A multidisciplinary study of allergy

Mouse models, immune modulation and lifestyle

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Contents

Contents

List of abbreviations

αCD3:	Antibody directed against CD3
αCD28:	Antibody directed against CD28
AA:	Amino acid
APC:	Antigen-presenting cells
Ca-I:	Calcium ionophore
ConA:	Concanavalin A
IgE:	Immunoglobulin type E
LPS:	Lipopolysaccharide
LBP:	LPS binding protein
PMA:	Phorbol myristate acetate
PRR:	Pathogen-recognition receptor
Tc:	Cytotoxic T cell
TCR:	T cell receptor
Th:	T helper cell
TLR:	Toll-like receptor

List of definitions

Allergen:
An antigen that causes an allergic response.

Allergic reaction:
A symptomatic response to a normally innocuous environmental antigen leading to the production of IgE.

Allergic march:
The natural history of atopic diseases, characterized by a typical sequence of sensitization and manifestation of symptoms which appear during a certain age period, persist over years or decades, and often show a tendency for spontaneous remission with age.

Atopy:
Refers to those allergic conditions which tend to cluster in families, including hay fever, asthma, and eczema, and which are associated with the production of specific IgE antibodies to common environmental allergens.

Prevalence:
The percentage of the population with a disease or an abnormality.

CHAPTER

1

General introduction

General context: The multidisciplinary approach to allergy

More than 25% of the population in industrialized countries suffers from a type I allergy [1, 2]. This type of allergy is characterized by the increased production of allergen-specific IgE antibodies against per se harmless antigens from different sources like pollen or food [3]. The immediate symptoms like rhino-conjunctivitis or gastro-intestinal complaints are caused by allergen-induced cross-linking of effector cell-bound IgE antibodies and the release of inflammatory mediators [3]. A more detailed description of the allergic response will be given in section 2 of this general introduction.

Although the mechanism by which allergic complaints develop within an atopic individual is largely known, the reason why a person becomes predisposed to become atopic, and which (combination of) triggers are eventually leading to the development of allergic diseases remain unknown. However, since it is unlikely that the genetic background of the population has recently changed, it is more likely that the rise in allergic disorders is caused by changes in the environment [4].

Over the last decades, this multifactorial disease gained more and more attention as the prevalence of allergic diseases shows a continuous increase. At first the increase in allergic individuals mainly gained clinical interest, but the substantial increase of the public awareness towards allergies caused a broadening of the allergenic scope. Besides clinicians and immunologists, also society itself, local and national governments and industry are now confronted with the consequences of the growing allergic population [5-7].

Chapter 1

1 Immune cells

Allergic diseases are the result of an interplay between multiple immune cells. Traditionally, these immune cells are either belonging to the innate part or the adaptive part of the immune system. Innate immune cells provide a first line of defence against many common microorganisms and are essential for the control of these infections. Monocytes, dendritic cells, eosinophils, neutrophils and basophils are examples of innate immune cells. Infectious organisms that cannot be eliminated by the innate immune cells, will be processed by the monocytes or dendritic cells, and thereafter presented to and attacked by the further evolved T and B cells of the adaptive immune system [8].

All blood cells originate from the pluripotent hematopoietic stem cell. This stem cell can differentiate into two common progenitors: the lymphoid progenitor and the myeloid progenitor. The lymphoid progenitor gives rise to the T cells and the B cells, whereas granulocytes and monocytes originate from the myeloid progenitor [8].

1.1 Antigen-presenting cells

The initiation of an immune response requires the presentation of the invading antigen to the other immune cells. In mammals, antigens are taken up by antigen-presenting cells (APC) that process the antigens into smaller peptides. These peptides are subsequently presented on the cell surface of the APC in a class II major histocompatibility complex (MHC-II). The dendritic cells, macrophages and B cells are generally all three considered as professional APC [8]. Antigen presentation in *in vitro* peripheral blood mononuclear cell (PBMC) cultures is mainly accounted for by monocytes. Differentiation of monocytes can lead to myeloid dendritic cells [9] or macrophages [10]. In this thesis, macrophages are considered as monocytes that migrate from the blood into the tissue. However, monocytes isolated from whole blood and cultured *in vitro* will adhere to the plastic and thereafter also behave as macrophages [11]. Macrophages are APC that play an important role in the initiation of an immunological response, as further described in section 1.2.1.

To mimic the activation of monocytes in an *in vitro* cell culture, artificial stimulation through distinct families of pattern-recognition receptors (PRR) can initiate intracellular signaling events. These intracellular events will lead to complex immune responses designed to eliminate the invading pathogen. The PRR include the membrane-bound and intracellular Toll-like receptors (TLR), that recognize conserved molecular patterns derived from bacteria, viruses, protozoa and fungi, and the C-type lectine receptors, that sense

fungi. Interaction of the TLR with pathogen-associated molecular patterns on the surface of the micro-organisms releases pro-inflammatory cytokines, including TNF-α, IFN-γ, IL-1β, IL-6 and IL-12, which induce maturation of T helper 1 cells, as opposed to the T helper 2 cells which are associated with allergic responses [12]. Th subsets are described in more detail in section 1.2.

Lipopolysaccharide (LPS) from the bacterial cell wall is often used as a polyclonal stimulus in *in vitro* PBMC cultures. LPS can only effectively activate a monocyte/macrophage when it is first bound by LPS-binding protein (LBP). Thereafter, the LPS-LBP complex can bind to the CD14 molecule on the surface of an APC. After association with CD14, TLR4 needs to form a complex with the CD14-LPS complex to initiate the intracellular signaling pathway [12].

1.2 T cells

T cells are divided into two classes: Cytotoxic T (Tc) cells and T helper (Th) cells. Until recently, Th cell subsets were characterised based on their cytokine profile, segregating them into Th1 and Th2 cells. More specifically, Th1 cells produce IL-2, IFN-γ and lymphotoxin-α, whereas IL-4, IL-5, IL-9 and IL-13 are regarded as cytokines produced by Th2 cells [13]. In addition, segregation of Th1 and Th2 cells based on transcription factors have been described; STAT-4 and T-bet are characteristic for Th1 and they antagonize

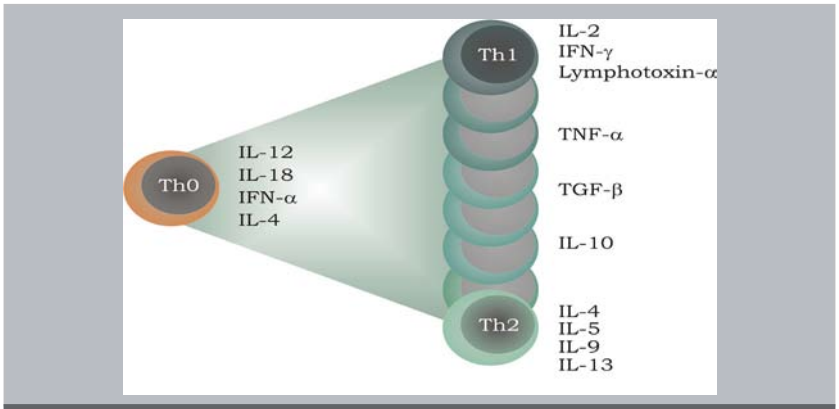

Figure 1. Effector Th1 and Th2 populations with their signature cytokines as differentiation end stages from activated Th0 cells. The figure shows that these Th1 and Th2 populations are the extremes in a whole cascade of intermediate subpopulations. All of these populations are characterized by the cytokine production profile they display after activation.

