mg/ml	5 min	37°C	IMDM/10%FCS
$1x10^5$	CS	FD	10 mg/ml	1 h	37°C	IMDM/10%FCS
$1x10^5$	CS	FD	10 mg/ml	5 min	37°C	IMDM/10%FCS
$4x10^5$	PS	FD	10 mg/ml	1 h	37°C	IMDM/10%FCS
$4x10^5$	PS	FD	10 mg/ml	5 min	37°C	IMDM/10%FCS

Result

The differences between the longer and the shorter incubation times were visible in vesicle size as well as in their positions. At the 5 min time point vesicles were located at the entry site near the cellular membrane while after 1 h they are found near the nucleus. In addition a difference in size and intensity could be seen. Endosomes were smaller at the 5 min time point compared to the bigger vesicles with brighter staining that were seen after 1 h (data not shown).

5.1.3 Endocytosis of protein dissolved in serum-free IMDM

To exclude the possibility that components of the FCS interfere with the labelled proteins that should be examined, DCs were incubated with FITC-Dextran in various concentrations di-

luted either in IMDM + 10% FCS or at the same concentrations in serum-free IMDM. At the serum-free approach, the cells were incubated with serum-free IMDM only before the FITC-Dextran was added in order to allow clearance of the bound components from intracellular receptors during receptor recycling.

Cells	Surface	Protein	Conc.	Incub time	Temp	Medium
$4x10^5$	CS	FD	5 mg/ml	1 h	37°C	IMDM/10%FCS
$4x10^5$	CS	FD	500 µg/ml	1 h	37°C	IMDM/10%FCS
$4x10^5$	CS	FD	50 µg/ml	1 h	37°C	IMDM/10%FCS
$4x10^5$	CS	FD	5 µg/ml	1 h	37°C	IMDM/10%FCS
$4x10^5$	CS	FD	5 mg/ml	1 h	37°C	serum-free IMDM
$4x10^5$	CS	FD	500 µg/ml	1 h	37°C	serum-free IMDM
$4x10^5$	CS	FD	50 µg/ml	1 h	37°C	serum-free IMDM
$4x10^5$	CS	FD	5 µg/ml	1 h	37°C	serum-free IMDM

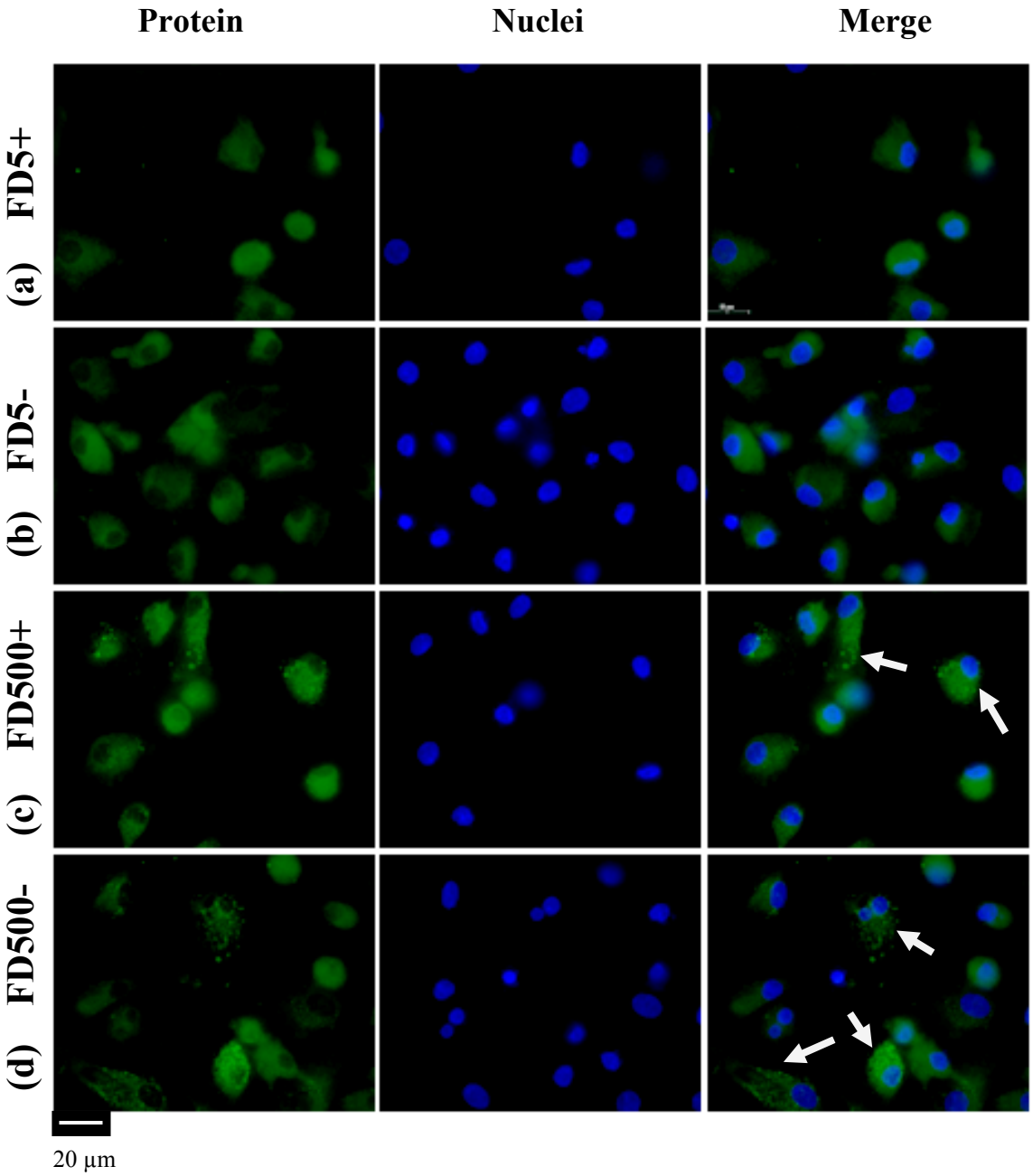

Figure 4 Incubation of DCs with different concentrations of FITC-Dextran (FD) in the presence (+) or absence (-) of serum in the medium at 37°C.

The two upper rows (a) and (b) show DCs incubated with 5 µg of FD. In (a), FCS was present in the serum in order to provide the cells with e.g. growth factors and, thus, stabilizing them. In (b), serum-free medium was used during incubation with FD in order to avoid possible interference of serum factors with the uptake of FD. In both assays no signal is visible compared to the negative control (see Figure 5 Incubation of DCs with different concentrations of FITC-Dextran (FD) in the presence (+)
or absence (-) of serum in the medium at 37°C. c, d). (c) and (d) show analogous incubation with 500 µg of FD being the lowest concentration at which a signal could be detected. Colour enhancement was done using Photoshop. In order to ensure comparability all pictures were equally altered.

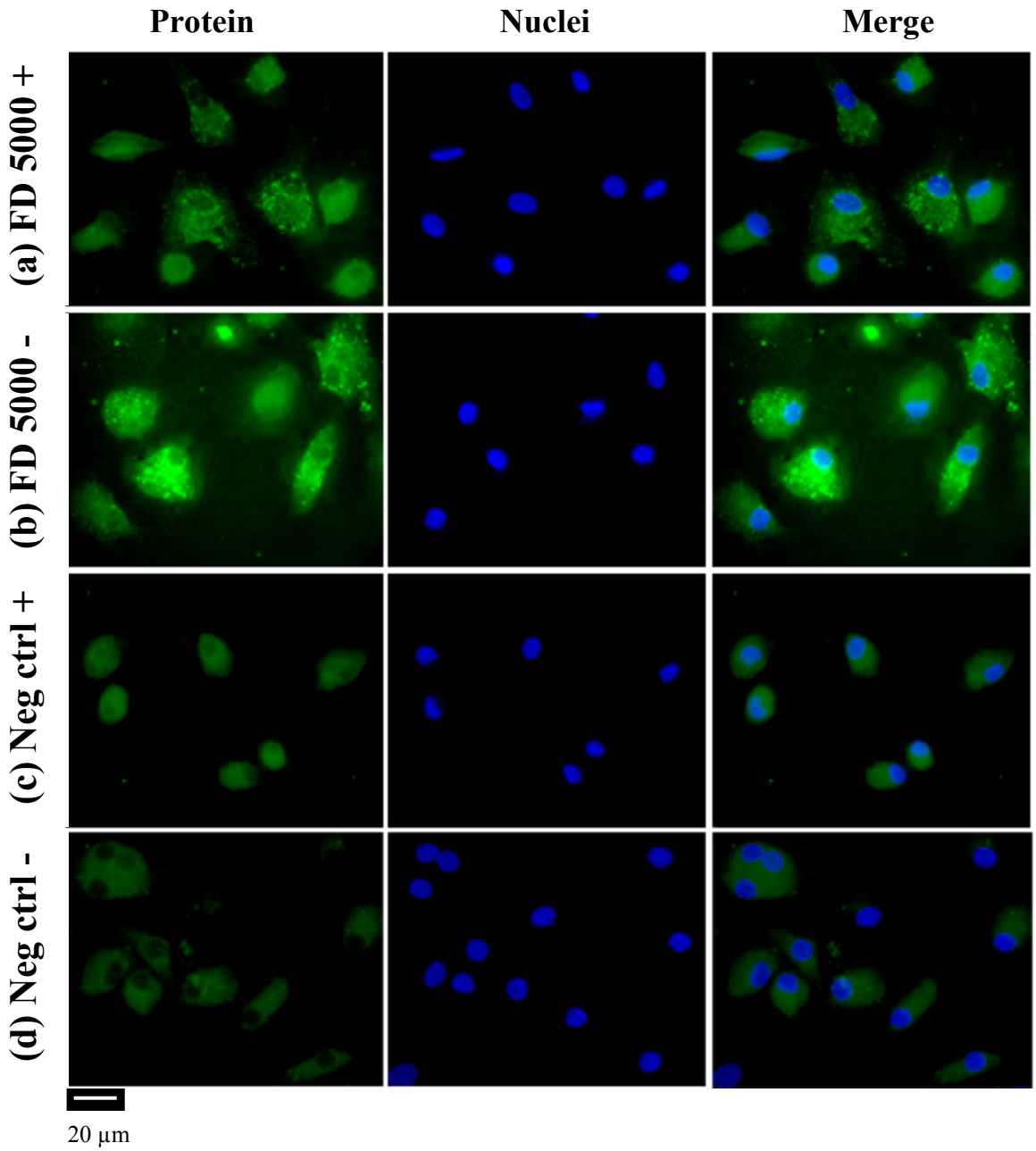

Figure 5 Incubation of DCs with different concentrations of FITC-Dextran (FD) in the presence (+) or absence (-) of serum in the medium at 37°C.
(a) and (b) show the highest concentration of FD used for incubation with (+) and without (-) serum. Many cells show visible staining. (c) and (d) represent only DCs grown in the presence (+) or absence (-) of serum. Colour enhancement was done using Photoshop. In order to ensure comparability all pictures were equally altered.

Results

Incubation with 5 µg/ml (Figure 4a, b) and 50 µg/ml (not shown) of FD did not give any detectable signal, neither in serum-containing nor in serum-free medium. At 500 µg/ml for both approaches in serum-containing (Figure 4c) and in serum-free medium (Figure 4d) signals were detectable. Incubation with 5 mg/ml FD gave a clear, strong signal of endocytosis in serum-containing (Figure 5a) as well as serum-free medium (Figure 5b).

5.1.4 Receptor binding of labelled Transferrin dissolved in serum-free IMDM

The same assay as in *5.1.3* was performed at 4°C to visualize receptor binding. Instead of FITC-Dextran, labelled Transferrin was used. Transferrin is internalized by DCs by binding to transferrin receptors on DCs [95].

Cells	Surface	Protein	Conc.	Incub time	Temp	Medium
$4x10^5$	CS	FT	50 µg/ml	1 h	4°C	IMDM/10%FCS
$4x10^5$	CS	FT	10 µg/ml	1 h	4°C	IMDM/10%FCS
$4x10^5$	CS	FT	2 µg/ml	1 h	4°C	IMDM/10%FCS
$4x10^5$	CS	FT	50 µg/ml	1 h	4°C	serum-free IMDM
$4x10^5$	CS	FT	10 µg/ml	1 h	4°C	serum-free IMDM
$4x10^5$	CS	FT	2 µg/ml	1 h	4°C	serum-free IMDM

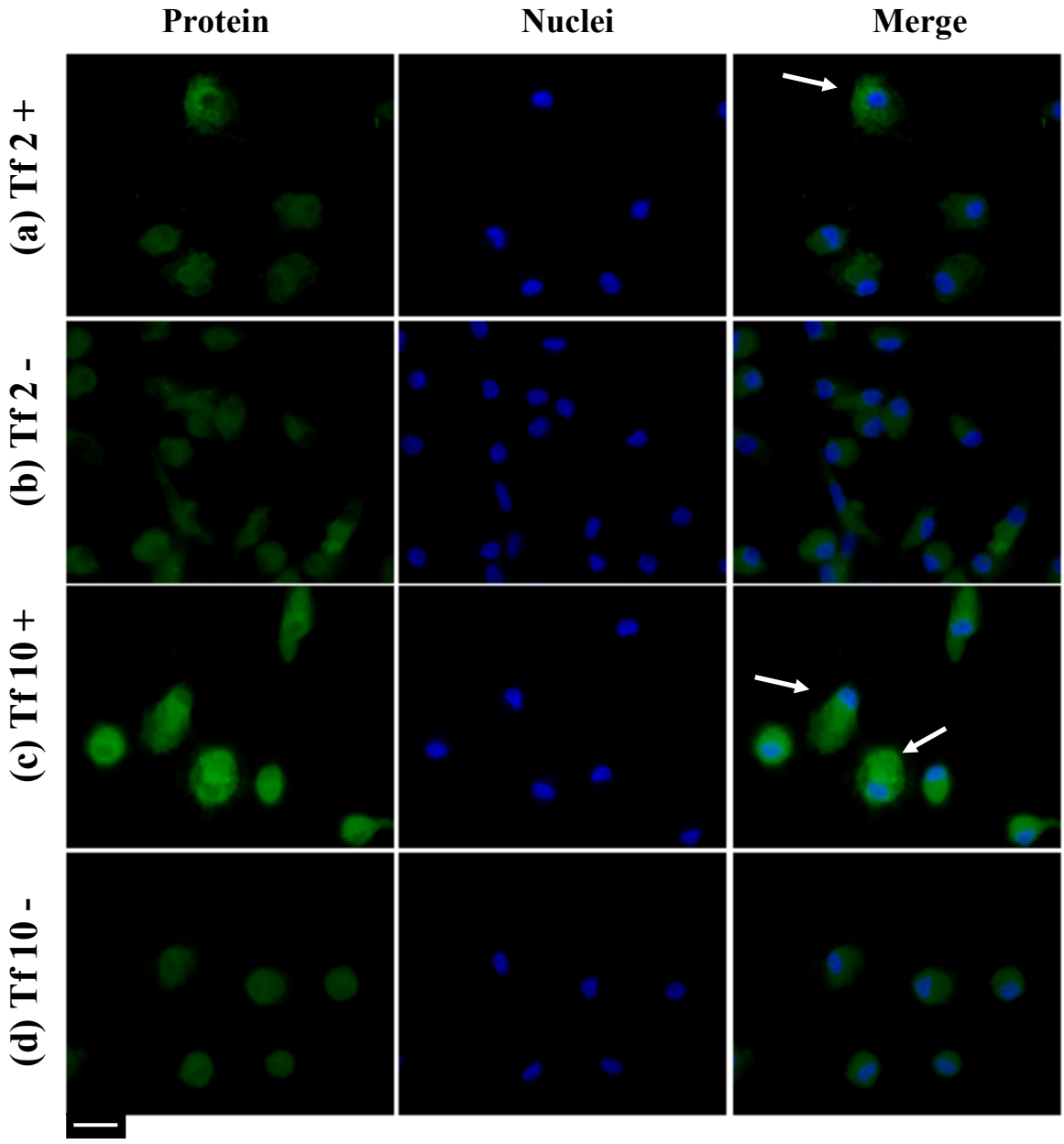

Figure 6 Incubation of DCs with different concentrations of labelled Transferrin (Tf) in the presence (+) or absence (-) of 10% FCS in the medium at 4°C
In (a) and (b) cells are shown that were incubated with 2 µg/ml of labelled Tf in presence and absence of FCS, respectively. (c) and (d) show incubation of DCs with 10 µg/ml of labelled Tf with or without FCS. In both cases the assay using serum during incubation delivers a brighter staining. Colour enhancement was done using Photoshop. In order to ensure comparability all pictures were equally altered.