Chapter 1

the transcription factors STAT-6, GATA-3 and c-maf in Th2 cells [13].

Although not investigated in this thesis, both Th1 and Th2 cells are regulated by a heterogenous family of regulatory cells. Within this family, Th3, Tr1 and $CD4^+CD25^+Foxp3^+$ can be discriminated (see Figure 1). Th3 cells are induced by oral antigen administration and their main suppressor activity is regulated by the production of TGF-β. Tr1 cells are induced by IL-10 and also exert their immunosuppressive action through their own IL-10 production. The function and origin of $CD4^+CD25^+Foxp3^+$ cells is still debated [13].

To complete the currently known T cell subsets section, recently, a novel Th subset has been described; the Th17 cells. These cells are probably induced by the presence of IL-23, and they produce IL-17 and IL-22 that both evoke inflammation. Since the Th17 cells do not express T-bet or GATA-3, the cells are probably originating as a distinct subset from Th1 and Th2 cells [14]. Undoubtedly, research groups in the allergic asthma and food allergy field will investigate this new Th subset in the upcoming years.

1.2.1 T cell activation

T cell activation requires at least three signals: 1) the association of the MHC-peptide complex with the T cell Receptor (TCR), 2) the binding of CD28 to B7-1/2 (CD80/CD86) expressed on the APC (also called co-stimulation), and 3) cytokine signaling. After the first two signals, an immunological synapse is formed, resulting in the activation of several tyrosine kinases and recruitment of adapter proteins and specific downstream signaling leading to T cell activation and specific cytokine production. When a naïve, activated T cell is exposed to IL-4, it will differentiate into a Th2 cell, whereas IL-12, IL-23, and

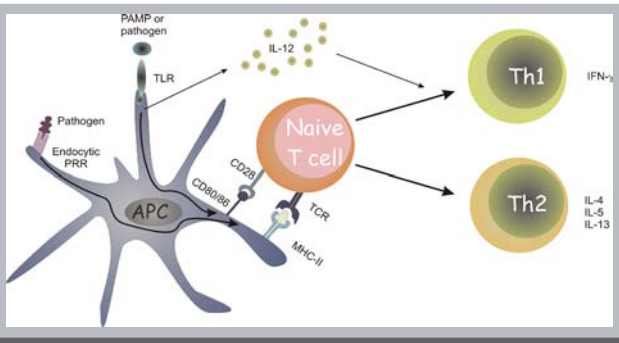

Figure 2. Schematic representation of the interactions between essential cell types in the antigen-specific adaptive immune response and the cross-talk with activities of the innate immune system.

Modified after Medzhitov R (2001), Nature Rev Imm. 1: 135-145.

TGF-β and IL-6 will let the T cell differentiate into a Th1, Th17 or Treg, respectively (see Figure 2) [13].

These differentiating cytokines are produced in the micro-environment of, or by the APC involved in the cell-cell contact with, the naïve T cell. Until now, it remains unknown whether it is the T cell that directs the type of cytokine production by the APC, or whether the APC type is responsible for the differentiation of the Th subsets.

In antigen-specific responses, APC can only present the antigen to T cells that can recognize the combination of the MHC-II molecule and the presented peptide. Although the percentage of T cells in peripheral blood is around 70%, during a strong viral infection the percentage of antigen-specific T cells within the total T cell population might increase to approximately 5%. However, in less severe infections or allergic responses, these antigen-specific T cell numbers may be undetectable in the peripheral blood [15]. To overcome this, amplification of the response by the use of polyclonal stimuli is often used. For example, for an *in vitro* polyclonal activation of T cells, phorbol myristate acetate (PMA) plus calcium ionophore (Ca-I), concanavalin A (ConA) or anti-CD3 plus anti-CD28 (αCD3/αCD28) are widely used.

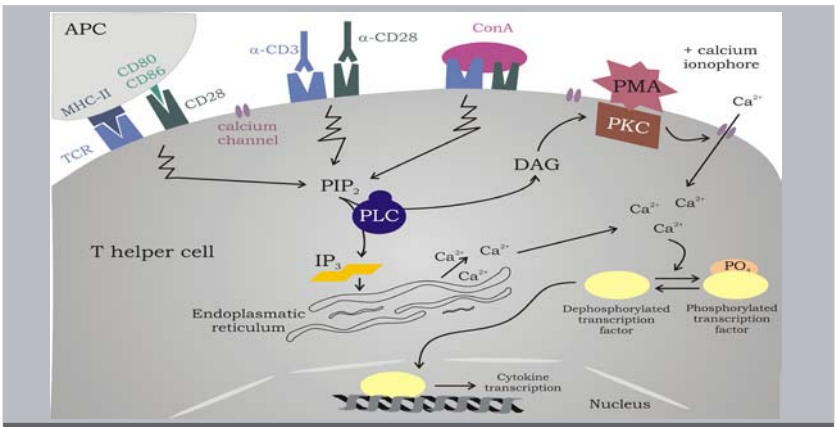

Figure 3. Polyclonal T cell activation routes. In the *in vivo* situation, the T helper cell becomes activated by the physical contact with an antigen presented in the MHC-II molecule on the APC, in combination with the co-stimulatory signal provided by CD80 or CD86 to CD28. In the case of antibody-regulated activation, the combination of anti-CD3 plus anti-CD28 antibodies will provide the required activation signals. The binding of ConA to both the TCR and CD28 will also provide the required activation signals for the initiation of the activation pathway. In contrast, PMA/Ca-I does not activate the T cells by means of a receptor mediated pathway, but by the direct activation of PKC, subsequently leading to the activation of the downstream pathway.

All three stimuli act at different sites and in different ways to activate the T cell receptor pathway [16-18] as depicted in Figure 3. Anti-CD3 and anti-CD28 have been widely used to provide all T cells with the required activation signals [19-21]. ConA is a lectin that binds to glycoproteins expressed on the T cell surface. ConA cross-links the T cell receptor, thereby mimicking the T cell receptor activation which bypasses the requirement of co-stimulatory signals [22]. PMA directly activates cytoplasmic protein kinase C resulting in phosphorylation of the calcium channels and influx of extracellular calcium which is provided by addition of exogenous calcium ionophore [23].

1.3 B cells

Humoral immune responses in mammals are mediated by at least three mature B cell subsets: follicular (FO) B-2 B cells in the spleen, lymph nodes and Peyer's patches; marginal zone (MZ) B-2 B cells in the spleen; and B-1 B cells, mainly in the peritoneal and pleural cavity. The FO B cells mainly produce antibodies in response to T cell dependent antigens, whereas MZ and B-1 B cells respond to T cell independent challenges [24, 25]. Furthermore, MZ B cells are better equipped to present antigens and to activate T cells than FO B cells, the B-1 antibody repertoire is more restricted than the B-2 repertoire, B-2 cells can undergo affinity maturation of the antibody response and B-1 B cells in mice make up about 1-5% of the total B cells [25-27].

The B-1 B cells can be further subdivided into B-1a and B-1b B cells [26]. B-1a B cells are the main producers of IgM natural antibodies and thereby contribute to the innate immune responses, whereas the B-1b B cells are more contributing to the adaptive immune response [25, 26]. B-1a and B-1b B cells are believed to originate from a B-1a or B-1b progenitor, respectively [25, 26], whereas FO and MZ B cells (both considered as B-2 B cells) originate from B-2 progenitors [28]. However, both B-1 and B-2 B cells are capable of switching to other isotypes, including IgE [29].

1.3.1 Isotype switching

B cell activation can result in a switch of antibody heavy-chain isotypes from IgM/IgD expression to IgG, IgE or IgA expression [30]. Isotype switching is regulated by Th cells through CD40L (CD154), which is upregulated after activation, interacting with CD40 on B cells through physical contact and specific cytokines produced by the Th cells [31]. Especially in mice, the effects of IL-2, IL-4, IL-5, IL-6, IL-13, TGF-β and IFN-γ have been extensively investigated leading to the common knowledge that IL-4 and IL-13 provide isotype switching towards IgE, IL-4 and IL-5 synergize to enhance the IgG1

response, whereas optimal IgG1 responses also require IL-2. In addition, IL-6 rapidly enhances IgG1 class switching. TGF-β inhibits the production of many immunoglobulin isotypes like IgM, IgG1, and IgG2a, whereas IFN-γ inhibits the switch to IgG2b, the ability of IL-4 to enhance IgG1 and IgE synthesis, and stimulates IgG2a secretion in mice [29, 31]. In humans, IL-4 and IL-13 induce IgG4 and IgE switching, whereas TGF-β directs IgA switching [32]. In addition, both IL-21 [33] and IL-10 are involved in the isotype switching to IgG1 and IgG3, whereas IL-27 exclusively regulates the production of IgG1 [34].

Like all antibody molecules, also IgE antibodies are able to recognize two identical epitopes simultaneously (see Figure 4). Unlike IgG molecules, however, IgE antibody molecules are rather inflexible. This gives restrictions to the three-dimensional orientation of the epitopes on the allergen molecule and thereby explains why the spacer length between the epitopes contributes to the allergenicity of a protein. The optimal amino acid (AA) length of a B cell epitope is 5 AA or less [35], whereas the optimal $CD4^+$ T cell epitope consists of an AA length between 12 – 18 AA [36]. A more detailed description of the differences between T and B cell epitopes will be provided in section 3.5.

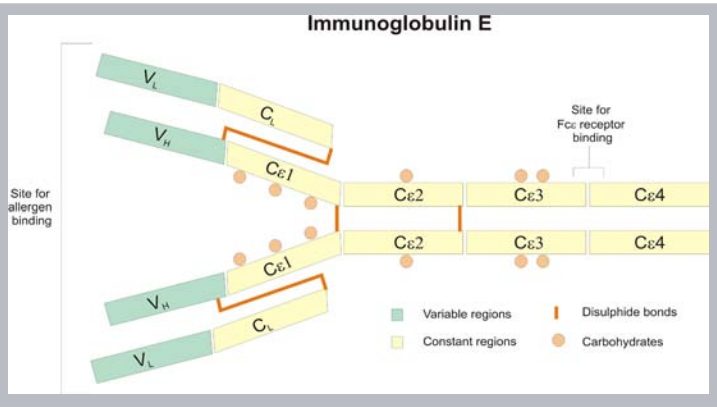

Figure 4. Schematic representation of the structure of an IgE molecule, showing the typical domain structure of the antibody molecule. The molecule consists of a single variable and multiple constant domains that carry carbohydrate residues. The two variable domains are involved in binding the two identical epitopes present on the allergen molecule. In the constant Fc part is involved in Fc receptor binding to mast cells and basophils.

2 Allergy

As described in section 1, the human immune system protects the body against harmful antigens from the environment. When an antigen enters our body, our immune system produces antibodies against those antigens. In allergy, the antigens are called allergens. An allergen itself is not harmful for the body; it is the reaction of the immune system that is harmful [37]. Genetics play a critical role in making people predisposed to develop allergies, also called atopic predisposition [38]. Atopic persons have a genetic predisposition to develop an allergic reaction after contact with an allergen. When there is a history of atopy in a family, a child has a higher chance to become allergic than a child without a family-history of atopy. Without atopic parents, a child has a 10% chance of becoming allergic, with one atopic parent this increases to a 20% chance, and two atopic parents with the same allergy give their child a 70% chance of becoming allergic [39].

2.1 The immunogenetic basis

The genetic risk to develop an allergic disease can be determined by analysing the family history and the presence of polymorphisms or mutations in selective cytokine genes. Extensive research on asthma and allergy have revealed conditions in which various genetic hits that are individually mild may be capable of major phenotypic effects when acting in concert within a permissive environmental context. This multifactorial pathogenesis differentiates complex diseases from Mendelian disorders, which are caused by single gene mutations, and renders their genetic components more elusive and difficult to trace [38].

Since allergic diseases are considered to be a Th2-driven immunopathology, several Th2-linked genes were analysed for the presence of promoter polymorphisms or exon mutations occurring in patients. Such polymorphisms or mutations could be responsible for the risk to develop allergic symptoms and provide possible markers predicting allergic diseases. Moreover, they can be used to monitor the development of allergic symptoms and potential effective treatment. So far, several exon mutations were found in allergic patients: IL-4Receptor (Q576R, I50V) [40], IL-10 (C-571A) [38], TNF-α (C368A) [38], CD14 (C159T) [41], FcεRIB (I181L, I183V, E237G) [42]. In addition, promotor polymorphisms were described for TNF-α (-308G/A) [38], IL-4 (-589C/T) [38], IL-10 (-571C/A, -1082A/G) [38], and IL-9 (-345A/G) [43].

Many of these genes are located on defined positions on several different chromosomes and genetic linkage has been proposed in allergic individuals with several chromosomes, like 5q3-33 (cytokine gene cluster), 5q32 (beta-2 receptor), 6p21.3 (TNF-α), 11q13 (FcεRIB), 12q15-24.1 (IFN-γ) and 1q32.1

(IL-10) [38]. Despite these interesting linkages, the multigenic nature of allergic diseases and the variability in disease expression among affected individuals, these data are not yet validated in large patient populations or used on a routinely basis.