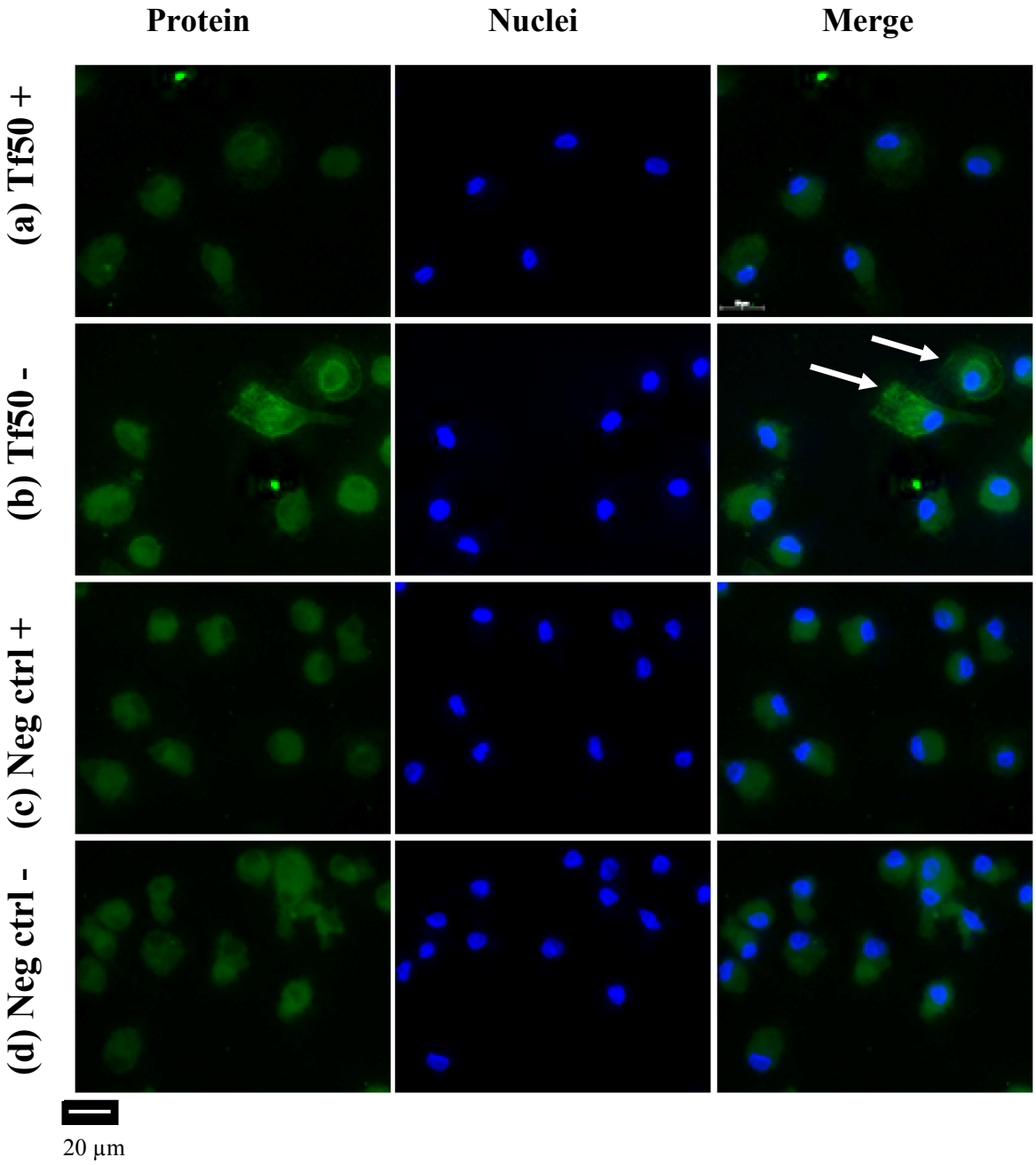

Figure 7 Incubation of DCs with different concentrations of labelled Transferrin (Tf) in the presence (+) or absence (-) of 10% FCS in the medium at 4°C
In (a) and (b) DCs were incubated with 50 mg/µl of labelled Tf in presence and absence of FCS, respectively. Incubation in presence of FCS resembles the negative controls (c) and (d) while serum-free incubation gave a nice signal on the surface of some cells. Colour enhancement was done using Photoshop. In order to ensure comparability all pictures were equally altered.

Results

The receptor binding at 4°C could be visualized, the cells were intact, staining was visible at only 2 µg/ml (Figure 6a, b) and the optimal concentration for Tf was determined 10 µg/ml (Figure 6 c, d). This concentration is also adopted for the labelled proteins. While the 2 µg/ml and 10 µg/ml concentrations gave a brighter signal when incubated in the presence of serum, in the 50 µg/ml approach the serum-free incubation (Figure 7a) resulted in a brighter signal compared with the 10% FCS/IMDM incubation (Figure 7b).

5.1.5 Concentration of labelled proteins

The 2S albumins from Brazil nut, Ber e 1, and sunflower, SFA 8, each labelled with Oregon Green 488 (OG) emitting in the FITC range, and with pHrodo, a red dye emitting only in an environment of low pH as present in late endosomes, were added to the cells in different concentrations. In order to simulate the case when a smaller amount of cells are available, OG and pH labelled proteins of the same source were added to the same well (*Ber mix* and *SFA mix*, respectively). The result should show whether or not the detection of the proteins is possible in the respective channels. Serum-free medium was used for incubation.

Cells	Surface	Protein	Conc.	Incub time	Temp	Medium
$4x10^5$	CS	FD	10 mg/ml	1 h	37°C	serum-free IMDM
$4x10^5$	CS	Ber mix	10 µg/ml	1 h	37°C	serum-free IMDM
$4x10^5$	CS	SFA mix	10 µg/ml	1 h	37°C	serum-free IMDM

In parallel, an approach at 4°C was done to show the binding of the labelled protein to membrane-bound receptors on dendritic cells.

Cells	Surface	Protein	Conc.	Incub time	Temp	Medium
$4x10^5$	CS	Ber mix	10 µg/ml	1 h	4°C	serum-free IMDM
$4x10^5$	CS	Ber mix	2 µg/ml	1 h	4°C	serum-free IMDM
$4x10^5$	CS	SFA mix	2 µg/ml	1 h	4°C	serum-free IMDM
$4x10^5$	CS	SFA mix	10 µg/ml	1 h	4°C	serum-free IMDM
$4x10^5$	CS	FT	2 µg/ml	1 h	4°C	serum-free IMDM
$4x10^5$	CS	FT	10 µg/ml	1 h	4°C	serum-free IMDM

Result

The cells were torn after the staining procedure (data not shown). Thus, the experiments were carried out using 12-well plates again for both cultivation of DCs and incubation with proteins. In addition, the cells were incubated with the proteins in separate wells depending on the label and the type of protein. This was decided to make sure future experiments would deliver intact cells.

5.1.6 Endocytosis and receptor binding of labelled proteins

Previous studies showed that binding to receptors could also be detected when incubating with medium containing serum. To provide the cells with a minimal amount of serum and at the same time reduce the amount of possibly interfering components, proteins were diluted in 1% FCS for the endocytosis assay as well as for the binding assay. Since pHrodo shows fluorescence only at low pH it was not used for the binding experiment. The neutral environment provided by the medium would not allow detection of the pHrodo-labelled protein bound to the cells.

Cells	Surface	Protein	Conc.	Incub time	Temp	Medium
$4x10^5$	CS	Ber-pH	10 µg/ml	1 h	37°C	IMDM/1%FCS
$4x10^5$	CS	Ber-OG	10 µg/ml	1 h	37°C	IMDM/1%FCS
$4x10^5$	CS	SFA-pH	10 µg/ml	1 h	37°C	IMDM/1%FCS
$4x10^5$	CS	SFA-OG	10 µg/ml	1 h	37°C	IMDM/1%FCS
$4x10^5$	CS	Ber-OG	10 µg/ml	1 h	4°C	IMDM/1%FCS
$4x10^5$	CS	SFA-OG	10 µg/ml	1 h	4°C	IMDM/1%FCS

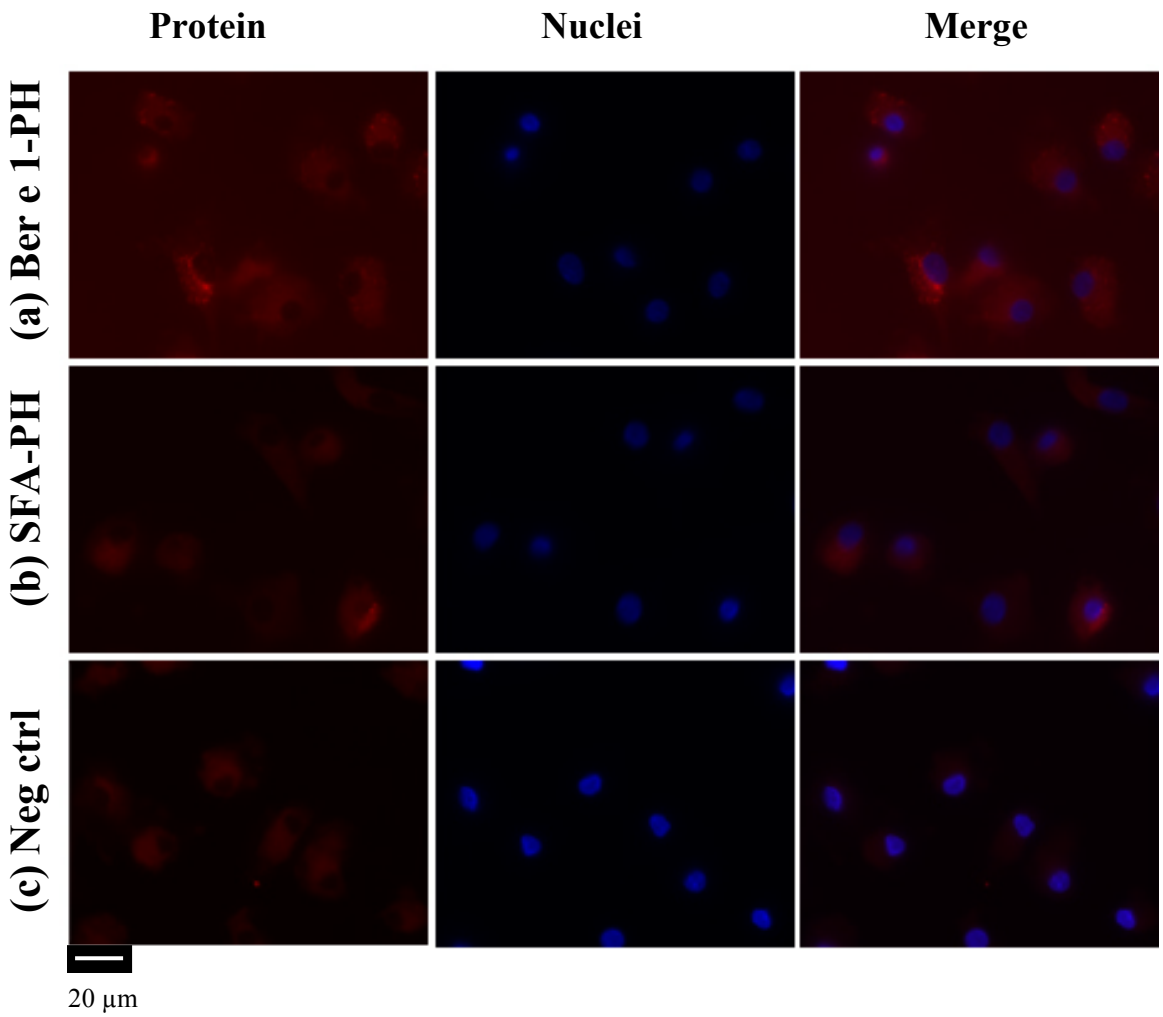

Figure 8 Endocytosis of Ber e 1 and SFA-8 labeled with a pH-dependent dye.
In (a) pHrodo-labeled Ber e 1 is shown to give a detectable signal within some DCs whereas for SFA the signal is weaker, but still detectable (b). In (c) the negative control can be seen giving a faint background. Colour enhancement was done using Photoshop. In order to ensure comparability all pictures were equally altered.

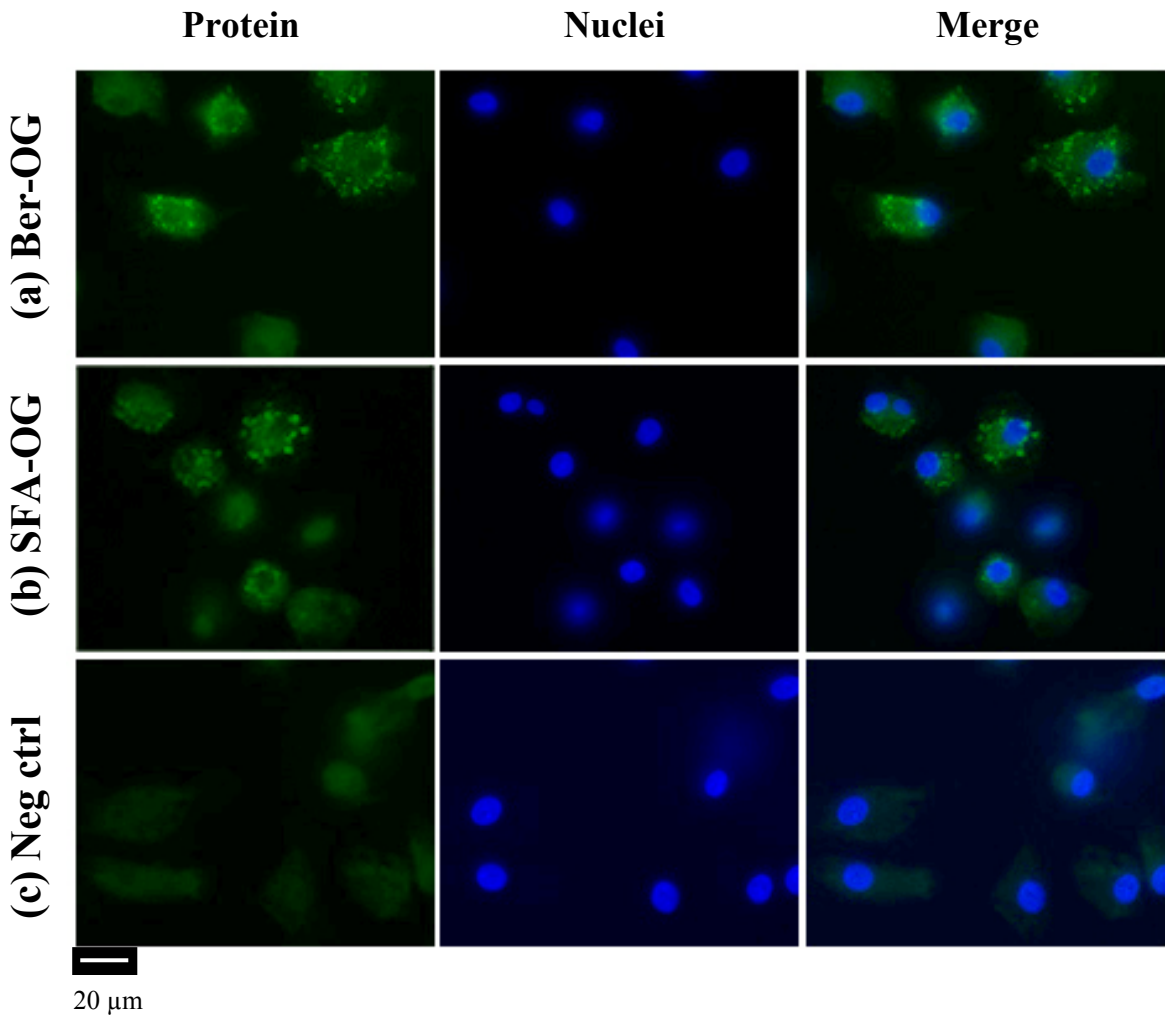

Figure 9 Endocytosis of proteins labeled with a pH-independent dye.
Both Ber e 1 (a) and SFA8 (b) give a clear signal upon endocytosis. In (c) the negative control can be seen giving a faint background. Colour enhancement was done using Photoshop. In order to ensure comparability all pictures were equally altered.

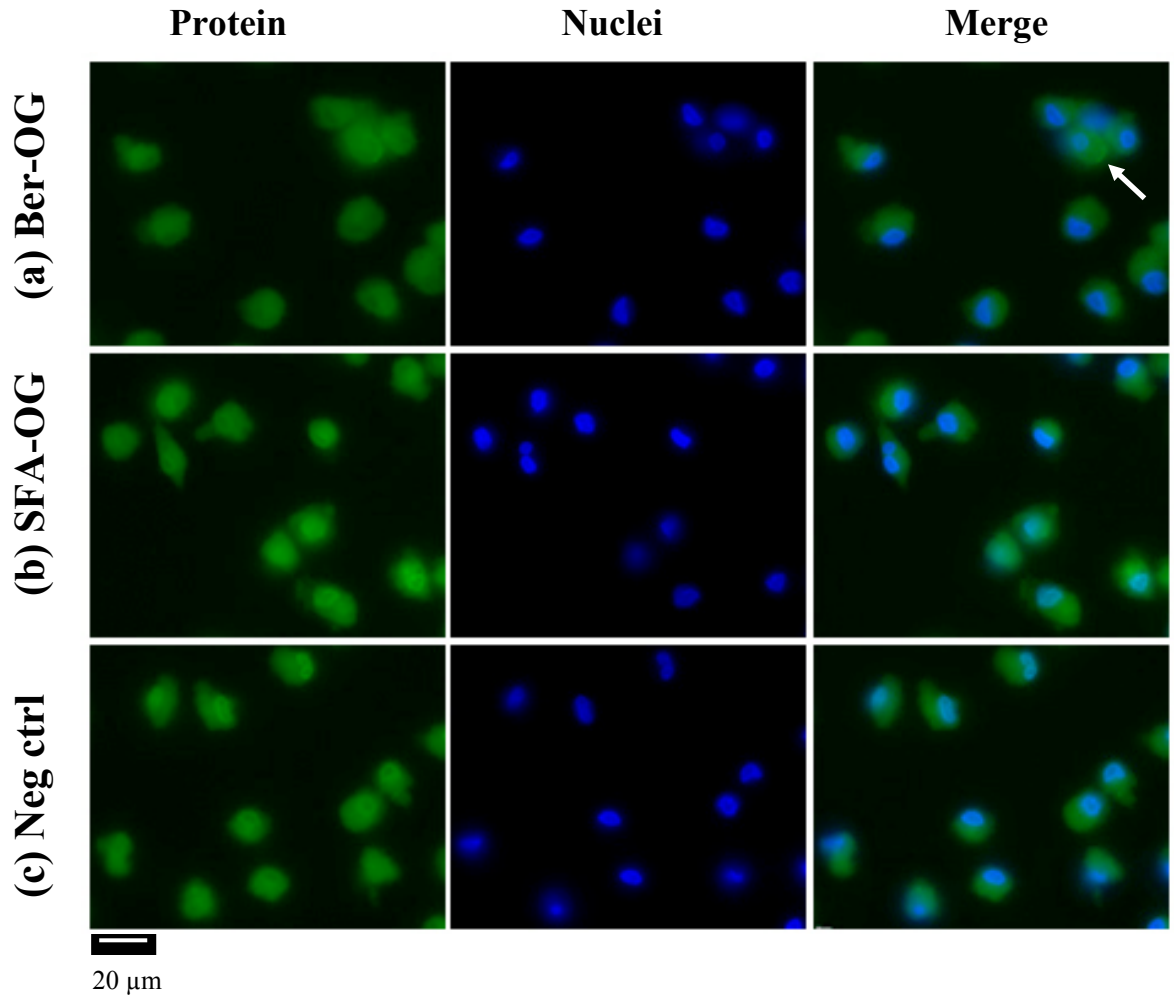

Figure 10 Incubation of DCs with labelled protein at 4°C.
In (a) DCs were incubated with Ber e 1-OG was incubated in order to show receptor binding. The arrow indicates possible receptor binding of Ber e 1, other cells appear unstained. In (b) DCs were incubated with SFA8-OG which did not give any signal. In (c) the negative control can be seen giving a faint background. Colour enhancement was done using Photoshop. In order to ensure comparability all pictures were equally altered.

Result

The endocytosis of Ber e 1-OG could be observed well (Figure 9a). Ber e 1-PH was visible in some single cells (Figure 8a) and also SFA-OG (Figure 9b) could be detected inside the cells indicating that adequate conditions for this staining were chosen. However, no convincing pictures showing endocytosis of SFA-pH (Figure 8b) were found. Although there is staining in some cells, it is weak and very rare.

Ber-OG and SFA-OG bound to cell surface receptors was not frequently detected. Only in very few cases of the Ber e 1-staining the characteristic pattern of receptor-bound protein could be assumed (Figure 10a), although the signal was very low and could not be clearly distinguished from background noise.

5.1.7 Route of internalization of a labelled protein

Incubation times of 5 min, 10 min, and 30 min were chosen to follow the route of a 2S albumin after internalization more closely. As a model protein Ber e 1-OG was chosen because in previous experiments this was the protein that gave the best signal.

Cells	Surface	Protein	Conc.	Incub time	Temp	Medium
$4x10^5$	CS	Ber-OG	10 µg/ml	5 min	37°C	serum-free IMDM
$4x10^5$	CS	Ber-OG	10 µg/ml	10 min	37°C	serum-free IMDM
$4x10^5$	CS	Ber-OG	10 µg/ml	30 min	37°C	serum-free IMDM
$4x10^5$	CS	FT	10 µg/ml	5 min	37°C	serum-free IMDM
$4x10^5$	CS	FT	10 µg/ml	10 min	37°C	serum-free IMDM
$4x10^5$	CS	FT	10 µg/ml	30 min	37°C	serum-free IMDM

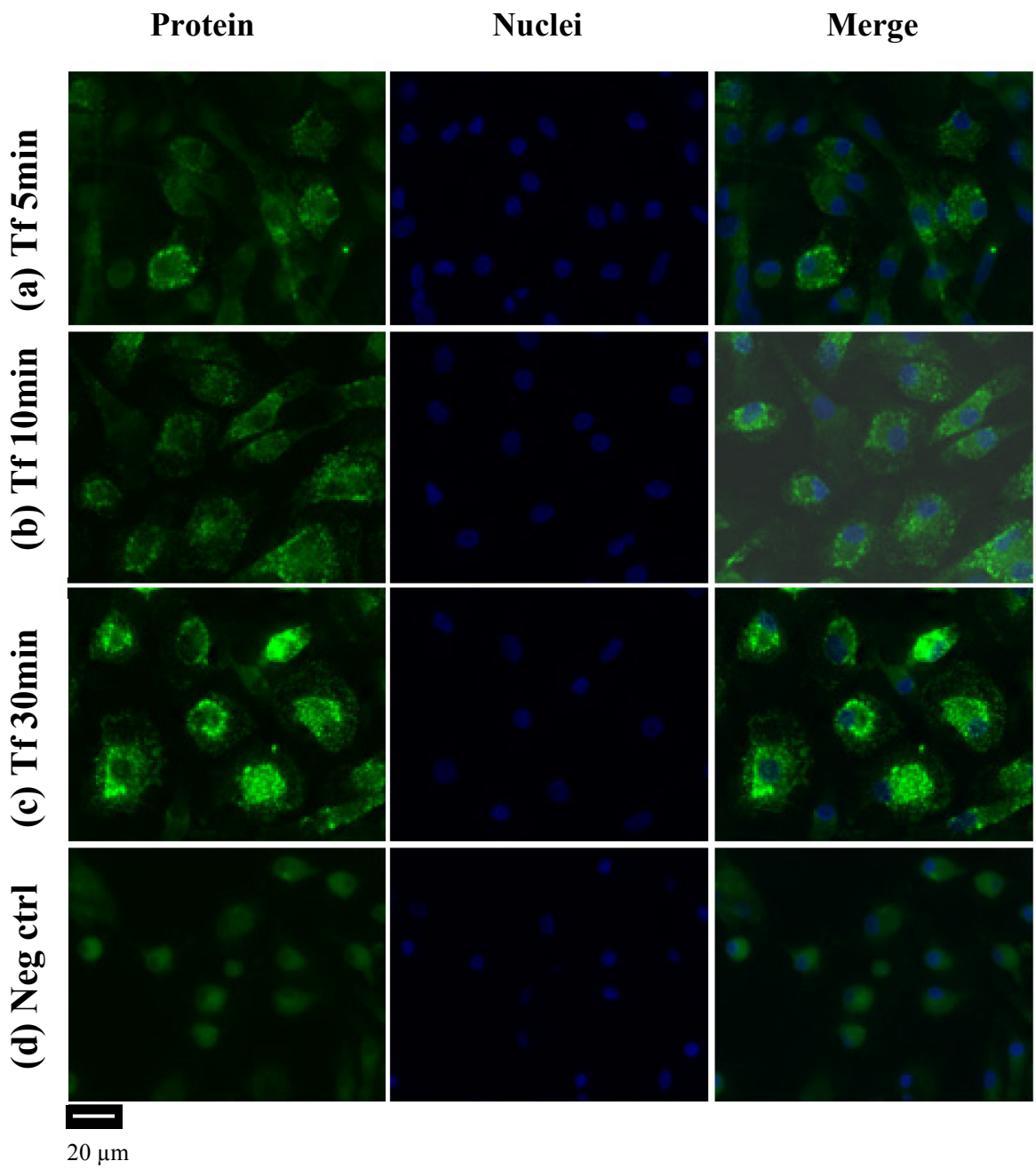

Figure 11 Endocytosis of Tf at different time points.
At 5 min the signal is visible at the outer regions of the cells close to the cell membrane (a). After 10 min the signal is more diffuse and closer to the nucleus (b). At 30 min the signal is very bright and concentrated around the nucleus while at the outer areas there is hardly any staining left (c). In comparison, the negative control shows only a faint background staining (d). Colour enhancement was done using Photoshop. In order to ensure comparability all pictures were equally altered.

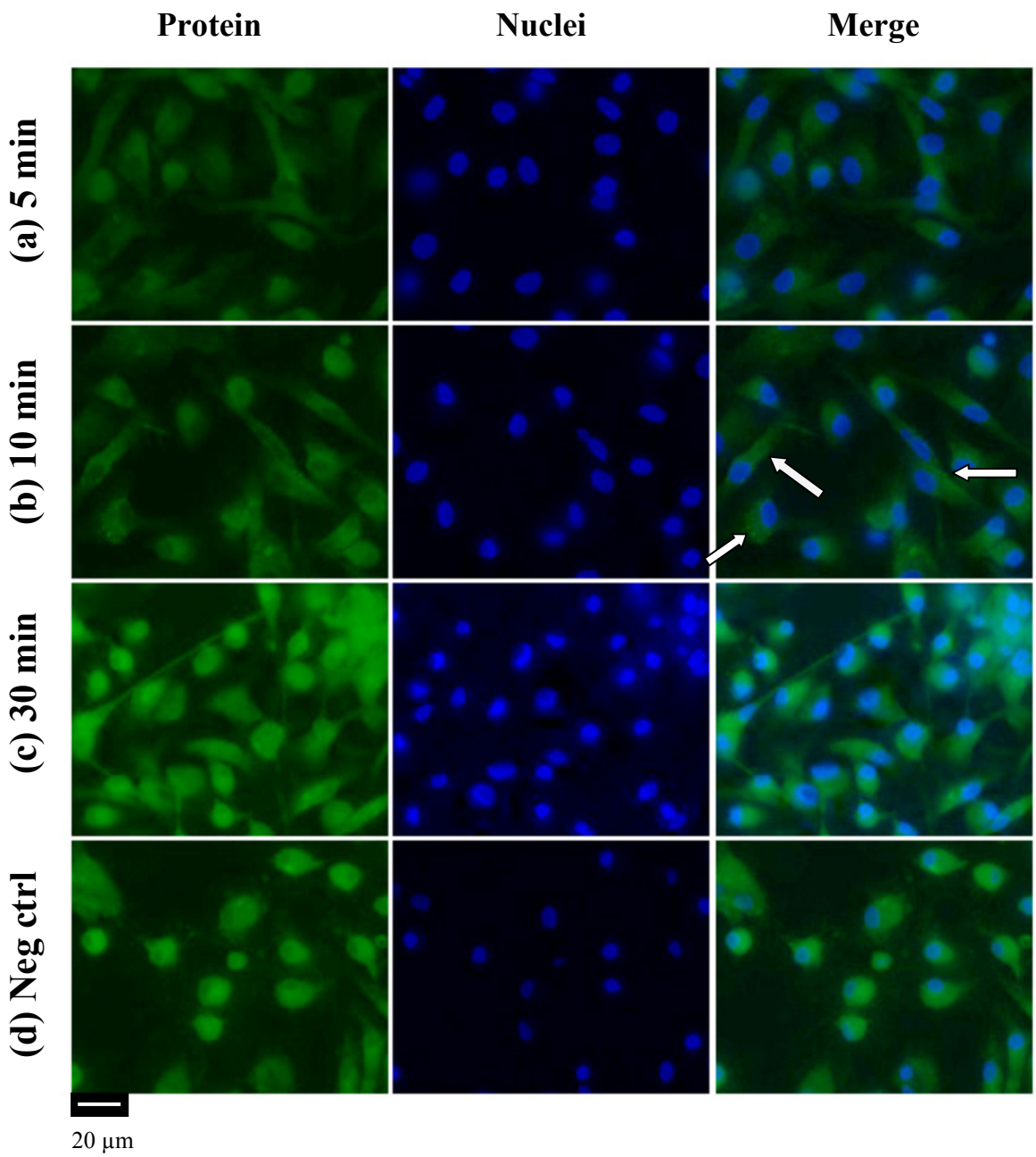

Figure 12 Endocytosis of Ber e 1 by DCs at different time points.
At 5 min of incubation with labelled protein no signal can be seen (a) whereas at 10 min a weak signal within endocytosed vesicles can be seen as indicated by the arrows (b). At 30 min it was again difficult to make out a signal (c) compared to the negative control (d). Colour enhancement was done using Photoshop. In order to ensure comparability all pictures were equally altered.

Result

While the positive control Tf was taken up by the cells well and the pictures of different incubation times were also distinguishable (Figure 11), Ber-OG gave hardly any signal at 5 and 10 min (Figure 12). Only at 30 min distinct vesicles were visible.

5.1.8 Repetition of receptor binding experiment

The 4°C assay was repeated using serum-free IMDM in order to obtain convincing pictures of receptor binding of the protein. Thus, the possibility of serum components blocking the receptors on the cell surfaces was eliminated.

Cells	Surface	Protein	Conc.	Incub time	Temp	Medium
$4x10^5$	CS	Ber-OG	10 µg/ml	1 h	4°C	serum-free IMDM
$4x10^5$	CS	FT	10 µg/ml	1 h	4°C	serum-free IMDM

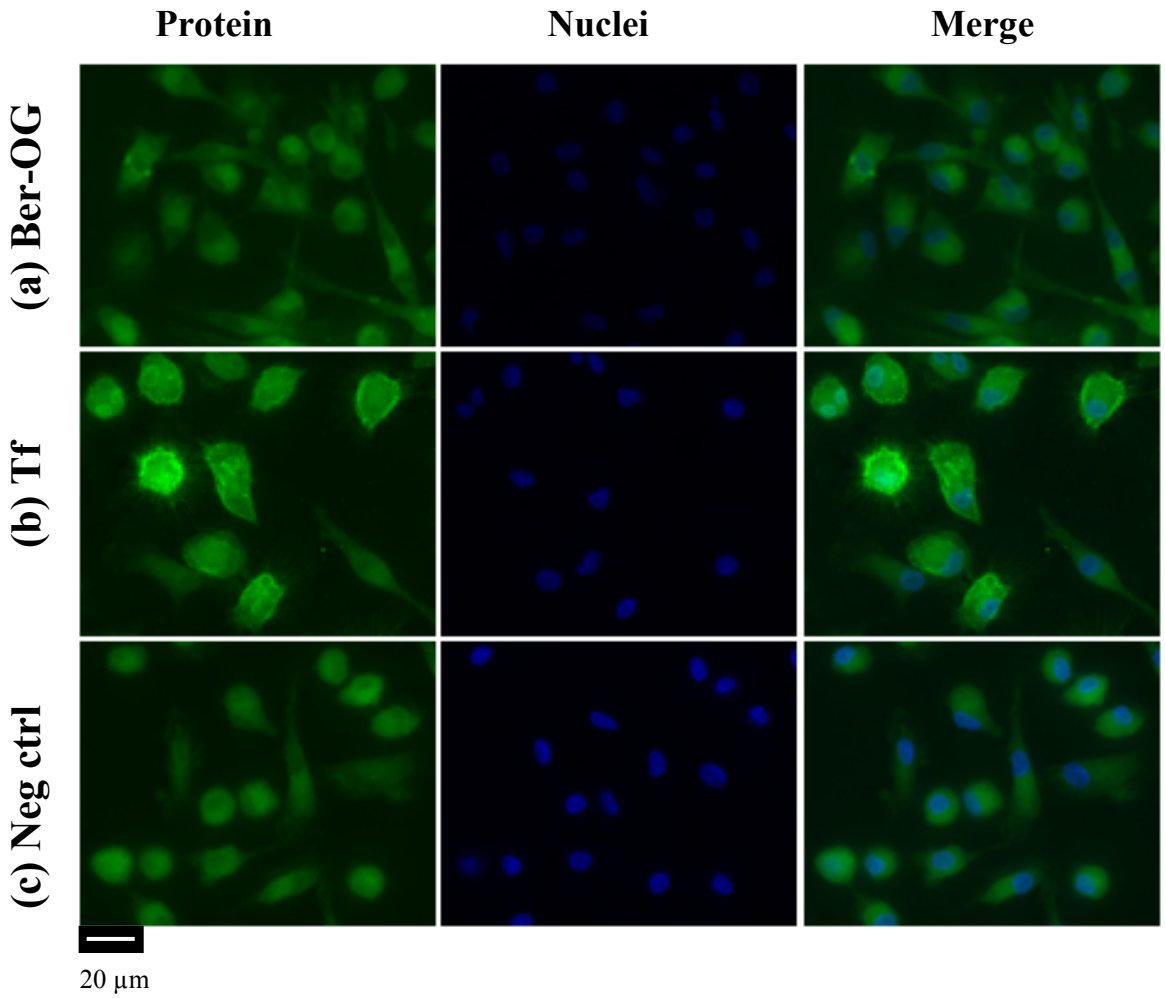

Figure 13 Receptor binding of Ber e 1.
Incubation of DCs with labelled protein, Ber e 1-OG (a), a labelled positive control, Tf (b), and w/o protein (c) at 4°C in serum-free medium. While receptor binding of the positive control was very bright and clear, Ber e 1-OG did not show surface staining.

Results

Receptor binding could be well shown on cells incubated with the positive control (Figure 13b). Ber-OG, however, was not detectable (Figure 13a). This could be because too low concentrations of the protein were used. Since Ber e 1 and Tf are supposedly not taken up by the same receptor, protein concentrations can not be compared.

Another explanation could be that the protein is simply not taken up by the cells in a receptor-mediated manner. To exclude this possibility the experiment needs to be repeated using higher amounts of labelled protein.