Besides the direct effect of polymorphisms and differences in genetic backgrounds in mice and humans, also the overall influence of the genetics in mice is a serious point of consideration, since the mouse is often used as a model for humans. For example, Kaminski *et al.* showed that significant B cell-intrinsic differences in antibody class switching can be observed when comparing different stains of inbred mice. They observed that splenocytes from mouse strain 129 have a defective proliferative response to IL-5 in the presence of blocking antibodies to CD38 due to a lower expression of the IL-5Rα and γ chain in 129 B cells compared to C57Bl/6 mice. In addition, splenic B cells from SJL and 129 mice undergo poor IgG3 and IgG2b, but similar IgG1 class switching compared with B cells from C57Bl/6 mice [44]. Since antibody isotype switching is an important step in the sensitization phase of an atopic individual, genetic differences amongst individuals remain an important research topic.

2.1 Sensitization phase

Allergic sensitization describes the genetically determined propensity of certain individuals to react with the induction of Th2 cells and subsequent allergen-specific IgE-antibody formation upon repeated low-dose exposure of allergens at mucosal surfaces. In more detail, when an allergen intrudes the body, the peptides/MHC-II complex will bind to the TCR on naïve T cells (Figure 4). As described before, three signals are required for T cell activation, eventually resulting in the formation of an immunological synapse, leading to T cell activation and specific cytokine production. In Th2 cells, GATA3 is activated by APC produced prostaglandin E2, which direct naïve T cells to become Th2 cells. This skewing disturbs the balance between Th1 and Th2 cells, resulting in an overproduction of Th2 cells. Therefore, allergy is called a Th2 disease. Th2 cells produce cytokines like IL-4, IL-5, IL-9 and IL-13, that are involved in the class-switching of B cells to IgE synthesis or the recruitment of mast cells. Secreted IgE attaches to high-affinity IgE receptors (FCεRI) on mast cells, basophilic and eosinophilic granulocytes. IgE levels in the serum are usually relatively low (0.05 mg/ml), partly due to a short half-life of IgE antibodies in the serum (less than a day). However, a markedly prolonged half-life (up to 14 days) is obtained when the IgE antibodies are bound to the high-affinity Fc receptors due to the protection of the IgE antibodies from proteolytic cleavage and clearance.

Whenever the allergen-specific IgE antibodies are bound to the FCεRI on the mast cell, a person is called 'sensitized' (Figure 5) [3]. This allergic sensitization can last for many months to years, but does not necessarily lead to an immediate clinical allergic response. It is possible that people sensitized against birch pollen will never experience any signs of birch pollen-induced hay fever [45]. However, most individuals that are sensitized will experience allergic symptoms during a subsequent exposure, called the 'challenge phase'.

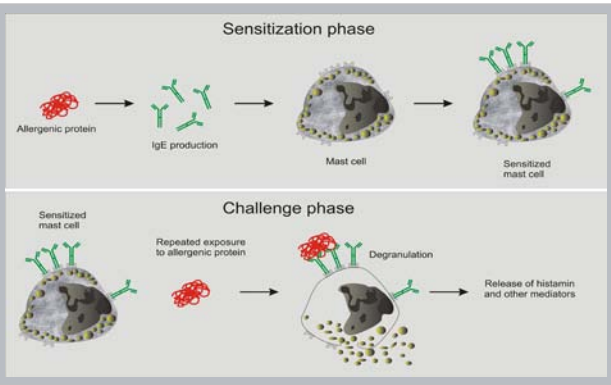

Figure 5. Schematic representation of the basic immune mechanisms of an immediate type I allergic reaction, resulting in the rapid release of histamine and other substances inducing the typical clinical allergic pattern of symptoms.

2.2 Challenge phase

A subsequent exposure to the same allergen will cause the binding of the protein to the mast cell-bound IgE. Due to multiple binding sites on the protein, neighbor IgE molecules will bind to the same protein. In this way, the IgE antibodies are cross-linked, which results in an intracellular signal in the mast cell. This intracellular signal will cause the mast cell to degranulate and release much of the inflammatory mediators stored inside the mast cell. These inflammatory mediators, like histamine and leukotrienes, cause vasodilatation and triggering of nerve cells, leading to e.g. swollen eyes and itching sensations (see Figure 5). These mediator caused symptoms are the hallmark of IgE-mediated allergic responses, called type I allergies, which generally result in fast occurring clinical complaints (1-30 minutes). In addition, the release of cytokines and chemokines from the mast cell result in an influx of macrophages, eosinophils and basophils that comprise the late response (within 6 -72 hours) [3].

Sometimes, regular diagnostic procedures cannot confirm the food allergic complaints due to the absence of allergen-specific IgE. Although still debated, it has been suggested that besides allergen-specific IgE, also allergen-specific IgG4 could play an important role in humans. The presence of IgG4 antibodies is considered to be the result of chronic exposure to allergens, and has been indicated to release histamine from basophils [46]. Recently, the group of Kleine-Tebbe has shown that food-specific IgG4 does not indicate food allergy, but rather a physiological response of the immune system after exposition to food components [47].

2.3 Clinical symptoms of allergies

Although all allergic diseases are based on the cross-linking of mast cell-bound IgE, the disease can be expressed in different organs like the skin (atopic dermatitis), gut (food allergy), the nose-and-eyes (rhinoconjunctivitis or hay fever), and lungs (allergic asthma). Atopic dermatitis is characterized by redness, itching and dryness, cracking or bleeding of the skin, food allergy by angioedema, hives, nausea, wheezing or even anaphylaxis, hay fever by swollen eyes and itching sensations in the nasal or oropharynxial cavities and asthma by broncho-constriction. Food allergens that cross-react with birch pollen (described in more details in section 3.4) result in the so-called oral allergy syndrome, characterized by an itching or burning sensation in the lips, mouth and/or pharynx. Also the eyes, nose and skin might be itching, whereas the tongue, lips and uvula might become swollen resulting in a sensation of tightness in the throat.

Although the allergic responses in the distinct organs might be unrelated, allergic patients often experience allergic complaints in different organs in a sequential manner, also called 'the allergic march'. The allergic march describes the age-related difference in expression of allergic symptoms in these different locations from newborns to adults [48]. It is widely believed that most babies display their first symptoms of allergy by suffering from food allergy within the first few months of their life, reflected as eczema. After 1-2 years of age, in most children this eczema resolves (60-70%), giving rise to respiratory symptoms in some (30%), whereas the remaining 30% stays symptom-free for years or even for life. At 6 years of age, allergic asthma is prevalent in children at risk to develop allergy, whereas at 12-16 years of age, it is primarily hay fever. Although still widely accepted, there are data to support that heterogeneity might exist in allergic children with some displaying skin-related symptoms (eczema), whereas others display respiratory-system-related symptoms (asthma and hay fever) [48]. However, although the mechanism of the allergic response is quite clear, why these allergens do cause an allergic response in one person, but not in another person, remains unknown.