5.1.9 Table of results

In this subproject methods were established to detect the receptor binding and internalization of labelled proteins. The results for each protein are summarized in Table 5.

Protein	Receptor binding	Internalization	Concentration
FITC-Tf	+	+	10 µg/ml
Ber e 1-OG	+/-	+	10 µg/ml
SFA8-OG	+/-	+	10 µg/ml
Ber e 1-pH	n.d.	+	10 µg/ml
SFA8-pH	n.d.	+/-	10 µg/ml

Table 5 Summary of the results obtained by DC experiments.

5.2 IgE-reactivity patterns of sera from patients with anaphylaxis to soy and wheat

5.2.1 Analysis of sera from soy allergic patients

Twenty patients suffering from soy allergy with symptoms ranging from weak manifestations like the oral allergy syndrome (OAS) to severe systemic symptoms. Fourteen out of 20 patients also had OAS to fruits or vegetables most of which are known to contain proteins of the PR10 family. In all cases of OAS apple was among the eliciting foods (n = 14) followed by peach (n = 9) and hazelnut (n = 8).

Total IgE had been determined in 19 of the 20 patients ranging from 25.8 to 1718 kU/l with a mean value of 281 kU/l.

In addition, RAST had been performed in most cases. Detected allergens or protein extracts were soy bean extract, the PR10 protein from soy, Gly m 4, birch extract, and the PR10 protein from birch, Bet v 1. Two of 15 tested sera were positive for soybean extract while 14 of 16 tests resulted positive for Gly m 4. For BP extract, 17 of 19 sera displayed sIgE and 11 of 12 tested positive for Bet v 1.

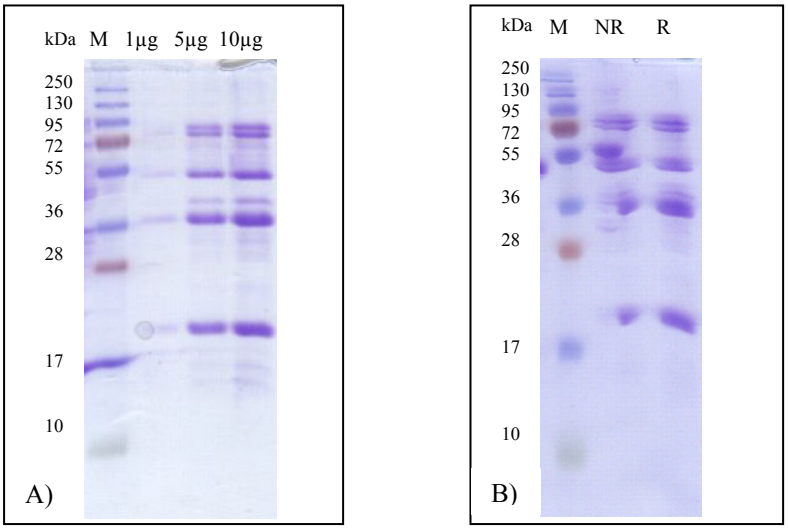

Figure 14 Soy extract loaded onto an SDS-PAGE.
A) Soy extract before lyophilization. Numbers indicate the different amount of extract loaded onto the gel.
B) Soy extract after lyophilization. Of each sample 5 µg were loaded.
M: marker, NR: non-reducing sample buffer, R: reducing sample buffer.

Soy extract

Purified soy proteins were available in the lab and a soy extract from soy flour was prepared. The extract was loaded onto an SDS-PAGE before and after lyophilization (see Figure 14) giving about the same pattern of bands in a M_W range from ~20 to 80 kDa. In the lane where the extract in non-reducing sample buffer had been loaded an additional band can be seen at ~60 kDa. According to Table 6 the allergens that might be represented in the extract are isoforms of the high M_W proteins Gly m 5 and Gly m 6 ranging between 47 and 65 kDa.

Allergen	Protein family	M_W
Gly m 1	Hydrophobic protein	7 kDa
Gly m 2	Defensin	8 kDa
Gly m 3	Profilin	14 kDa
Gly m 4	PR10	17 kDa
Gly m 5	7S globulin	47 – 65 kDa
Gly m 6	11S globulin	52 – 61 kDa

Table 6 Allergens from soy, the protein families they belong to and their size.
M_W can vary for one allergen due to different sizes of their isoforms.

ELISA

In order to identify the eliciting allergen for the soy allergic patients, ELISAs were performed screening for sIgE to the PR10 protein Gly m 4, the 7S globulin Gly m 5, and the 11S globulin Gly m 6 as well as an extract from soy flour (see Figure 15).

Nineteen of 20 sera were positive to Gly m 4 although the signal was very low in some of them. Seven displayed sIgE for Gly m 5 and five for Gly m 6 also including very low positive signals (in 23 cases the OD_{405} value was below 0.1).

Two sera were positive for proteins from the soy extract. Taking into concern that 19 sera were positive to Gly m 4, it seems likely that this PR10 homologue is underrepresented in the extract. This is supported by various papers stating that it can be difficult to produce a soy extract with a sufficient amount of Gly m 4 [96, 97] as well as the SDS gel of the extract that did not show bands in the lower molecular range.

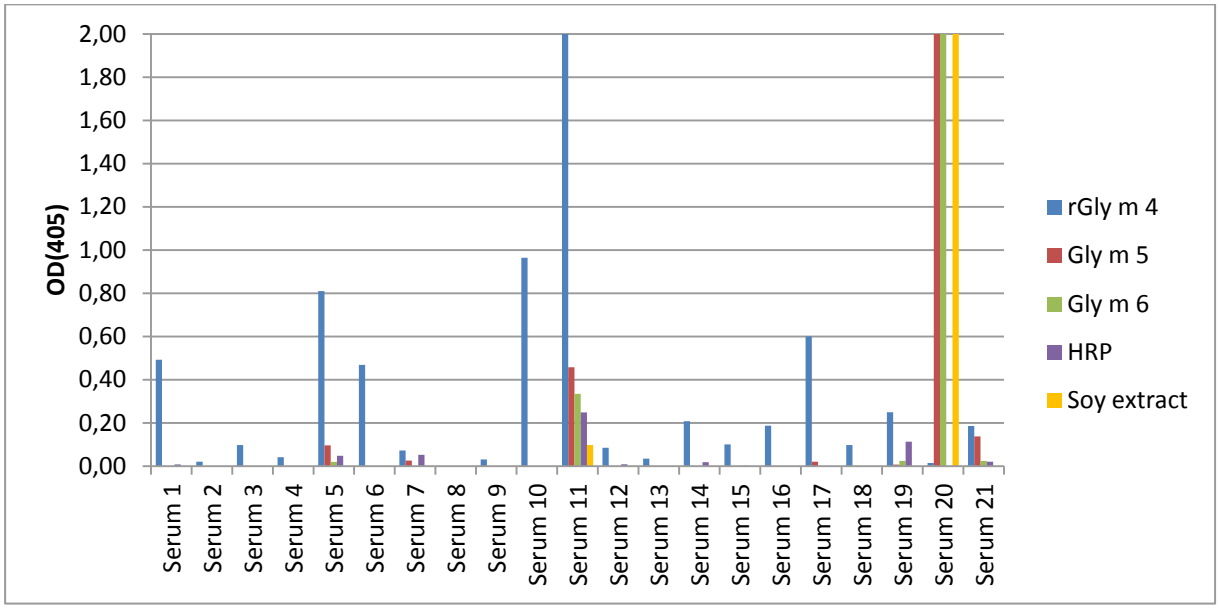

Figure 15 Summary of performed ELISAs with sera of 20 soy allergic patients. OD_{405} values were measured after 30 minutes, 1 µg/ml purified proteins and 100 µg/ml extract were coated, 10 µg/ml of HRP.

IMMUNOBLOT

Sera of the 20 soy allergic patients were applied to an immunoblot in order to detect sIgE to soy proteins contained in an extract from soy flour (see Figure 16).

In all 20 patients, no IgE binding to a 17 kDa band representing Gly m 4 could be detected. Thus, the ELISA results in Figure 15 that showed at least weak IgE reactivity to Gly m 4 in all sera except for serum 8, could not be confirmed by the immunoblot. Also, ELISA analysis using soy extract resulted positive in only 2 sera. In combination with the lack of a Gly m 4 band in the immunoblot, this indicates that Gly m 4 was underrepresented in the soy extract and that the positive ELISA results derive from other allergens in the extract. This is confirmed by immunoblot, where these 2 positive sera also show bands.

The strongly reactive serum 20 gave a signal in the 47 – 65 kDa range that represents the globulins Gly m 5 and Gly m 6. The lack of clear bands could be attributed to Gly m 5 that undergoes processing during maturation which results in protein variants of different size.

Serum 16 displays 3 bands in the range from 45 – 80 kDa. The 45 kDa band is visible also in sera 3, 11, 12, and 19 as well as in the non-allergic control while it is missing in the buffer control. This indicates that there is some unspecific IgE binding in this region.

Serum 7 displays 2 bands at approximately 30 kDa.

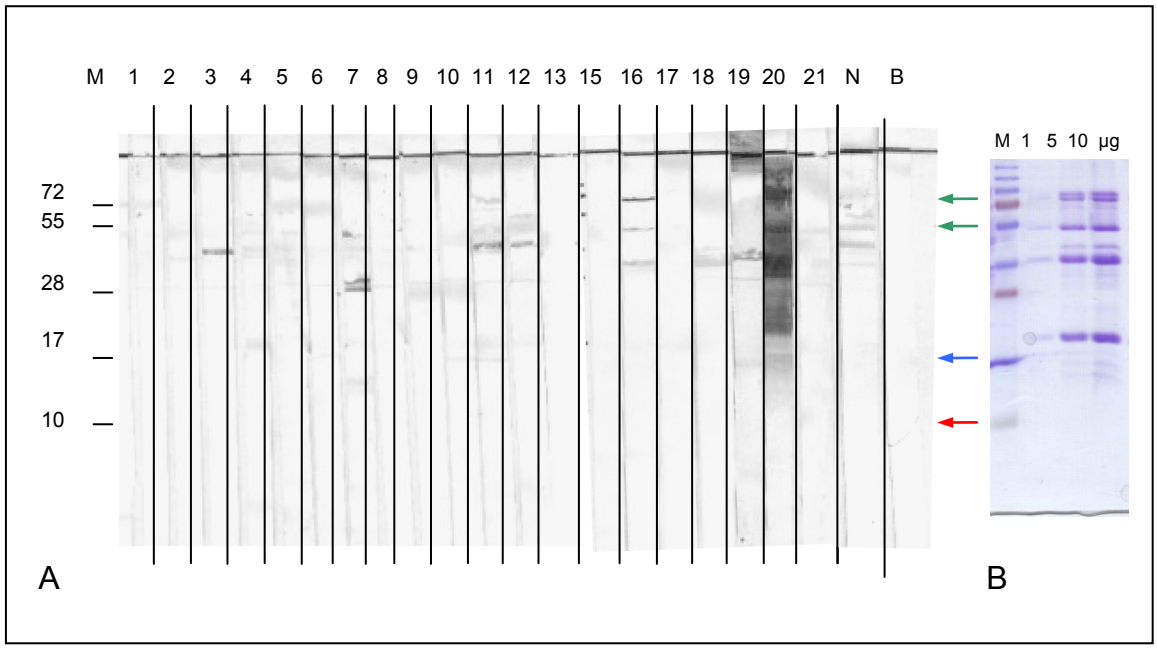

Figure 16 A) Immunoblot with sera from 20 soy allergic patients to soy extract. M: marker, 1-20: patients' sera, N: non-allergic control, B: buffer control.
B) SDS PAGE of soy extract before (right) and after (left) lyophilization. M: marker, NR: non-reducing sample buffer, R: reducing sample buffer.
Green arrows indicates MW area of Gly m 5 and 6 isoforms, blue arrow indicates MW of Gly m 4 and red arrow indicates MW area of Gly m 1 and 2.

ISAC

The next step was to test the sera in the latest version of the ImmunoCAP ISAC microarray presenting 112 purified natural or recombinant allergens and thus delivering a detailed sIgE pattern for each tested serum. The allergens immobilized on the chip can be divided into food, airborne, and cross-reactive components. Since the patients' collective is composed of 20 food allergic patients, the former allergen group was of major interest for this analysis.

Upon testing the sera by ISAC analysis, in 19 of the 20 patients similar allergen recognition patterns could be detected. All of these sera showed sIgE for at least 7 of the ten PR10 proteins on the allergen chip. The allergens Aln g 1, Bet v 1, Cor a 1.0101, Cor a 1.0401, Mal d 1, Pru p 1, and Ara h 8 deriving from alder, birch pollen, hazel pollen, hazelnut, apple, peach and peanut respectively were detected in all sera from this group. The representative from soy within the PR10 family, Gly m 4, was one of the least frequently recognized allergens with 14 positive outcomes in 20 sera. This indicates that it might not be essential to have IgE to Gly m 4 to develop PR10-dependent soy allergy or that the sensitivity of the assay is too low.

Seven of the 20 sera displayed sIgE to at least 1 profilin which is a highly cross-reactive protein family whose members are found in various foods.

Other food allergens were nsLTPs, legumes and nuts, and seeds. One serum was tested positive for allergens from each group.

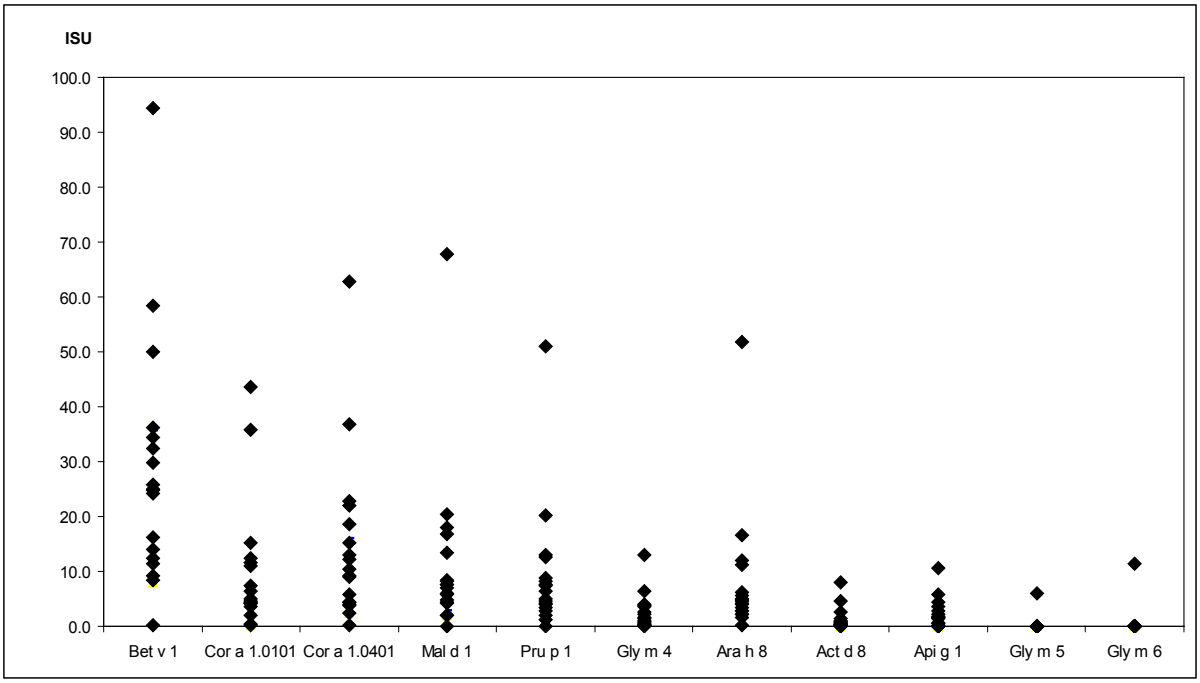

Figure 17 Dot plot of 20 sera from soy allergic patients analyzed by ISAC microarray. ISU levels of the sera expressing the amount of sIgE to the respective PR10 or soy allergen are shown.

While in the majority of the patients the most important eliciting agents seem to be PR10 proteins, one serum only displayed slight IgE reactivity to one PR10 protein (Cor a 1.0401) while it tested positive for other food allergen groups like profilins, nsLTPs, and 7S and 11S globulins including the 2 soy allergens Gly m 5 and Gly m 6. These results indicate that 19 patients have birch pollen-related food allergy and thus had been primarily sensitized for Bet v 1 via the respiratory route and had afterwards developed food allergy elicited by homologous PR10 proteins.

A further observation was that the only serum reactive to Gly m 5 and Gly m 6 showed also by far the highest level of total IgE (1718 kU/l). Results are shown in Figure 17 and were published in [98].

5.2.2 Analysis of the sera from wheat allergic patients

By conventional diagnosis, 22 cases of wheat anaphylaxis had been identified in the patients' collective. These cases could be divided into patients suffering from wheat-dependent exercise-induced anaphylaxis (WDEIA), additive-related wheat-induced anaphylaxis (WIA) and wheat-induced anaphylaxis without co-factors. 20 of 22 sera had been tested in

ImmunoCAP for sIgE to wheat extract and/or Tri a 19, a major wheat allergen. Of the Tri a 19 tested sera, 13 had sIgE while 6 were negative, see Table 7.

	Co-factor	Patient	SPT	CAP		ISAC	ELISA
				Wheat	Tri a 19	Tri a 19	Tri a 19
WDEIA	Exercise	11	+	+	+	1.7	0.8
		18	+	+	-	< 0.3	0.07
		22	+	nd	+	< 0.3	0.99
		27	+	+	+	< 0.3	2.8
		28	+	+	+	0.6	0.26
		29	+	+	+	1.4	1.14
		39	+	nd	+	0.8	1.23
		43	+	nd	+	0.9	1.31
		51	+	+	+	< 0.3	0.14
		54	+	nd	+	< 0.3	0.16
		55	+	nd	-	< 0.3	0
		56	-	nd	-	< 0.3	0
		58	+	nd	+	< 0.3	0.47
		59	+	+	+	0.3	0.76
WIA + co-factors	Exercise+Clindamycin	60	-	nd	nd	< 0.3	0
	Pantozol+co-factor	6	-	-	nd	< 0.3	0.04
	Unclear	7	+	+	+	4	1.18
	ASS	31	-	+	-	< 0.3	0
WIA	None	5	+	+	-	< 0.3	0
		10	+	+	-	< 0.3	1
		40	-	nd	nd	< 0.3	0.06
		53	+	+	+	40	3.57

Table 7 Characteristics of wheat anaphylactic patients and the outcome of the tests.
Patient 60 was grouped as WIA + co-factor because the allergic reaction is triggered by exercise only with previous consumption of clindamycin.
SPT: skin prick test, CAP: specific IgE determination by ImmunoCAP, ISAC: sIgE determination by Immuno-solid phase allergy chip expressed in ISU (compare table), ELISA: sIgE expressed in OD_{405}, nd: not done.

Upon analysis by the ISAC microarray, 8 of the 22 sera were tested positive for Tri a 19. The highest ISU-values were detected for the only 2 patients suffering from WIA or co-factor related WIA who also showed sIgE to Tri a 19 on the chip (39.52 ISU and 3.97 ISU respectively) whereas the 6 sera in the WDEIA group, in which sIgE to Tri a 19 could be detected, presented lower levels (range: 0.32 ISU – 1.67 ISU), see Table 7.

In addition to the ISAC, also ELISAs were performed To compare their sensitivities for all wheat allergic patients. In total, 16 of the 22 sera were tested positive for Tri a 19. For the WDEIA group 85% of the sera were positive using the ELISA approach, 50% of the WIA + co-factor group and 75% of the WIA group. In comparison, the ISAC method led to 43% positive tests in the WDEIA group and 25% positives for each WIA + co-factors and WIA groups. Hence the sensitivity of the ELISA method was higher in every group, see Table 7**.**

In conclusion, for 36% (n=8) of the patients with diagnosed wheat anaphylaxis sIgE to the major wheat allergen Tri a 19 could be detected using the ISAC microarray while the ELISA method was able to detect sIgE to Tri a 19 in 77% (n = 17) of the patients.
The ELISA results were also in better agreement with the ImmunoCAP tests than those of the ISAC due to the apparent higher sensitivity of the former method.

5.3 ISAC microarray for the diagnosis of food anaphylaxis

Conventional diagnosis

The 55 patients were previously diagnosed of their respective food allergy based on skin prick tests (SPT), determination of total IgE levels and sIgE to selected allergens or allergen extracts by ImmunoCAP, and oral food challenge. Based on these findings, a final diagnosis was made. The procedure to get to these diagnoses was rather complicated since the tests were expensive, time-consuming and/or required a clinician. The goal of this project was to analyze these serum samples by the microarray approach and compare the test regarding sensitivity and specificity to established in vitro methods.

Considering the diagnosis resulting from the conventional methods described above, 67 cases of anaphylaxis were identified in 55 allergic patients. The majority of the cases (n = 62) was related to plant food allergens whereas 5 cases displayed reactions due to food allergens from

animal products. Among the plant food elicitors wheat (n = 22) was by far the most frequently identified source which can be explained by a pre-selection for wheat anaphylactic patients that had been done. In 16 patients the eliciting food could not be definitely determined and they were thus declared unclear. Further detected elicitors were celery (n = 7) and soy (n = 5) as well as others like hazelnut, lupine (n = 2 for each), horseradish, curry/chili, carrot, pumpkin seed, peanut, cashew, potato, and apple (n = 1 for each), see Figure 18.

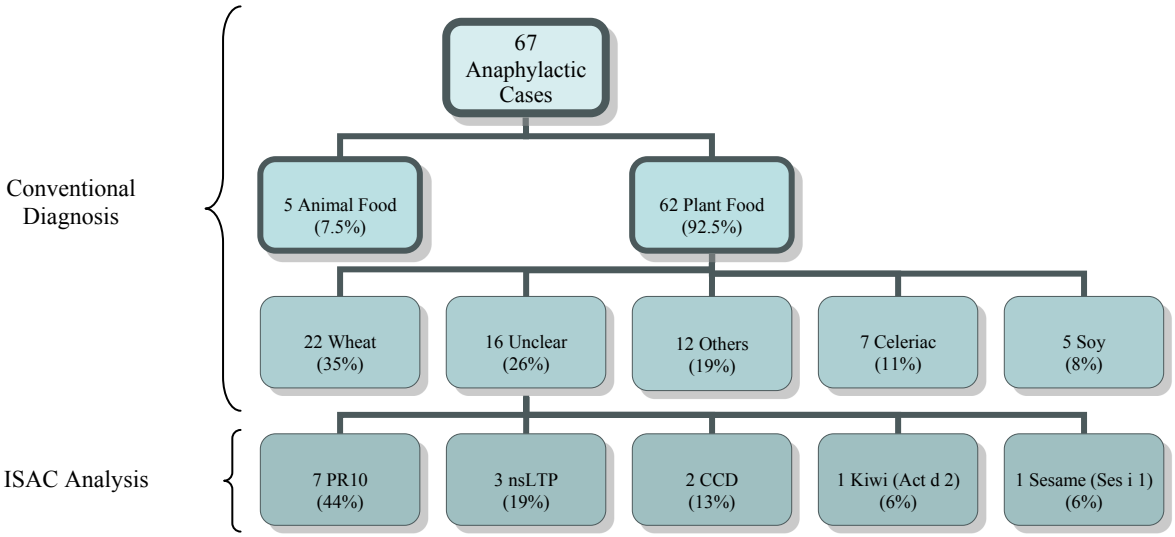

Figure 18 Classification of 55 patients' sera based on clinical history displaying the distribution of 67 reported anaphylactic cases to the most frequent food sources of their respective eliciting allergens and further analysis of cases with unclear elicitors by ISAC microarray.

ISAC analysis

All 55 sera were analyzed using the ISAC microarray. Of these, 41 tested positive for IgE reactivity to one or more allergens on the chip whereas 31 sera showed IgE specific for food allergens. In 14 sera no sIgE with reactivity to any allergen on the chip could be detected. Most of the patients were sensitized to more than 1 allergen while only 6 patients displayed mono-sensitization. Of these mono-sensitized patients 3 showed reactivity to Tri a 19, two to Art v 1 and one to Bet v 1. Additional 4 patients were tested positive for IgE to more than 1 allergen although all allergens to which they had reactive IgE were from one single source (2x grass pollen, 1x animal, 1x mite allergens, see Figure 20).

Of the 14 sera that did not give any result in the microarray, 5 patients suffered from anaphylaxis to wheat and in another 5 the elicitor was unknown. Two patients with anaphylaxis to soy, one to cashew and one to pumkinseed also did not display sIgE to any of the proteins on the microarray (see Figure 19).

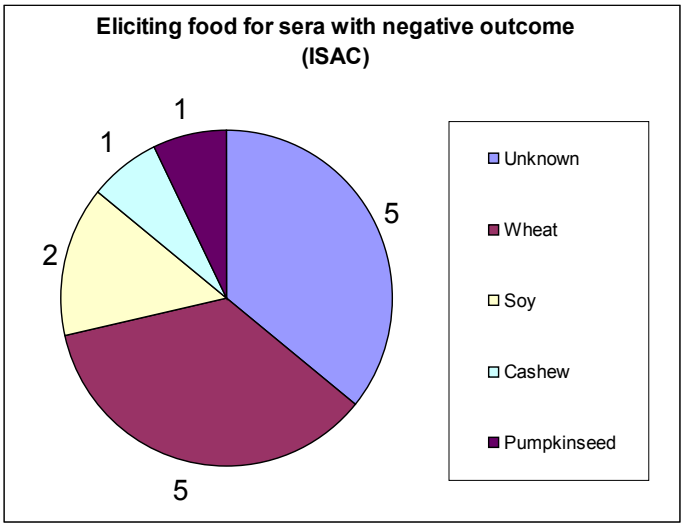

Figure 19 Sources of food allergens that cause anaphylactic reactions according to the anamnesis, and their distribution among patients that were tested negative on the ISAC micro-array. Numbers of cases elicited by the respective food are found next to the corresponding fragment.

The β-expansin from Bermuda grass pollen, Cyn d 1, was the most frequently recognized allergen with 22 patients having sIgE reactive to this protein. The PR10 protein, Bet v 1, was recognized by sIgE from 19 patients ranging on the second place. The food allergen giving the most positive tests were the PR10 proteins Pru p 1 and Cor a 1.0401 (n = 18) from peach and hazelnut, respectively while 31 allergens on the chip did not give a positive reaction with any of the 55 sera that were tested.

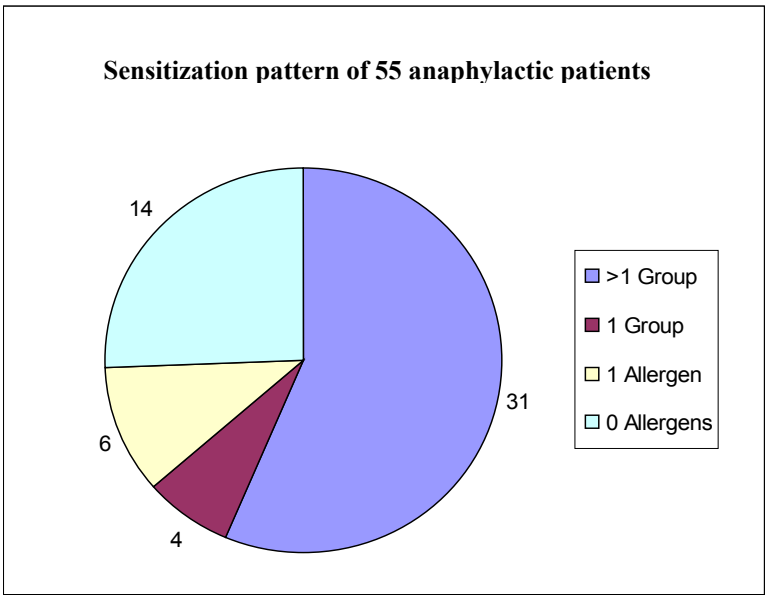

Figure 20 Sensitization pattern within the patients' collective according to ISAC analysis. The diagram shows the prevalence of polysensitization among the tested patients. Numbers next to the segments indicate the number of patients grouped as sensitized to no allergen on the chip / 1 allergen / more than 1 allergen within 1 group / allergens from more than 1 group.

The most frequent group of allergens giving a signal with 32 sera were pollen proteins (25 sera reacted to grass pollen, 24 to tree pollen and 15 to weed pollen allergens) followed by plant food allergens (31 sera).

Taking into concern that the cross-reactive protein families are present in food as well as pollen allergens, PR10 is the leading protein family being recognized by sIgE from 19 sera followed by ω-5-gliadin from wheat (n = 8), and nsLTPs (n = 6), see Table 8.

	Protein family	Source	n
	PR10	Celery, soy, hazelnut, apple	19
	ω-5-gliadin	Wheat	8
	nsLTP	Peach, hazelnut, curry/chili	6
	7S globulin	Peanut, soy	6
	Profilin	Latex	3
	2S albumin	Peanut, sesame	3

Table 8 The most frequent allergen groups as analyzed by the ISAC microarray and their respective food-relevant sources.

6 DISCUSSION

In the development of allergy the sensitizing phase is the critical event. During this process the allergen is taken up, processed and presented to Th2 cells by APCs. Due to interaction of the Th2 cells with B cells, IgE antibodies are produced and released to the circulation.
In the first part of this work this sensitization phase was analyzed. In particular, the optimal experimental settings for the examination of allergen uptake and endocytosis by DCs were established.
For this purpose, several cell culture conditions had to be determined.
In a first attempt 1 x 10^5 cells/ml and 4 x 10^5 cells/ml were seeded and the latter proved to be an adequate concentration to see a great number that would be sufficient to visualize the endocytosis of the sample protein since it could not be expected to be seen in each cell.

To determine if the quality of microscopy images varies using different surfaces to grow the cells on, glass coverslips and plastic slides were tested. No significant differences were noticed, background noises were equally low for both materials. Since the surface of the glass coverslips was bigger, they were chosen in the following experiments.

DCs were incubated with a sufficient amount of the positive control FITC-Dextran (10 mg/ml), which was shown to be taken up by DCs before [99] for 5 min and 60 min and the localization was checked by fluorescence microscopy. At 5 min FITC-Dextran had already been taken up resulting in a dotted staining close to the cell membrane.

All previous experiments had been done in the presence of 10% FCS in the medium, but due to suspected interference of serum components with the receptor binding properties, the effect of incubation with serum-free medium on the DCs was analzyed for the endocytosis assay as well as for the receptor binding assay.

Internalization could be detected with the positive control FITC-Tf and also with Ber e 1 and SFA8 labelled with OG and pH although the latter gave a weaker signal for both proteins. For a stronger signal the used concentration of pHrodo-labelled protein might have to be increased.

A time-scale experiment using FITC-Tf and Ber e 1-OG over 5, 10, and 30 min resulted in a nice, strong signal for FITC-Tf that grows stronger and locates closer to the nucleus the more time passes. For OG-Ber, however, the signal can only be seen at the 10 min time point. Internalization could be seen after 1 h so either the protein needs more time to be taken up by the DCs or the concentration was too low.

In order to answer the question whether or not the internalization is receptor-mediated, endocytosis was stopped at 4°C and binding of Ber e 1-OG, SFA8-OG, and FITC-Tf was detected. While FITC-Tf could be shown to bind to the surface of DCs, the result for Ber e 1-OG and SFA8-OG were not so clear. This could be due to a probably lower amount of receptors on the surface or a lower affinity of the proteins to the receptor. In this case the use of higher concentrations, again, might lead to a recognizable signal.

In the future, receptor binding experiments have to be performed for both Ber e 1 and SFA8 using higher concentrations and the endocytic route has to be examined closely. If any differences are visible that could be a further step towards the answer to the question: What makes an allergen an allergen?

Once an allergic disease is established in an individual, contact with the sensitizing allergen results in symptoms that can be severe and even lead to death. Thus, it is important to diagnose the patient and identify the eliciting allergen. However, the search for the concerning allergen is sometimes difficult due to various factors.
For the in vitro and in vivo diagnosis of allergy, allergen extracts used to be important. However, due to the different properties of allergens, some might be hard to extract and, thus, be underrepresented in an allergen extract. Thus, it has been a great achievement to introduce the component-resolved diagnostics. Here, instead of allergen extracts, purified allergens are used. This is an improvement because different allergens could be attributed different properties, which also determines their allergenic potential.

In a subproject, 20 sera from soy allergic patients were analyzed using ELISA and immunoblot and the results were compared to the outcomes of ISAC analysis. ELISA testing indicated involvement of the member from the PR-10 family in soy, Gly m 4 since specific IgE to Gly m 4 could be detected in 19 out of 20 sera.

The clinical relevance of PR-10 allergens in severe allergic reactions to food is subject to debate since they have been shown to be labile to peptic digestion and heat [82]. However, cases were reported in which Gly m 4 was involved in the development of severe allergic symptoms [83, 100].
Compared to the results obtained from testing with soy extract, CRD gave more specific and also more sensitive results. In ELISA, sIgE to soy extract could only be detected in 2 sera and in immunoblot they were 7 reactive sera. Due to some unspecific binding in the non-allergic control, however, no definite conclusions could be made.
A possible argument for the disadvantageous outcome of soy extract testing in respect to detection of Gly m 4 could be that in soy milk, an important soy product, lipids are present that stabilize the proteins while in aqueous extracts this matrix is absent [101]. This emphasizes the importance of CRD in allergy diagnosis.

In addition, sera from wheat allergic patients were screened for sIgE to the major wheat allergen Tri a 19 using ELISA and the ISAC microarray. While by ELISA testing 16 out of 22 sera showed sIgE reactivity, the microarray detected sIgE in only 8 sera.
Recently published studies stated the low sensitivity of the ISAC while its specificity was high [102, 103]. Brans and co-workers compared the sensitivity of the old version of the ISAC microarray to the newer 112 allergen version in the detection of Tri a 19. While they also obtained rather poor sensitivity using the older ISAC, the newer version delivered more positive results with the same sera [104].

Comparing patients' history to ISAC results, especially in cases for which the eliciting allergen source was unknown, ISAC provided valuable additional information.

In conclusion, the set-up for cell culture experiments using labelled allergens could be established and the internalization of Ber e 1 and SFA8 as well as the positive control could be shown nicely. However, the receptor binding of the two allergens remains to be confirmed.
Using the ISAC microarray in addition to conventional diagnostic methods, Gly m 4 could be shown to cause severe symptoms in soy allergic patients while the diagnosis of wheat allergy proved to be complex due to many negative outcomes in ELISA as well as in ISAC.
Although it lacks sensitivity in some cases, the ISAC microarray has the potential to be a powerful support in the diagnosis of allergic diseases.