3 Allergens

Allergens are almost always proteins that are widely present in the diet and the environment. Few proteins are allergens by themselves, but they can become allergens under special conditions or in atopic individuals [37]. In this thesis, the studied allergens originate from birch trees, apple, carrot, celery and hen's eggs. This section gives detailed information about these allergens.

3.1 Aeroallergens

The birch tree is a major sensitizing tree in The Netherlands and the rest of Europe [49]. The most common birch species in Europe is the Betula pendula, which has been widely investigated in relation to hay fever. The birch tree is a wind pollinating tree, leading to high concentrations of birch pollen in the environment during the flowering season. Birch pollen contain several allergens, termed Bet v 1 to Bet v 8 [50-55]. Although Bet v 1 is the major sensitizer in Europe, there are geographic differences in the occurrence of sensitization to these allergens [56]. More than 70% of the birch pollen allergic individuals also becomes allergic to foods like stone-fruits, nuts or certain vegetables [57]. This cross-reactive response will be described in more detail in section 3.4.

3.2 Food allergens

Food allergens are individually relatively resistant to processing (temperature, pressure, vacuum, enzymes) and relatively digestion-resistant. Currently, there is profound interest within the food industry in the allergenicity of their products with respect to the new labelling requirements that are issued by EU regulations. This requires that the food industry will establish the potential of proteins within their products to provoke an allergic response in an atopic individual. The allergenicity of a food allergen is determined by the biochemical properties of the allergen, innate immune response stimulating substances around the allergen at the time of exposure (within the same extract or from co-exposure), stability of the allergen in the tissues, digestive system, skin or mucosa, and finally dose and time of stay in lymphatic organs during the interaction with the immune system [58]. Examples of the biochemical properties include the abundance of the protein in the food, high numbers of linear IgE binding epitopes, and the resistance of the protein to digestion and processing of the food [37].

A large number of food-allergen sequences is described in literature, distributed over 20 protein families of the 3849 possible families. More than 65% of the known food allergens belong to only 4 families: Bet v 1 superfamily, profilins, prolamin superfamily and cupin superfamily [59].

3.3 Allergens within the Bet v 1 superfamily

An extended sequence comparison revealed that the Bet v 1 superfamily comprises at least 4 families with low levels of sequence similarity between each other: 1) the PR-10 family, 2) the family of major latex proteins and ripening-related proteins, 3) the norcoclaurine synthases, and 4) cytokinin-binding proteins from legumes [60]. The allergen Bet v 1 is a member of the cytoplasmic disease resistance-related proteins within the PR-10 family. This family comprises many homologous proteins with well-known members like Bet v 1 from birch, as well as Dau c 1 from carrot, Pru av 1 from cherry, Api g 1 from celery and Mal d 1 from apple [57]. In plants, the PR-10 proteins have functions in the defence against bacterial and fungal infections [57].

3.4 Cross reactivity between PR-10 proteins

Food allergy can result from either direct sensitization to foods or cross-reactive IgE responses to inhalant allergens [5]. A typical example of cross-reactive food allergy is the 'birch-fruit-vegetable-syndrome'. More than 70% of the birch-pollen allergic individuals develop allergies to stone-fruits, nuts or certain vegetables over time, leading to orophanyngial complaints [57]. More than 95% of the birch pollen-allergic individuals respond to the major birch pollen-allergen Bet v 1. The PR-10 family contains several other proteins that show a high sequential homology to the Bet v 1 protein, leading to a similar tertiary protein structure (see Figure 6) [61]. Therefore, antibodies formed against Bet v 1 can easily bind to the homologous PR-10 proteins found in apple, hazelnut, celery and carrot. Sera from birch pollen allergic individuals showed binding of 99% to Mal d 1, 93% to Cor a 1, 59% to Api g 1, and 38% to Dau c 1 [57].

In addition, the group of Bohle described that the extent of cross-reactivity between Bet v 1 and homologous food allergens is different at the T and B cell levels. For example, Bet v 1-specific IgE antibodies react preferentially with PR-10 like allergens in Rosaceae fruits, and less with the vegetables of the Apiaceae family. In contrast, the Apiaceae vegetables induced much stronger responses in Bet v 1-specific T cell lines and T cell clones than the cross-reactive allergens in Rosaceae fruits [62]. Furthermore, it has been shown that Bet v 1-specific Th cells persist in the peripheral blood for a long time period, whereas these Th cells do not seem to be intrinsically long-lived. It is

Chapter 1

therefore thought that the uptake of birch pollen-related food proteins might activate the Bet v 1-specific Th cells when patients do not encounter the pollen allergen [63]. Whether the cross-reactivity is only due to the cross-reactive binding of the food allergens to the Bet v 1-directed IgE antibodies, or also food-specific T cells might occur over time, remains to be further elucidated.

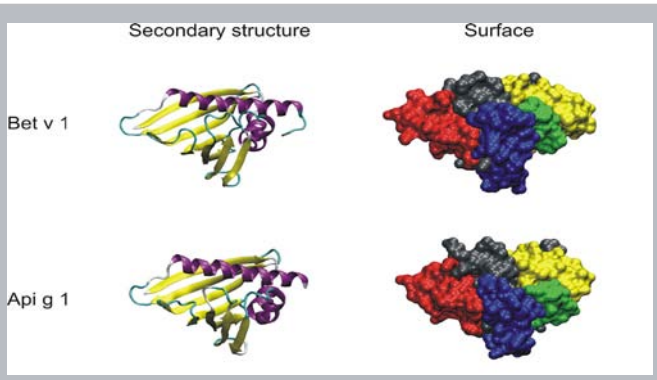

Figure 6. Structural homology between the major allergens in birch pollen and celery, Bet v1 and Api g1, respectively. Structural homology can explain cross-reactive IgE binding, but does not necessarily imply similar chemical stability or activity in the induction of allergic responses.

3.5 T cell and B cell epitopes of the Bet v 1 protein

In allergens, T cell epitopes essentially consist of linear peptide sequences processed from phagocytised proteins by APC with an AA length between 12 – 18 AA. These linear T cell epitopes are generally different from the conformational B cell epitopes comprising IgE-binding to the allergen. Only comparison of amino-acid sequences of T and B cell epitopes of different patients can reveal the structure of these epitopes in allergens [62]. The allergen Bet v 1 contains 10 distinct T cell epitopes, of which the epitope Bet v 1142-156 was shown to be the dominant T cell epitope [62]. This immune-dominant T cell epitope was found to be highly conserved among the Bet v 1 cross-reactive foods, ranging from 60 – 80% similarity. In addition, the T cell epitope Bet v 1109-126 shares 61% AA identity with the dominant Api g 1109-126 T cell epitope [63].