7 REFERENCES

1. Johansson, S.G., et al., *A revised nomenclature for allergy. An EAACI position statement from the EAACI nomenclature task force.* Allergy, 2001. **56**(9): p. 813-24.

2. Descotes, J. and G. Choquet-Kastylevsky, *Gell and Coombs's classification: is it still valid?* Toxicology, 2001. **158**(1-2): p. 43-9.

3. Abbas, A.K., A.H. Lichtman, and S. Pillai, *Cellular and molecular immunology*. 6th ed2007, Philadelphia: Saunders Elsevier. viii, 566 p.

4. Rajan, T.V., *The Gell-Coombs classification of hypersensitivity reactions: a re-interpretation.* Trends Immunol, 2003. **24**(7): p. 376-9.

5. Mahnke, K., et al., *Tolerogenic dendritic cells and regulatory T cells: a two-way relationship.* J Dermatol Sci, 2007. **46**(3): p. 159-67.

6. Kumar, S., et al., *Molecular mechanisms of IgE mediated food allergy.* Int Immunopharmacol, 2012. **13**(4): p. 432-9.

7. Novak, N., et al., *Dendritic cells in allergy.* Allergy, 1999. **54**(8): p. 792-803.

8. Steinman, R.M. and Z.A. Cohn, *Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution.* J Exp Med, 1973. **137**(5): p. 1142-62.

9. Naik, S.H., *Demystifying the development of dendritic cell subtypes, a little.* Immunol Cell Biol, 2008. **86**(5): p. 439-52.

10. Naik, S.H., et al., *Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo.* Nat Immunol, 2007. **8**(11): p. 1217-26.

11. Shortman, K. and S.H. Naik, *Steady-state and inflammatory dendritic-cell development.* Nat Rev Immunol, 2007. **7**(1): p. 19-30.

12. Liu, K. and M.C. Nussenzweig, *Development and homeostasis of dendritic cells.* Eur J Immunol, 2010. **40**(8): p. 2099-102.

13. Caux, C., *Pathways of development of human dendritic cells.* Eur J Dermatol, 1998. **8**(6): p. 375-84.

14. Wu, L. and Y.J. Liu, *Development of dendritic-cell lineages.* Immunity, 2007. **26**(6): p. 741-50.

15. D'Amico, A. and L. Wu, *The early progenitors of mouse dendritic cells and plasmacytoid predendritic cells are within the bone marrow hemopoietic precursors expressing Flt3.* J Exp Med, 2003. **198**(2): p. 293-303.

16. Chicha, L., D. Jarrossay, and M.G. Manz, *Clonal type I interferon-producing and dendritic cell precursors are contained in both human lymphoid and myeloid progenitor populations.* J Exp Med, 2004. **200**(11): p. 1519-24.

17. Santana-de Anda, K., et al., *Plasmacytoid dendritic cells: Key players in viral infections and autoimmune diseases.* Semin Arthritis Rheum, 2013.

18. Martin, P., et al., *Characterization of a new subpopulation of mouse CD8alpha+ B220+ dendritic cells endowed with type 1 interferon production capacity and tolerogenic potential.* Blood, 2002. **100**(2): p. 383-90.

19. Arpinati, M., et al., *Role of plasmacytoid dendritic cells in immunity and tolerance after allogeneic hematopoietic stem cell transplantation.* Transpl Immunol, 2003. **11**(3-4): p. 345-56.

20. Rescigno, M., et al., *Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria.* Nat Immunol, 2001. **2**(4): p. 361-7.

21. West, M.A., et al., *Enhanced dendritic cell antigen capture via toll-like receptor-induced actin remodeling.* Science, 2004. **305**(5687): p. 1153-7.

22. Mellman, I. and R.M. Steinman, *Dendritic cells: specialized and regulated antigen processing machines.* Cell, 2001. **106**(3): p. 255-8.

23. Trombetta, E.S. and I. Mellman, *Cell biology of antigen processing in vitro and in vivo.* Annu Rev Immunol, 2005. **23**: p. 975-1028.

24. Yamazaki, S., et al., *Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells.* J Exp Med, 2003. **198**(2): p. 235-47.

25. Steinman, R.M., D. Hawiger, and M.C. Nussenzweig, *Tolerogenic dendritic cells.* Annu Rev Immunol, 2003. **21**: p. 685-711.

26. Hemmi, H., et al., *Skin antigens in the steady state are trafficked to regional lymph nodes by transforming growth factor-beta1-dependent cells.* Int Immunol, 2001. **13**(5): p. 695-704.

27. Tew, J.G., et al., *Follicular dendritic cells: beyond the necessity of T-cell help.* Trends Immunol, 2001. **22**(7): p. 361-7.

28. Cerovic, V., et al., *New insights into the roles of dendritic cells in intestinal immunity and tolerance.* Int Rev Cell Mol Biol, 2009. **272**: p. 33-105.

29. Conner, S.D. and S.L. Schmid, *Regulated portals of entry into the cell.* Nature, 2003. **422**(6927): p. 37-44.

30. Underhill, D.M. and A. Ozinsky, *Phagocytosis of microbes: complexity in action.* Annu Rev Immunol, 2002. **20**: p. 825-52.

31. Wills-Karp, M., *Allergen-specific pattern recognition receptor pathways.* Curr Opin Immunol, 2010. **22**(6): p. 777-82.

32. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nat Immunol, 2010. **11**(5): p. 373-84.

33. Trompette, A., et al., *Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein.* Nature, 2009. **457**(7229): p. 585-8.

34. Tan, A.M., et al., *TLR4 signaling in stromal cells is critical for the initiation of allergic Th2 responses to inhaled antigen.* J Immunol, 2010. **184**(7): p. 3535-44.

35. Royer, P.J., et al., *The mannose receptor mediates the uptake of diverse native allergens by dendritic cells and determines allergen-induced T cell polarization through modulation of IDO activity.* J Immunol, 2010. **185**(3): p. 1522-31.

36. Garrett, W.S., et al., *Developmental control of endocytosis in dendritic cells by Cdc42.* Cell, 2000. **102**(3): p. 325-34.

37. Savina, A. and S. Amigorena, *Phagocytosis and antigen presentation in dendritic cells.* Immunol Rev, 2007. **219**: p. 143-56.

38. Rock, K.L., S. Gamble, and L. Rothstein, *Presentation of exogenous antigen with class I major histocompatibility complex molecules.* Science, 1990. **249**(4971): p. 918-21.

39. Johansson, S.G., *The History of IgE: From discovery to 2010.* Curr Allergy Asthma Rep, 2011. **11**(2): p. 173-7.

40. Bennich, H.H., et al., *Immunoglobulin E: a new class of human immunoglobulin.* Immunology, 1968. **15**(3): p. 323-4.

41. Stone, K.D., C. Prussin, and D.D. Metcalfe, *IgE, mast cells, basophils, and eosinophils.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S73-80.

42. Kraft, S. and J.P. Kinet, *New developments in FcepsilonRI regulation, function and inhibition.* Nat Rev Immunol, 2007. **7**(5): p. 365-78.

43. Saini, S.S., et al., *Down-regulation of human basophil IgE and FC epsilon RI alpha surface densities and mediator release by anti-IgE-infusions is reversible in vitro and in vivo.* J Immunol, 1999. **162**(9): p. 5624-30.

44. Novak, N., et al., *Evidence for a differential expression of the FcepsilonRIgamma chain in dendritic cells of atopic and nonatopic donors.* J Clin Invest, 2003. **111**(7): p. 1047-56.

45. Kubota, T., et al., *Different stabilities of the structurally related receptors for IgE and IgG on the cell surface are determined by length of the stalk region in their alpha-chains.* J Immunol, 2006. **176**(11): p. 7008-14.

46. MacGlashan, D.W., Jr., *Endocytosis, recycling, and degradation of unoccupied FcepsilonRI in human basophils.* J Leukoc Biol, 2007. **82**(4): p. 1003-10.

47. Platzer, B., et al., *Soluble IgE receptors--elements of the IgE network.* Immunol Lett, 2011. **141**(1): p. 36-44.

48. Acharya, M., et al., *CD23/FcepsilonRII: molecular multi-tasking.* Clin Exp Immunol, 2010. **162**(1): p. 12-23.

49. Armitage, R.J., L.K. Goff, and P.C. Beverley, *Expression and functional role of CD23 on T cells.* Eur J Immunol, 1989. **19**(1): p. 31-5.

50. Kikutani, H., et al., *Structure and function of Fc epsilon receptor II (Fc epsilon RII/CD23): a point of contact between the effector phase of allergy and B cell differentiation.* Ciba Found Symp, 1989. **147**: p. 23-31; discussion 31-5.

51. Defrance, T., et al., *Human recombinant interleukin 4 induces Fc epsilon receptors (CD23) on normal human B lymphocytes.* J Exp Med, 1987. **165**(6): p. 1459-67.

52. Larche, M., C.A. Akdis, and R. Valenta, *Immunological mechanisms of allergen-specific immunotherapy.* Nat Rev Immunol, 2006. **6**(10): p. 761-71.

53. Hart, P.H., *Regulation of the inflammatory response in asthma by mast cell products.* Immunol Cell Biol, 2001. **79**(2): p. 149-53.

54. Grimbaldeston, M.A., et al., *Effector and potential immunoregulatory roles of mast cells in IgE-associated acquired immune responses.* Curr Opin Immunol, 2006. **18**(6): p. 751-60.

55. Shea-Donohue, T., et al., *Mast cells.* Curr Gastroenterol Rep, 2010. **12**(5): p. 349-57.

56. Fulkerson, P.C. and M.E. Rothenberg, *Targeting eosinophils in allergy, inflammation and beyond.* Nat Rev Drug Discov, 2013. **12**(2): p. 117-29.

57. Sicherer, S.H. and H.A. Sampson, *Food allergy.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S116-25.

58. Cianferoni, A. and J.M. Spergel, *Food allergy: review, classification and diagnosis.* Allergol Int, 2009. **58**(4): p. 457-66.

59. Naclerio, R., *Clinical manifestations of the release of histamine and other inflammatory mediators.* J Allergy Clin Immunol, 1999. **103**(3 Pt 2): p. S382-5.

60. Kraneveld, A.D., et al., *The two faces of mast cells in food allergy and allergic asthma: the possible concept of Yin Yang.* Biochim Biophys Acta, 2012. **1822**(1): p. 93-9.

61. Lieberman, P., et al., *Epidemiology of anaphylaxis: findings of the American College of Allergy, Asthma and Immunology Epidemiology of Anaphylaxis Working Group.* Ann Allergy Asthma Immunol, 2006. **97**(5): p. 596-602.

62. Simons, F.E., *Anaphylaxis.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S161-81.

63. Matsuo, H., et al., *Exercise and aspirin increase levels of circulating gliadin peptides in patients with wheat-dependent exercise-induced anaphylaxis.* Clin Exp Allergy, 2005. **35**(4): p. 461-6.

64. Palosuo, K., et al., *Transglutaminase-mediated cross-linking of a peptic fraction of omega-5 gliadin enhances IgE reactivity in wheat-dependent, exercise-induced anaphylaxis.* J Allergy Clin Immunol, 2003. **111**(6): p. 1386-92.

65. Boyce, J.A., et al., *Guidelines for the diagnosis and management of food allergy in the United States: summary of the NIAID-Sponsored Expert Panel Report.* Nutrition, 2011. **27**(2): p. 253-67.

66. Antunes, J., et al., *Skin prick tests and allergy diagnosis.* Allergol Immunopathol (Madr), 2009. **37**(3): p. 155-64.

67. Sampson, H.A., *Food allergy. Part 2: diagnosis and management.* J Allergy Clin Immunol, 1999. **103**(6): p. 981-9.

68. Gerez, I.F., et al., *Diagnostic tests for food allergy.* Singapore Med J, 2010. **51**(1): p. 4-9.

69. Asero, R., et al., *IgE-mediated food allergy diagnosis: Current status and new perspectives.* Mol Nutr Food Res, 2007. **51**(1): p. 135-47.

70. Berni Canani, R., M. Di Costanzo, and R. Troncone, *The optimal diagnostic workup for children with suspected food allergy.* Nutrition, 2011. **27**(10): p. 983-7.

71. Garcia, B.E. and M.T. Lizaso, *Cross-reactivity syndromes in food allergy.* J Investig Allergol Clin Immunol, 2011. **21**(3): p. 162-70; quiz 2 p following 170.

72. Breiteneder, H. and C. Radauer, *A classification of plant food allergens.* J Allergy Clin Immunol, 2004. **113**(5): p. 821-30; quiz 831.

73. Sun, S.S., S.B. Altenbach, and F.W. Leung, *Properties, biosynthesis and processing of a sulfur-rich protein in Brazil nut (Bertholletia excelsa H.B.K.).* Eur J Biochem, 1987. **162**(3): p. 477-83.

74. Alcocer, M., L. Rundqvist, and G. Larsson, *Ber e 1 protein: the versatile major allergen from Brazil nut seeds.* Biotechnol Lett, 2012. **34**(4): p. 597-610.

75. Pastorello, E.A., et al., *Sensitization to the major allergen of Brazil nut is correlated with the clinical expression of allergy.* J Allergy Clin Immunol, 1998. **102**(6 Pt 1): p. 1021-7.

76. Kean, D.E., et al., *Differential polarization of immune responses by plant 2S seed albumins, Ber e 1, and SFA8.* J Immunol, 2006. **177**(3): p. 1561-6.

77. Murtagh, G.J., et al., *In vitro stability and immunoreactivity of the native and recombinant plant food 2S albumins Ber e 1 and SFA-8.* Clin Exp Allergy, 2003. **33**(8): p. 1147-52.

78. Dearman, R.J., M.J. Alcocer, and I. Kimber, *Influence of plant lipids on immune responses in mice to the major Brazil nut allergen Ber e 1.* Clin Exp Allergy, 2007. **37**(4): p. 582-91.

79. Wensing, M., et al., *IgE to Bet v 1 and profilin: cross-reactivity patterns and clinical relevance.* J Allergy Clin Immunol, 2002. **110**(3): p. 435-42.

80. van Loon, L.C., M. Rep, and C.M. Pieterse, *Significance of inducible defense-related proteins in infected plants.* Annu Rev Phytopathol, 2006. **44**: p. 135-62.

81. Radauer, C. and H. Breiteneder, *Evolutionary biology of plant food allergens.* J Allergy Clin Immunol, 2007. **120**(3): p. 518-25.

82. Schimek, E.M., et al., *Gastrointestinal digestion of Bet v 1-homologous food allergens destroys their mediator-releasing, but not T cell-activating, capacity.* J Allergy Clin Immunol, 2005. **116**(6): p. 1327-33.

83. Kleine-Tebbe, J., et al., *Severe oral allergy syndrome and anaphylactic reactions caused by a Bet v 1-related PR-10 protein in soybean, SAM22.* J Allergy Clin Immunol, 2002. **110**(5): p. 797-804.

84. Ausukua, M., et al., *Oral Allergy Syndrome (OAS). General and stomatological aspects.* Med Oral Patol Oral Cir Bucal, 2009. **14**(11): p. e568-72.

85. Vieths, S., *Allergenic cross-reactivity, food allergy and pollen.* Environ Toxicol Pharmacol, 1997. **4**(1-2): p. 61-70.

86. Kazemi-Shirazi, L., et al., *Quantitative IgE inhibition experiments with purified recombinant allergens indicate pollen-derived allergens as the sensitizing agents responsible for many forms of plant food allergy.* J Allergy Clin Immunol, 2000. **105**(1 Pt 1): p. 116-25.

87. Vieths, S., S. Scheurer, and B. Ballmer-Weber, *Current understanding of cross-reactivity of food allergens and pollen.* Ann N Y Acad Sci, 2002. **964**: p. 47-68.

88. Pascual, C., M. Martin Esteban, and J.F. Crespo, *Fish allergy: evaluation of the importance of cross-reactivity.* J Pediatr, 1992. **121**(5 Pt 2): p. S29-34.

89. Shanti, K.N., et al., *Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes.* J Immunol, 1993. **151**(10): p. 5354-63.

90. Carrillo, T., et al., *Squid hypersensitivity: a clinical and immunologic study.* Ann Allergy, 1992. **68**(6): p. 483-7.

91. Mari, A., et al., *Specific IgE to cross-reactive carbohydrate determinants strongly affect the in vitro diagnosis of allergic diseases.* J Allergy Clin Immunol, 1999. **103**(6): p. 1005-11.

92. Aalberse, R.C., J. Akkerdaas, and R. van Ree, *Cross-reactivity of IgE antibodies to allergens.* Allergy, 2001. **56**(6): p. 478-90.

93. Ishihara, H., et al., *Complete structure of the carbohydrate moiety of stem bromelain. An application of the almond glycopeptidase for structural studies of glycopeptides.* J Biol Chem, 1979. **254**(21): p. 10715-9.

94. Chung, C.H., et al., *Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose.* N Engl J Med, 2008. **358**(11): p. 1109-17.

95. Brinkmann, M., et al., *Expression of iron transport proteins divalent metal transporter-1, Ferroportin-1, HFE and transferrin receptor-1 in human monocyte-derived dendritic cells.* Cell Biochem Funct, 2007. **25**(3): p. 287-96.

96. Natarajan, S.S., et al., *An efficient extraction method to enhance analysis of low abundant proteins from soybean seed.* Anal Biochem, 2009. **394**(2): p. 259-68.

97. Mittag, D., et al., *Soybean allergy in patients allergic to birch pollen: clinical investigation and molecular characterization of allergens.* J Allergy Clin Immunol, 2004. **113**(1): p. 148-54.

98. Berneder, M., et al., *Allergen Chip Diagnosis for Soy-Allergic Patients: Gly m 4 as a Marker for Severe Food-Allergic Reactions to Soy.* Int Arch Allergy Immunol, 2013. **161**(3): p. 229-33.

99. Kato, M., et al., *Expression of multilectin receptors and comparative FITC-dextran uptake by human dendritic cells.* Int Immunol, 2000. **12**(11): p. 1511-9.

100. Fukutomi, Y., et al., *Clinical relevance of IgE to recombinant Gly m 4 in the diagnosis of adult soybean allergy.* J Allergy Clin Immunol, 2012. **129**(3): p. 860-863 e3.

101. Grimshaw, K.E., et al., *Presentation of allergen in different food preparations affects the nature of the allergic reaction--a case series.* Clin Exp Allergy, 2003. **33**(11): p. 1581-5.

102. Javaloyes, G., et al., *Performance of different in vitro techniques in the molecular diagnosis of peanut allergy.* J Investig Allergol Clin Immunol, 2012. **22**(7): p. 508-13.

103. Olivieri, M., et al., *Wheat IgE profiling and wheat IgE levels in bakers with allergic occupational phenotypes.* Occup Environ Med, 2013. **70**(9): p. 617-22.

104. Brans, R., et al., *Microarray-based detection of specific IgE against recombinant omega-5-gliadin in suspected wheat-dependent exercise-induced anaphylaxis.* Eur J Dermatol, 2012. **22**(3): p. 358-62.

8 SUPPLEMENT: FOOD ALLERGIC PATIENTS WITH ANAPHYLACTIC EPISODES IN THEIR CASE HISTORY

No.	Age	Anaphylaxis-eliciting allergen	Affected system	Skin-prick test		Total IgE [kU/l]	sIgE [kUA/l]	ISAC [ISU]				ELISA
1	56	**Hazelnut**	Respiration	Hazelnut 4 Mussel 2 Walnut 3 Peanut 3 Apple 4 Carrot 3 Celeriac4 Tomato 2 Lupine flour 2 Hazel pollen 7 Alder 8	Birch 8 Grass 3 Mugwort 7 Parietaria 2 Dog 2 Cashew 3 Sultana 2	58,5	Hazelnut 2,24 Birch 2,79 Peanut 0,11	nPhl p 4 2,6 rBet v 1 15 rAln g 1 3,4 rCor a 1.0101 1,6 nArt v 1 0,7 rCor a 1.0401 4,5 rMal d 1 3,2 rPru p 1 2 rGly m 4 0,8 rAra h 8 3,1				Ara h 1: neg Ara h 3/4: neg Ara h 6: neg Dau c 1 neg Api g 1 neg
2	33	**Seafood**	**Unknown**	Mussel >6 Crab >6	Squid 6 Birch 2-4	101	Shrimps 0,00 Krabbe 0,06	rFel d 4 4,6 nMus m 1 3				Bet v 1 neg.
3	32	**Celeriac**	Respiration	Celeriac 7 Mussel 2 Apple 2 Carrot 2 Celeriac 7 Grass 4 Ambrosia 2	Aspergillus fumigatus 1 D. farinae 4 D. Pteronyssinus 4 Rye 4 Mugwort 4 Alder 2	65,6	Celeriac 0,06 Apple 0,08 Carrot 0,07 Mussel 0,06	nCyn d 1 0,5 rPhl p 1 1,1 rPhl p 2 0,8 nPhl p 4 1,7 rPhl p 11 1,2 nOle e 1 1,6	rEur m 2 0,3 nArt v 1 14,2 nDer p 1 0,7 nDer f 1 0,8 nDer p 2 1,4 rDer f 2 1,2			BBQ 0.01 Mal d 1 0,1 Dau c 1 0,15 Api g 1 0,15
4	26	**Lupine flour**	Respiration and Circulation	Lupine flour5		241	n.d.	nAct d 2 2 nCyn d 1 4,7 rPhl p 1 4,9 nPhl p 4 4,3 rPhl p 5 24 rPhl p 6 1,3 rPhl p 11 3,3 rBet v 1 13	rAln g 1 4,1 rCor a 1.0101 3,3 nOle e 1 1,4 nArt v 1 0,8 rAlt a 1 5,1 rCor a 1.0401 6,7 rMal d 1 3,2 rPru p 1 1,2	rAra h 8 0,6 rBet v 2 0,4 nOle e 2 0,5 rHev b 8 0,4 rMer a 1 0,9 rPhl p 12 0,5	Ara h 1: neg Ara h 3/4: neg Ara h 6: neg
5	44	**Wheat**	Respiration	Hazelnut 3 Barley flour 5 Rye flour 4	Oat flour 5 Wheat grain 4 Wheat flour 6	34	ω-5 Gliadin 0,03 Lupine seed 0,03 Wheat flour					Tri a 19 0.07

						6,06			
6	33	**Wheat + co-factors**	Circulation	Pepper 2		17,2 Wheat flour 0,01 Tomato 0,0 ω-5 Gliadin 0			BBQ neg. Tri a 19 0.04
7	55	**Wheat + co-factors**	**Unknown**	Wheat grain 5 Wheat flour 10	191	Wheat flour 2; Wheat flour 1,12 C 0,73 ω-5-Gliadin 4 Der p 0,76	rTri a 19.0101 4		Tri a 19 1,18
8	26	**Celeriac**	Respiration and Circulation	Hazelnut (native) 6 Peanut 7 Soybean 5 Apple (native) 5 Carrot (native) 11 Celeriac (native) 13 Tomato (native) 5 Camomille 6 Curry 4	554	Celeriac 10,3 Seafood 0.02 Carrot 9,03 Tomato 0,09 Nuts 95,00 Grass + Der p 0,05 Der f 0,01 Rye 12,2 Alder 47,7 Birch 51,1 Hazel pollen 32,3 Oak 34,7 Ambrosia 2,74 Mugwort 44,2	nAct d 2 2,5 nCyn d 1 14 rPhl p 1 7,9 rPhl p 2 17 rPhl p 5 1,2 rBet v 1 26 rAln g 1 26 r Cor a 1.0101 12 nArt v 1 45	rAlt a 1 2,1 r Cor a 1.0401 17 rMal d 1 11 rPru p 1 7,1 rGly m 4 14 rAra h 8 30 rApi g 1 13 rDau c 1 1,5	Ara h 1: neg Ara h 3/4: neg Ara h 6: neg BBQ neg. Cor a 8 neg Cor a 9 neg Cor a 11 neg Cor a 14 neg
9	59	**unclear**	Respiration and Circulation	Bell pepper 4	199	Bell pepper 1,22 Tomato 1,35 ω-5-Gliadin 0,11 Sesame 1,17 Celeriac 1,09 Birch 1,14 Mugwort 2,15 Timothy 1,44	nAct d 2 0,6 nCyn d 1 2,2 nPhl p 4 2,3 nPla a 2 1,3 nArt v 1 2,4 rAlt a 1 5,3 rAsp f 3 3,3	nApi m 1 1 nAna c 2 0,9 rFel d 1 22 rFel d 4 2,1 rCan f 1 0,9 nMus m 1 1,5	BBQ 0,06 Api g 1 neg
10	64	**Wheat**	Circulation	Hen's egg 3 Cow's milk 3 Pork 3 Mussel 3 Walnut 3 Hazelnut 9 Peanut 3 Soybean 3 Barley flour 4 Rye flour 3 Wheat flour 4 Oat flour 5 Apple 5 Carrot 3 Celeriac 5 Tomato 3 Pepper 2	247	Wheat 1,72 Rye 5,52 ω-5-Gliadin 0 Pork 0,06 Egg white 0,05 Birch 66,9 Alder 55,3 Timothy 4,37	nCyn d 1 0,8 rPhl p 1 1,2 rPhl p 2 2,1 nPhl p 4 0,9 rBet v 1 18 rAln g 1 7,5 rCor a 1.0101 5,8 rFel d 1 0,6 rCor a 1.0401		Api g 1 neg Dau c 1 neg Tri a 19 1

				Wheat flour (native) 10 Hazel pollen 20 Alder 12	Gluten 9 Birch 15 Grass 13			6,4 rMal d 1 4,6 rPru p 1 8,6	
11	45	**Wheat + exercise**	Respiration	Rye flour 2 Wheat flour 10	Wheat grain 8	405	ω-5-gliadin 7,95 Der p 5,59 Der f 5,70 Wheat flour 1,24 Seafood 0,10	rTri a 19.0101 1,7 rAsp f 1 1,5	Tri a 19 0,8
13	65	**unclear**	Circulation	Hazel pollen 11 Alder 8 Birch 11 Grass 4 Mugwort 6 Ambrosia 7 Hen's egg 3 Cow's milk 3 Pork 3 Herring 3 Mackerel 5 Crab 4 Mussel 4 Walnut 4 Hazelnut 10	Rye flour 3 Wheat flour 5 Oat flour 5 Apple 4 Carrot 7 Celeriac 6 Tomato 5 Lupinen flour 13 Almond 5 Coconut 2 Sesame 5 Peanut 6 Soybean 5 Barley flour 4	132	Egg white 0,01 Milk protein 0,06 Celeriac 1,2 Nuts 12,3 Seafood 0,00 Grass 0,01 Birch 18,9 Hazel pollen 10,2 Herbs 0,26 Peanut 0,00 Hazelnut 11,4 Almond 0,02 Coconut 0,01 Wheat flour 0,01 ω-5-gliadin 0,00 Sesame 0,09	rBet v 1 17 rAln g 1 9,9 rCor a 1.0101 7,1 nAmb a 1 1,6 nArt v 1 0,4 rFel d 1 11 rCor a 1.0401 11 rPru p 1 2,8 rGly m 4 1,7 rAra h 8 3,1 rApi g 1 1,2	BBQ neg. Cor a 8 neg Cor a 9 neg Cor a 11 neg Cor a 14 neg
14	37	**Horseradish**	Respiration and Circulation	Grapes 2 Plane 3 Grass 4 Wheat flour 4	Mugwort 4 Mussel 2 Barley flour 2 Tomato 2	59,3	Latex 0,03 Rye flour 0,04 Celeriac 0,02 Tomato 0,05 ω-5-gliadin 0,00 Birch 0,02 Mugwort 0,03 Timothy 0,64 Wheat flour 0,03 Seafood 0,00 Tryptase 1,59	nCyn d 1 0,4 rPhl p 1 0,5	BBQ neg.
15	21	**unclear**	Respiration	Sunflower seed 3 Daisy 3 Artemisia 2	Celeriac 5 Soybean 2	139	Peach 0,00 Birch 0,03 Mugwort 29,8 Apple 0,20 Celeriac 0,18 Soybean 0,02	nArt v 1 6,3	BBQ 0,35
16	45	**Celeriac**	Respiration and Circulation	Cherry 7 Camomille 6	Hazelnut 4 Peanut 5	555	Birch 0,45 Mugwort >100	nArt v 1 28 nPru p 3 5,3	BBQ 0,07 Bet v 1 0,05

				Bell pepper spice 3 Curry mix 3 Lupine flour 3	Oat flour 3 Carrot 3 Celeriac 7 Onion 4		Grass 6,67	rCor a 8 2,3 nArt v 3 47			
17	67	**unclear**	Circulation	Alder 4 Mugwort 5 Ambrosia 3 Derm. pteronyssinus 10 Derm. farinae 6	Mussel 3 Peanut 4	360	Timothy 1,03 Derm. pteronyssinus 10,1 Derm. farinae 15,7 Nuts neg.	nDer p 1 2,7 nDer f 1 2,3 nDer p 2 5,5 rDer f 2 5,4 rEur m 2 1,2			Ara h 1: neg Ara h 3/4: neg Ara h 6: neg BBQ neg.
18	27	**Wheat + exercise**	**Unknown**	Wheat 2 - 5 Rye 5		24,5	Gluten 1.59 Rye flour 1.42 Wheat flour 0.59 ω-5-gliadin 0				Tri a 19 0,07
20	35	**unclear**	Respiration and Circulation	Hazelnut 2		256	n.d.				
21	30	**Lupine, Soy, Peanut**	Respiration and Circulation	Hazel pollen 21 Alder 15 Birch 8 Grass 10 Alternaria 18 Hazelnut 6 Peanut 25	Soy 5 Lupine 3 Bell pepper spice 3 Curry 25 Apple 4 Carrot 7 Celeriac 9	585	Birch 75,7 Timothy 21,2 ω-5-gliadin 0 Wheat flour 0,34 Celeriac 1,94	nAct d 2 3 nAra h 1 20 nAra h 2 31 nAra h 3 7,9 nGly m 5 0,3 nGly m 6 0,9 nCyn d 1 11 rPhl p 1 7,9 rPhl p 2 0,8	rPhl p 5 8,4 rPhl p 6 0,7 rBet v 1 42 rAln g 1 39 rCor a 1.0101 15 rFel d 1 6,8 rCan f 1 3,6 nMus m 1 2,2 rAlt a 1 7,6	rCor a 1.0401 24 rMal d 1 23 rPru p 1 21 rGly m 4 1,5 rAra h 8 14 nAct d 8 1,2 rApi g 1 0,7	
22	39	**Wheat + exercise**	Circulation	Wheat flour 6 Wheat grain 5		145	ω-5-gliadin 3,68	nGly m 5 0,5 rFel d 1 1	rFel d 4 0,5 n Fel d 2 0,3		Tri a 19 0,99
23	21	**unclear**	Respiration	Wheat flour 5 Wheat grain 2	Rye 3 Oat 3	67,5	**n.d.**				Tri a 19 2,48
25	33	**unclear**	Respiration	Mugwort 12		17,9	ω-5-gliadin 0	nArt v 1 0,3			
26	27	**unclear**	Respiration and Circulation	Mussel 4 Soybean 3 Barley 3 Rye 3 Carrot 4 Celeriac 5 Tomato 4 Lupine flour 3	Kiwi 7 Mustard 8 Almond 3 Walnut 4 Hazelnut 8 Strawberry 4 Spinach 4 Mango 4	723	ω-5-gliadin 0,1	nCyn d 1 23 rPhl p 1 15 rPhl p 2 11 rPhl p 5 9,6 rPhl p 6 2,9 rBet v 1 35 rAln g 1 24 rCor a 1.0101 15 nOle e 1 2,1	nCup a 1 0,7 nArt v 1 0,9 rFel d 1 2,8 rAlt a 1 0,5 rCor a 1.0401 8,3 rMal d 1 11 rPru p 1 8 rAra h 8 2,2 nAct d 8 0,9	nPru p 3 4,3 nArt v 3 30 rBet v 2 1,5 nOle e 2 1 rHev b 8 1,1 rMet a 1 2,2 rPhl p 12 0,9 rApi g 1 3,3 nCry j 1 0,4	
27	33	**Wheat + exercise**	Respiration and Circulation	Wheat flour 5	Wheat grain 2	210	ω-5-gliadin 10,80				Tri a 19 2,8

					Mussel 3		Wheat flour 0,60				
28	36	**Wheat + exercise + co-factors**	Respiration	Mussel 4 Soybean 3 Wheat flour 3	Oat flour 3 Tomato 4	145	ω-5-gliadin 4,34 Wheat flour 0,46	rTri a 19.0101 0,6			Tri a 19 0,26
29	40	**Wheat + exercise**	Circulation	Grass 5 Wheat grain 4	Mussel 2 Wheat flour native 10 Oat flour 4	96,4	Timothy 3,34 Barley flour 0,30 Oat flour 0,07 ω-5-gliadin 9,85 Rye flour 3,59 Wheat flour 1,43 Tryptase 5,03	rTri a 19.0101 1,4 nCyn d 1 0,4 rPhl p 1 1 nPhl p 4 0,8 rPhl p 5 2,2			Tri a 19 1,14
30	47	**unclear**	Respiration and Circulation	Hen's egg 3 Walnut 4 Hazelnut 8 Soybean 5 Barley flour 4 Rye flour 5 Wheat flour 5 Apple 6 Carrot 10	Celeriac 19 Tomato 6 Lupine 4 Hazel pollen 16 Alder 9 Birch 10 Grass 9 Mugwort 9 Ambrosia 6	89,7	Celeriac 7,51 Lupine seed 1,22 Gly-m-4 2,13	nSes i 1 3 nCyn d 1 2,2 nPhl p 4 1,2 rPhl p 11 0,7 rBet v 1 7,6 rAln g 1 3,9 rCor a 1.0101 2,8 rOle e 1 0,8 rPla a 2 1,4	nArt v 1 0,9 rFel d 1 3,1 nDer p 1 7,9 nDer f 1 10 nDer p 2 14 rDer f 2 19 rEur m 2 2,2 nApi m 1 1,1 rCor a 1.0401 5,4	rPru p 1 3,1 rGly m 4 0,4 rAra h 8 1,6 rApi g 1 9,2 rDau c 1 8,3 nAna c 2 1 rMal d 1 3,5 nCup a 1 0,3	Cor a 8 neg Cor a 9 neg Cor a 11 0.1 Cor a 14 neg
31	51	**Wheat + co-factors**	Respiration and Circulation	Apple 3 Carrot 3 Celeriac 5 Tomato 3 Lupine flour 3	Hazel pollen 10 Alder 6 Birch 7 Grass 6 Mugwort 13	488,3	Tryptase 1,36 ω-5-gliadin 0,02 Wheat flour 1,37 Rye flour 0,93 Alder 0,58 Birch 1,65 Mugwort 9,17	nCyn d 1 1,7 rPhl p 2 0,5 rBet v 1 2,9 rAln g 1 0,6 rCor a 1.0101 0,7 rCor a 1.0401 1,2 nArt v 1 44	rPru p 1 1,1 nArt v 3 49		Dau c 1 neg Tri a 19 neg
32	60	**Soy**	Circulation	Hazelnut 3 Peanut 3 Soybean 3 Barleyn flour 3 Rye flour 3 Wheat flour 3	Carrot 3 Celeriac 3 Tomato 3 Lupine 4 Pepper 5 Apple 3	262	Tryptase 5,17 Mugwort 1,07 Peanut 0,47 Lupine flour 0,98 Buckwheat flour 4,15 Spelt 4,78				Ara h 1: neg Ara h 3/4: neg Ara h 6: neg BBQ neg. Bet v 1 neg Tri a 19 neg
34	47	**Celeriac**	Circulation	Alder 3 Birch 3 Plane 3	Soybean 5 Rye flour 4 Carrot 5	86,6	Tryptase 4,62 Gly-m-4 0,85	nCyn d 1 2 nPhl p 4 1,9 nOle e 1 1	nPla a 2 1,3 nAna c 2 0,5		