Besides T cell epitopes, an allergenic molecule can carry one or more (up to ± 25) epitopes constituting potential binding sites for allergen-specific IgE antibodies. It is generally hypothesized that a sequence region that is rich in

charged residues is likely to be surface exposed in the folded protein, and would therefore be more likely to form a B cell epitope [64]. Three regions on Bet v 1 protein have been shown to contain B cell epitopes [65]. In addition, at least one Bet v 1 B cell epitope must be classified as discontinuous, since the residues 42 – 52 constitute approximately 80% of the contact surface of Bet v 1 and all eight involved intermolecular hydrogen bonds are also found in this part of the residue region. However, the other 20% is not present within this linear sequence [66]. In addition, the level of conservation of the binding surface in the Bet v 1 homologues is considerably higher than the level of sequence identity. More specifically, Mal d 1 and Gly m 4 (from wheat) show a conservation of ± 71% and 60% in the surface compared to Bet v 1, respectively, compared to an overall sequence identity with Bet v 1 of 56% and 47%, respectively [59]. Despite their homology in amino-acid sequence (mainly the serine amino acid at position 122 is crucial) and therefore three-dimensional structure, these proteins can behave similarly in a biochemical way. For example, Mal d 1 and Api g 1 are rather thermo labile, whereas Dau c 1 is relatively thermo stable. In heat labile proteins, both conformational and linear epitopes might be destructed leading to their inactivity. Conformational epitopes can be conformation-dependent and therefore temperature- and processing-sensitive, whereas linear epitopes can be enzyme activity-sensitive to, for example, trypsin. Therefore, many different approaches to mutate the epitopes have been undertaken to reduce the allergenicity of these allergens. We need to realize, however, that no specific structure or allergen can account for and predict the major allergenicity of a product, as shown in e.g. milk and peanut, since functional and/or structural homology cause the allergy.

To study differences in genetic background, exposure effects and cellular communication processes, mouse models for inhalation and food allergy are frequently used. An allergen that has been extensively used for allergy studies in the mouse is derived from chicken eggs, and is called ovalbumin.

3.6 Ovalbumin

Ovalbumin is used as an artificial allergen in mouse models for allergic asthma [67, 68] and food allergy [69, 70]. Ovalbumin is one of the five major allergens found in egg white, making up approximately 60% of the total protein [71, 72]. Ovalbumin is made up of 385 AA, and its relative molecular mass is 42.7 kDa [73]. Ovalbumin is a phosporylated glycoprotein with four sites of glycosylation and it belongs to the serpin superfamily of proteins [73, 74]. Ovalbumin is often given in combination with Alum adjuvance [67-70] which promotes the adaptive immune system by the release of uric acid, leading to the differentiation of recruited monocytes into inflammatory dendritic cells [75]. In mice, the combination of ovalbumin and alum leads to

e.g. the influx of eosinophils into the allergen exposed site, allergen-specific IgE synthesis and mast cell degranulation [67-70]. Also in humans, allergic responses to ovalbumin are described. Approximately 2.4% of the 2-year old food allergic children displays an allergic response to hen's egg that usually decreases with age, but also often represents the start of the allergic march [76].

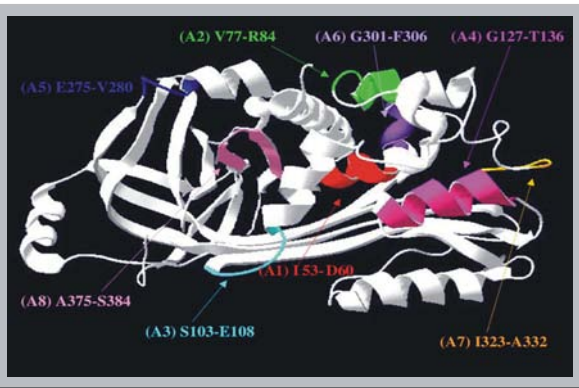

Figure 7. Representation of the ovalbumin sequential epitopes on a 3D model. The picture represents the tertiary structure of hen ovalbumin. The eight epitope regions identified after oral sensitization are represented in eight different colors on the ribbon structure.

From: Mine and Yang, Biochimica et Biophysica Acta, 1774 (2007)

A specific peptide of ovalbumin, OVA323-339, was found to be an allergenic and antigenic epitope [71], containing both T and B cell epitopes [77]. However, Mine *et al.* showed that the administration route also determines which peptides within this protein will serve as an allergenic peptide. For example, when a oral challenge with ovalbumin was performed, 8 epitopes were recognized by allergen-specific IgE antibodies, whereas intraperitoneal and subcutaneous challenge led to 2 and 5 epitopes, respectively. However, the 2 and 5 epitopes found in intraperitoneal and subcutaneous challenge did overlap with the seven epitopes unravelled in the oral challenge mouse model. Interestingly, Mine *et al.* showed that not all IgE binding regions within the ovalbumin protein were common between mice and man, but at least two of them show epitope similarities [64]. Figure 7 shows the murine B and T cell epitopes present in the ovalbumin protein. The epitope (A7)I323-A332 was originally described as a T cell epitope, but is also recognized by allergen-specific IgE antibodies [64].

4 Immunomodulation

As described previously, genetic predisposition plays an important role in the development of allergic diseases. After the onset of an allergic disease, often more allergy types occur in the same individual (the allergic march). Sensitized and challenged allergics are often treated with antihistamines or corticosteroids to prevent that the compounds that are set free during the degranulation will cause clinical symptoms. In this whole allergic cascade three possibilities to intervene the allergic cascade are defined: Primary, secondary and tertiary intervention. Primary intervention aims for the elimination or reduction of factors related to the allergy incidence, the goal of secondary intervention is to detect to disease in an early stage and to reduce the morbidity (number of individuals affected by the disease), whereas tertiary intervention is used to mitigate the disease complications. In other words, primary intervention aims to prevent the allergic sensitization, secondary prevention tries to keep allergic diseases limited in people that are already sensitized, and tertiary intervention aims to mitigate the clinical symptoms.

Both mice models and *in vitro* cultures of human PBMC enable the investigation of possibilities to modulate the immune response, possibly functioning as primary, secondary or tertiary intervention measures. As described in section 2, several immune cells are involved in the allergic cascade. On the one hand this multicellular system complicates the research in multifactorial disease like allergy, whereas on the other hand it offers many opportunities to modulate the immune response. Although allergen-specific immunotherapy agents, pre- and probiotics, spice and fungal derived components display their immunomodulating activity on different cells or in different ways, they are all examples of immune modulating compounds [3, 78-80]

Lull *et al.* reviewed literature concerning medicinal mushrooms with an established history of use in traditional oriental therapies. Since mushroom metabolites from e.g. *Ganoderma lucidum* (Reishi) and *Lentinus edodes* (shiitake) can be added to the diet and used orally, and they display immunomodulating activities, they are interesting candidates for allergologic research. Bioactive metabolites have already been isolated from fruiting bodies, pure culture mycelia and culture filtrates. These bioactive metabolites comprise polysaccharides, polysaccharopeptides, polysaccharide proteins, and proteins and have shown to affect the mitogenicity and activation of immune cells by an altered production of cytokines. For example, both a polysaccharide from *G. lucidum* and a β-glucan from *G. frondosa* induced the release of IL-1β, IL-6 and TNF-α from macrophages [81]. Although much is known about the effects of fungal polysaccharides, relatively little information is available on the immunomodulating activity of fungal proteins.

Chapter 1

An example of the immunomodulating role of a fungal protein is the purified Fungal Immunomodulatory Protein (FIP)-*Vvo* from *Volvariella volvacea*, which is suggested to evoke its immunomodulatory effects through the regulation of the cytokine production by human PBMC [82]. FIPs are classified into a distinct family since they are a group of fungal proteins defined by AA sequence similarity and their actions on immunological responses [83-86]. FIPs have been isolated and purified from *G. lucidum* [83], *F. velutipes* [84], *V. volvacea* [82], *Ganoderma tsugae* [87], and *V. volvacea* [86] and are designated as LZ-8, *Fve*, *Vvo*, *Gts*, and *Vvl*, respectively.

Studies in which the epigenetic regulation (heritable changes in gene expression that occur in the absence of alterations in DNA sequences) is investigated, like cohort-driven epigenetic research, have the potential to address key questions concerning the influence of timing of exposure, dose of exposure, diet, and ethnicity on susceptibility to allergy development [88]. This means that besides the application of an immunomodulating compound, also alterations of the lifestyle might help to reduce the genetic predisposition and thereby the onset of allergic or cross-reactive responses.

5 Lifestyle and prevention

The genetic propensity to put an individual at risk of developing allergic disease is modulated by exposure to environmental factors. Such epigenetic factors include pollution of air and water [89], infectious load and vaccination strategies [90], globalization of foods and exposure to novel foods [6], induced chronic stress levels [91], level of exercise [92] and many others. Some of these factors are known for their added risk, some can be influenced by choosing an active and less allergenic lifestyle. Some factors, however, cannot be influenced by an individual action, and are therefore hard to control. The choice of lifestyle and the ability to control this with respect to modulate the risk to develop allergic diseases requires individualized advice and proper information. This is where preventive measures can help.

Preventive measures require a multidisciplinary approach to deliver the information and tools necessary for policy makers, regulators, clinicians and allergic consumers, together with food industry, to effectively manage allergies and the allergens that cause them. Therefore, many multidisciplinary studies are being funded by the European and national government. Examples of these large consortia are Europrevall, Top Institute of Food and Nutrition and Top Institute Pharma [5, 93, 94]. These multidisciplinary studies can focus on multiple levels. Primary prevention should provide the prediction of being at risk, and is based on general recommendations for society as well as on individual recommendations for individual people. These recommendations comprise behaviours like breast-feeding to children for a period of 4 to 6

months, and, if this is not possible, to provide newborns at risk with hypoallergenic-formula milk. Also late addition of solid food (after the 4th month), avoidance of pollutants (traffic exhaust), avoidance of indoor allergens (e.g. pets) and avoidance of irritating/sensitizing skin contacts together should decrease the pace of development and the severity of symptoms in allergic disease. Secondary prevention should be based on screening for IgE-mediated sensitization by blood assays and skin tests. This analysis should provide recommendations for sensitized individuals or patients with one allergic disease. Advice comprises allergen avoidance (in home, restaurant and school), pharmacotherapy (e.g. antihistamines, Epipen) and immunotherapy (successful for inhalant allergens, but not for food). Tertiary prevention comprises post-treatment follow-up and patient management and is based on adequate post-treatment follow-up (including skin care, psychosomatic counselling), educational programmes (asthma/eczema schools), label reading (especially urgent with new EC directives), the development of a concept of patient management, and rehabilitation in special clinics/programs.

Besides personal prevention measures, producers can aid by proper labeling food allergens. Taking the importance of the proper indication of the amount of a present allergen into account, extensive precautionary labeling with no regard to the actual risk should be avoided. Determination of the actual risk requires knowledge on the amounts of allergenic foods which do not trigger a reaction. Large population studies will be required to establish true individual thresholds, possibly dependent on the food matrix in which the allergen is present [5].

In conclusion, allergic diseases have an immunological component which is not only involved in sensitization, but also in the interaction between the genetic background and the modulation by the environment. Ultimately, a chronic inflammatory condition is apparent in an individually determined target organ linked to exposure with allergens, like skin, lungs, upper respiratory system and the gastro-intestinal tract. Allergic symptoms develop after a subsequent exposure to allergens like pollen, house dust mites or food proteins. Environmental conditions will modulate the disease's expression and consist of factors like tobacco smoke, air pollution, infections, vaccinations, food, exercise and factors linked to the profession. Knowledge of these principles, obtained in a multidisciplinary setting may result in advice and information on prevention of allergic diseases, both for the individual at risk and the society. However, the individual behaviour of an individual at risk and the quality of life that allergic persons want to achieve, determine to a large extent the impact of allergic diseases. Unfortunately, the perception of allergic disease due to the presence of clinical symptoms and diagnostic data might be different for various allergic diseases, especially for food allergy. Also the difference between IgE- and non-IgE-mediated allergic diseases

might compromise proper advice, treatment and management of the allergy.

6 Research aim and thesis outline

The research aim of this project was to study allergies by means of a multi-disciplinary approach, eventually leading to advice that will prevent the development of cross-reactive food allergies. By combining information on the genetic components, lifestyles and in vivo and *in vitro* assessment of the immune cells involved in allergy, a better insight in the possibilities of immunomodulation, or in the adaptation of the allergens itself, can be achieved. These issues were studied in mouse models and human *in vitro* PBMC cultures, and in addition, the lifestyles were assessed by means of a web-based questionnaire.

6.1 Mouse models

Since allergy has a multifactorial onset, first we investigated the influence of the genetic background. In Chapter 2, we used a recombinant congenic mouse strain in an ovalbumin model of allergic asthma to establish the impact of the genetic background on the onset of the allergic characteristics like e.g. airway hyper-responsiveness, eosinophils in the broncho-alveolar lavage fluid, Th2 cytokines (e.g. IL-4, IL-5 and IL-13) and allergen-specific IgE antibodies. A series of recombinant congenic mouse strains is generated by first crossing a recipient strain (BALB/c mice) with a donor strain (STS mice). Thereafter, the offspring is continuously back-crossed with the recipient strain to eventually generate approximately 20 homozygous strains, each of which contains on average 87.5% genes of the common recipient background and 12.5% of the donor background [95]. Together this set of 20 recombinant congenic strains contains approximately 95% of the genes of the donor strain. The essential feature that discriminates the recombinant congenic mouse strain system from the recombinant inbred strains is that unlinked genes of a multigenic trait are separated into individual recombinant congenic strains, thereby creating a number of single gene traits. In this way, the recombinant congenic mouse system transforms a multigenic difference into a set of mono- or oligogenic differences. Because the observed correlations (e.g. between airway hyperresponsiveness and allergen-specific IgE, or IL-5 and eosinophils) were not found in every individual strain, the asthma traits in this mouse model can be genetically dissociated.