				Grass 3 Ambrosia 3 Cockroach 3 Hazelnut 4	Celeriac 4 Tomato 4 Peanut 4						
35	50	**unclear**	Respiration and Circulation	Hazel pollen 4 Alder 4 Birch 8	Grass 7 Walnut 2 Hazelnut 5	105	Milk protein 0,05	nPhl p 4 0,3 rPhl p 5 6,4 rPhl p 11 1,5 rBet v 1 2,9	rAln g 1 0,4 rCor a 1.0401 1,4 rMal d 1 1,4 rPru p 1 1		Cor a 8 neg Cor a 9 neg Cor a 11 neg Cor a 14 neg
36	28	**Curry/Chili + exercise**	Respiration and Circulation	Hazelnut native 9 Peanut 5 Rye flour 3 Wheat flour4 Apple native 4 Celeriac native 5 Tomato native 3 Lupine flour native 5	Basil 3 Aubergine native 5 Curry native 15 Chili native 10 Bell pepper spice 8 Soy native 3 Coriander native 5	796	Celeriac 6,63 Nuts 42,4 Birch 69,1 Hazel pollen 35,6 Grass 18,6	nAct d 2 1,5 nCyn d 1 5,8 rPhl p 1 2,1 rPhl p 2 6,7 nPhl p 4 1 rPhl p 11 7,4 rBet v 1 80 rAln g 1 4 rCor a 1.0101 12	rFel d 1 35 nMus m 1 7,4 nDer p 1 7,8 nDer f 1 4,9 nDer p 2 7,3 rDer f 2 7,9 rEur m 2 2,5 rCor a 1.0401 24 rMal d 1 31	rAra h 8 23 nPru p 3 2 rCor a 8 1,9 nArt v 3 0,8 rPru p 1 31 rGly m 4 0,5 nOle e 1 4,9 nPla a 2 0,7	BBQ neg. Cor a 8 0.1 Cor a 9 neg Cor a 11 neg Cor a 14 neg
37	50	**Soy**	Respiration and Circulation	Grass 7		38,9	Tryptase 5,94				
38	38	**unclear**	Respiration and Circulation	Hazel pollen 4 Alder 3 Mugwort 1-2 Carrot 5 Celeriac 2 Tomato 2 Hazelnut 2 Chili 3	Coriander 8 Bay 4 Avocado 3 Soy 3 Curry 4 Camomille 3 Birch 4	38,9	Tryptase 3,19 Birch 17,2 Celeriac 1,99	rBet v 1 4,3 rAln g 1 3,7 rCor a 1.0101 7 rCor a 1.0401 2,6 rMal d 1 1,5 rPru p 1 1,2 rAra h 8 4,1 rApi g 1 0,9			BBQ neg. Dau c 1 neg
39	56	**Wheat + exercise**	Respiration and Circulation	Wheat flour 13 Wheat grain 5	Oat flour 6	54,9	ω-5-gliadin 16,5	rTri a 19.0101 0,8			Tri a 19 1,23
40	34	**Wheat, Potato, Celeriac, co-factors**	Respiration and Circulation	 Hazel pollen 3 Alder 4 Birch 4 Mugwort 3	Hazelnut 3 Apple 3 Tomato 4	80,7	Tryptase 3,10 Birch 0,36 Wheat flour 0,08 ω-5-gliadin 0,01 Celeriac 0,05 Gly-m-4 0,05 Potato 0,08	rBet v 1 0,4			Gly m 4 0,02 Tri a 19 0,06
41	69	**Celeriac**	Respiration and		Wheat grain 7	203	Birch 89,9	nCyn d 1 1,7	rCor a 1.0401	rApi g 1 1,3	BBQ neg.

			Circulation	Hazel pollen 10 Alder 10 Birch 20 Grass 8	Walnut 8 Celeriac 9 Soymilch 10		Timothy 1,39 Celeriac 2,44 Gly-m-4 21,5 Tryptase 5,04	rPhl p 1 0,6 rPhl p 2 0,6 rBet v 1 39 rAln g 1 33	24 rMal d 1 16 rPru p 1 17 rGly m 4 15 rAra h 8 20	nAct d 8 2,7 rCor a 1.0101 18	Tri a 19 neg
42	53	**Cashew**	Respiration and Circulation	n.d.		672	Tryptase 5,2 Latex 0,09 Cashew 0,04 Nuts 0,44 Celeriac 0,39 Milk protein 0,93 Egg white 1,05				
43	31	**Wheat + exercise**	Respiration and Circulation	Wheat (gluten) 8		660	ω-5-gliadin 12,8	rTri a 19.0101 0,9 rPen a 1 0,3 nPen i 1 0,4	rDer p 10 5,9 nBla g 7 0,6 nPen m 1 0,5	rAni s 3 0,7	Tri a 19 1,31
44	47	**Seafood, Soy**	Respiration and Circulation	Hazelnut 7 Peanut 6 Barley flour 2 Rye flour 2 Apple 5	Carrot 7 Celeriac 8 Tomato 8 Shrimps 6 Soy milk 4	1310	Tryptase 2,37 gly-m-4 7,33 Seafood 1,03 Celeriac 5,21	nAct d 1 0,6 nGly m 5 0,3 nCyn d 1 12 rPhl p 1 15 rPhl p 2 22 nPhl p 4 15 rPhl p 6 6,3 rPhl p 11 17 rBet v 1 85	rAln g 1 35 rCor a 1.0101 11 nPla a 2 1,3 nArt v 1 3,8 nGal d 1 0,5 rFel d 1 41 rDer f 2 8,6 rEur m 2 2,5 nApi m 1 0,5	rCor a 1.0401 31 rMal d 1 46 rPru p 1 17 rGly m 4 1,1 rAra h 8 2 rHev b 8 4,3 rPhl p 12 2,5 nAna c 2 0,3 rBet v 2 5,3 nOle e 2 5	BBQ 0.01
45	29	**unclear**	Respiration and Circulation	Hazel pollen 8 Alder 5 Birch 19 Grass 4 Ambrosia 3 Hazelnut 7 Peanut 5 Soybean 3 Rye flour 3	Apple 4 Carrot 4 Celeriac 3 Tomato 3 Lupine 4 Camomille 3 Bell pepper spice 3 Pepper 3 Curry 4	194	Tryptase 3,61 ω-5-gliadin 0,13	nCyn d 1 4,4 rPhl p 1 1,3 nPhl p 4 4,3 rBet v 1 27 rAln g 1 5,9 rCor a 1.0101 2,3 nPla a 2 2,1	nApi m 1 2 rCor a 1.0401 7 rMal d 1 8,9 rPru p 1 6,9 rGly m 4 2 rAra h 8 2,4 nPru p 3 3,4	rCor a 8 1,1 nAna c 2 1,5 nCup a 1 0,3 rFel d 1 0,8 rCan f 1 0,3	Tri a 19 0,02
46	49	**unclear**	Respiration and Circulation	Banana 3		<2	negative				
47	38	**unclear**	Respiration and Circulation	Mugwort 8 Ambrosia 2 Derm. Pteronyssimus 2	Cockroach 2 Camomille 2 Onion 2	49,7	Der p 0,33				BBQ neg.
48	40	**unclear**	Respiration and Circulation	negative		49,8	negative				Tri a 19 0,02

50	42	**unclear**	Respiration and Circulation	Hazel pollen 4 Alder 6 Birch 11 Grass 11 Mugwort 8 Cat 7 Dog 4 Cockroach 3	Soybean 5 Peanut 8 Hazelnut 12 Apple 8 Lupine flour 5 Fennel 7	282	Celeriac 0,91 Nuts 9,28 Birch 25,1 Grass 16,5 Hazel pollen 12,1 Ambrosia 3,09 Wheat flour 0,88 Lupine seed 1,88 Herbs 2,85 Der p 0,25 Cat dander 5,76 Dog dander 2,32	nCyn d 1 0,4 rPhl p 1 1,1 rPhl p 2 1 nPhl p 4 0,5 rPhl p 5 3,6 rPhl p 6 0,6 rPhl p 11 2,7 rBet v 1 17 rAln g 1 2	rCor a 1.0101 1,7 nArt v 1 0,6 rFel d 1 4,2 rCor a 1.0401 0,8 rMal d 1 3,8 rPru p 1 2 rAra h 8 1,2 nAct d 8 0,6 nPru p 3 1	nArt v 3 0,9	
51	59	**Wheat + exercise**	Respiration and Circulation	Walnut 4 Hazelnut 4 Peanut 5 Soybean 6 Wheat flour 5 Apple 4 Carrot 5	Celeriac 6 Tomato 5 Lupine 5 Hazel pollen 3 Alder 5 Plane 5 Ambrosia 5	27,4	Wheat flour 10,1 Mugwort 11,4 rPru p 3 1,00 ω-5-gliadin 0,85 Pork 0,95	nGly m 5 0,6 nCyn d 1 12 nPhl p 4 11 nOle e 1 2,7 nPla a 2 8,6 nApi m 1 4,7 nAna c 2 3,7			Cor a 8 neg Cor a 9 0,19 Cor a 11 1.2 Cor a 14 0.1 Tri a 19 0,14
53	49	**Wheat, Seafood**	Respiration and Circulation	Hazel pollen 5 Alder 6 Birch 5 Plane 3 Grass 8 Ambrosia 2 Cat 10 Dog 4 Der. Pteronyssinus 3 Derm farinae 3 Cockroach 4 Hen's egg 8 Cow's milk 10 Pork 6 Walnut 4	Hazelnut 6 Peanut 7 Soybean 10 Barley flour 15 Rye flour 15 Wheat flour 7 Oat flour 11 Celeriac 7 Lupine flour 5 Melissa 3 Chives 4 Rocket 6 Grapes red 5 Grapes white 3	>5000	Egg white 61,0 Milk protein >100 Celeriac 5,44 Nuts 60,1 Seafood 1,72 Spelt >100 Barley flour >100 Gluten (Gliadin) >100 Oat flour 75,2 Corn flour 18,4 ω-5-gliadin >100 Rice 22,2 Rye flour >100 Wheat flour >100 Curry 14,1	nAct d 1 2,7 rAna o 2 4,8 nCor a 9 1 nSes i 1 16 nAra h 2 0,3 nGly m 5 3,8 rTri a 19.0101 40 nTri a aA_TI 51 nCyn d 1 16	nBos d 8 9,2 rFel d 1 9,7 rFel d 4 3,4 nMus m 1 3 nApi m 1 1,1 rDer p 10 0,4 nBla g 7 1,1 rAni s 3 0,3	nPla a 2 3,5 nCry j 1 0,6 nCup a 1 1,7 rHev b 1 1,3 rHev b 6 2,2 nGal d 3 8,5 rPhl p 1 18 nPhl p 4 4,7	Tri a 19 3,57
54	66	**Wheat + exercise**	Circulation	Wheat grain 6		97,4	ω-5-gliadin 1,47				
55	57	**Wheat + exercise**	Respiration and Circulation	Grass +++ Rye +++ Birch +	Wheat grain 2 Wheat flour 4	23	Tryptase 4,41 Timothy 1,75 ω-5-gliadin 0,02 Bell pepper 0,00 Aspergillus n 0,00 Penicillium c 0,00 Tropomyosin 0,00	rPhl p 1 0,3 rPhl p 2 0,6 rPhl p 5 1 rFel d 1 3,2			Tri a 19 neg
56	30	**Wheat + exercise**	Circulation	Carrot 3	Grass 7	242	ω-5-gliadin 0,00	nAct d 2 0,4	rPhl p 2 6,6		Dau c 1

				Corn 3 Plane 3	Alternaria 5 Cat 9 Dog 3		Corn flour 0,26 Gluten (Gliadin) 0,01	nCyn d 1 0,9 rPhl p 1 3,4	rFel d 1 2 rAlt a 1 5,6		neg Tri a 19 neg
57	58	**Pumpkin**	Respiration and Circulation	Pumpkin 13		19,7	Seafood 0,00 Pumpkin seed 1,66 Birch 0,00				
58	45	**Wheat + exercise**	Respiration and Circulation	Hazel pollen 4 Alder 5 Birch 5 Ambrosia 5 Wheat flour 3		157	Gluten 3,55 ω-5-gliadin 0,18	nGly m 5 1,3 rBet v 1 18 rAln g 1 3,8 rCor a 1.0101 1,7	rGly m 4 1,6 rAra h 8 3,3 nPla a 2 0,5 nCry j 1 0,5	nCup a 1 0,7 rCor a 1.0401 6,4 rMal d 1 4,8 rPru p 1 3,9	Tri a 19 0,47
59	51	**Wheat + exercise**	Circulation	Tomato (native) 4 Soybean 3 Barley flour (native) 5	Rye flour (native) 5 Oat flour (native) 3 Wheat flour (native) 8	62,2	ω-5-gliadin 6,41 Wheat flour 0,43	rTri a 19.0101 0,3 rAsp f 1 0,6			Tri a 19 0,76
60	28	**Wheat +exercise + co-factors**	Respiration and Circulation	Grass 7 Ambrosia 3	Rye flour 4 Barley flour 3	79,5	Timothy 14,3 ω-5-gliadin 0,00 Rye flour 0,14	nCyn d 1 9,6 rPhl p 1 5,6	rPhl p 5 7,8 rPhl p 6 1,2		
61	34	**Hazelnut, Carrot, Apple, Seafood, Mushrooms, Soy, Celeriac**	Respiration and Circulation	Hazel pollen 14 Alder 12 Birch 10 Grass 14 Mugwort 7 Walnut 4	Peanut 12 Soybean 10 Carrot 11 Celeriac 20 Tomato 4 Hazelnut 5	127	Celeriac 2,86 Nuts 14,2 Grass 4,12 Birch 32,5 Hazel pollen 22,2 Herbs 1,17	nCyn d 1 2,4 rPhl p 1 0,7 rPhl p 2 2,5 rBet v 1 2 rAln g 1 6,5 rCor a 1.010 4,9	rFel d 1 11 rCan f 2 1,5 rCor a 1.0401 3,2 rMal d 1 0,5 rPru p 1 0,6 rGly m 4 6	rApi g 1 1,4 rAra h 8 6,2 nOle e 1 1,8	BBQ neg. Cor a 8 neg Cor a 9 neg Cor a 11 neg Cor a 14 neg

Legend: sIgE: allergen-specific IgE, ISAC: Immuno solid-phase allergen chip, ISU: ISAC standardized units, ELISA: Enzyme-linked immunosorbent assay, BBQ: barbecue extract, neg: negative, n.d.: not done

Curriculum Vitae

Maria Berneder

Scientific education

2005 High School Graduation

2005 – present Study of Molecular Biology, University of Vienna,

2010 Semester abroad at Universidad Autónoma de Madrid, Spain (ERASMUS)

Internships

2009 1 Month at the Max F. Perutz Laboratories, Vienna (Project Leader: Dr. Erwin Ivessa)

2010 3 Months at the Centro de Biología Molecular Severo Ochoa, Madrid (Project Leader: Dr. María Luisa Toribio)

1 Month at the Inst. of Mol. Biotechnology, Vienna (Project Leader: Dr. Arabella Meixner)

2011 Diploma Thesis at the Department of Pathophysiology and Allergy research, Vienna (Project Leader: Assoc. Prof. Dr. Karin Hoffmann-Sommergruber)

List of publications

Maria Berneder, Merima Bublin, Karin Hoffmann-Sommergruber, T Hawranek, Roland Lang (2013): Allergen Chip Diagnosis for Soy-Allergic Patients: Gly m 4 as a Marker for Severe Food-Allergic Reactions to Soy. Int Arch Allergy Immunol. 2013;161(3):229-33. doi: 10.1159/000345970. Epub 2013 Mar 15.

Poster presentations

Maria Berneder, Stephanie Claus, Margitta Worm, Karin Hoffmann-Sommergruber (2011): Food anaphylaxis diagnosis using the allergen CHIP approach.

Poster presentation at the Annual Meeting of the Austrian Society of Allergology and Immunology, September 2011, Graz, Austria

Maria Berneder, Stephanie Claus, Margitta Worm, Karin Hoffmann-Sommergruber (2011): Food anaphylaxis in adult patients: Allergen Chip analysis identifies PR10 proteins, Tri a 19 and nsLTPs as the most important elicitors