As described in chapter 1, an allergic reaction is a consequence of a gene by environment interaction. Since genetic differences can also influence immune cell function and maybe even their origin, and the absence of one cell type

can alter another immune cell type, in Chapter 3, we used mice in which the $CD4^+$ Th cells or the $CD8^+$ Tc cells were knocked out by homologous recombination. In addition, mice transgenic for the Th2 cytokine IL-5 have been used. Since IL-5 is known for its role in eosinophilopoiesis, B cell growth and B cell differentiation, it can be speculated that IL-5 transgenesis could modify the outcome of the allergic response in these mice. By also cross-breeding the knock-out mice with the IL-5 transgenic mice [96], a set of mice that is suitable to study the importance of T and B cells in the allergic mechanism has been created. However, there is still much debate about the heterogeneity of B cells, and in particular B-1 B cells, in terms of cell surface markers, Ig production on a per cell basis, migration and homing, and influence of the local microenvironment [97, 98]. We investigated whether $CD4^+$ Th cells are required to obtain IgE secretion, and whether different B cell subset could be involved in the production of allergen-specific versus total IgE levels.

6.2 Human *in vitro* PBMC studies

Chapters 2 and 3 describe how mouse models can be used to study the mechanistic aspects underlying the immunopathology characteristics of allergic responses. However, observations in mice always needs to be validated in human studies. In particular, phenotypic and functional characteristics of the molecular and cellular compartments of the B and T cell immune response need to be analyzed and compared in both allergic and non-allergic individuals. The allergic individuals need to be selected based on the diagnosis by an allergologist. Thereafter, peripheral blood from these individuals can be obtained and, subsequently, the peripheral blood mononuclear cell (PBMC) fraction can be isolated and tested *in vitro*. Isolated PBMC cultures often yield significantly higher number of positive results than whole-blood assays, since removal of the anticoagulant, erythrocytes, granulocytes, and/or plasma from the PBMC fraction favors *in vitro* proliferation [99]. Fresh samples of separated PBMC also result in higher proliferation yields compared with cryopreserved PBMC and for all stimulations of whole blood samples shipped overnight. This result was not surprising, and it probably reflects better preservation of cell viability and/or function in samples that were manipulated less [100]. Therefore, in Chapter 4, we describe a method to cryopreserve and stimulate human peripheral blood mononuclear cells in order to read out the immunophenotype, proliferation capacity and cytokine production. These parameters permit the qualitative and quantitative evaluation of the functional capacity of immune cells, and provided the background to study allergen-specific stimulations as shown in Chapter 5 and the screening of the immunomodulatory capacity of mushroom extracts in Chapter 6. In Chapter 5, allergen-specific cellular analysis (PBMC cultures) in addition to serological binding analysis (allergen-specific inhibition ELISA) have been performed to

study the effect of heating on the allergenicity potential of the major birch pollen allergen, Bet v 1, and its cross-reactive allergens Dau c 1 and Api g 1. The allergen-specific PBMC cultures were designed to analyze the $CD4^+$ Th cell response to the per definition linear epitopes within the Bet v 1, Api g 1 and Dau c1 proteins. In contrast, the ELISA was used to analyze the binding capacity and the effect of heat-treatment on these conformational epitopes, principally involved in the B cell response to these allergens. In addition, both the natural and recombinant forms of these PR-10 homologous proteins were compared to observe whether recombinant proteins provide a reliable resemblance of the allergenic proteins present in the natural source. More specifically, we investigated whether the presence of multiple isoforms within the natural extracts could complicates the comparison between purified native proteins and their recombinant counterparts.

When testing human PBMC, the *in vitro* experiment may be influenced by the method of PBMC preparation as well as the variable responsiveness of the donors. Once identified, however, these parameters/factors can be controlled by using standardized methodologies, allowing, on the one hand, to classify immunomodulatory compounds according to the *in vitro* differences in their interaction with human immunocompetent cells. On the other hand, to confirm in vivo the "protective capacity" of the best candidate molecules (e.g. showing reduction of inflammatory cytokines and increase in anti-inflammatory cytokines). Evidence has been accumulating that regularly ingested herbal and medicinal mushroom supplements may have beneficial effects. These supplements, unlike medicines, are not effective instantly, but must be taken over long periods of time in order to be effective. Stimulating the immune system is a useful application for such supplements, since the immune system can be improved gradually. Such an improved immune system can be beneficial in fighting infections, suppressing tumor development and reducing allergic symptoms. In Chapter 6, eight mushroom strains, *Agaricus blazei*, *Coprinus comatus*, *Flammulina velutipes*, *Ganoderma lucidum*, *Grifola frondosa*, *Volvariella volvacea*, *Lentinus edodes*, and *Pleurotus ostreatus* were tested for the immunomodulating activity of the isolated protein fractions and polysaccharides fractions present in mycelia and culture liquid. The immunomodulating activity of the isolated compounds altered the *in vitro* immune response of human PBMC. Based on the cytokine production, it can be concluded that mushroom proteins mainly influenced the monocyte activation, thereby indirectly altering the T cell-specific cytokine production.

6.3 Socio-economic data

All previous chapters aimed at the identification of parameters that can be used to develop strategies to prevent the onset of the allergic sensitization or the development of clinical (cross-reactive food) allergic complaints. However,

such strategies require interventions based on the lifestyle characteristics of allergic individuals. To get more insight in the possible influence of lifestyle factors on the development of allergic responses, we performed a literature review on the mutual influences of allergic responses and lifestyle factors, as shown in Chapter 7. Literature showed that hay fever is an indicating factor for the development of asthma in approximately 30% of the hay fever patients. In addition, 50-70% of the birch pollen allergic individuals develop a cross-reactive food allergy over time. Since hay fever is partially initiated by the influences of environmental factors (e.g. presence of birch pollen, living environment, family size, exposure to smoke, labor circumstances, and nutritional habits), the lifestyle of an individual might, in addition to genetic factors, modulate health related issues within the allergic immunopathology in a causal or curative way. In order to assess lifestyle differences between allergic and non-allergic individuals, the relevant lifestyle factors assessed in chapter 7 were thereafter used to develop the questionnaire used in the FREYAL study. By means of this FREYAL study we aimed to assess differences between lifestyle factors of non-allergic and allergic individuals. Besides the completed questionnaire, all participants donated serum in which the amount of allergen-specific IgE to birch, apple, carrot and celery was determined. The findings of the FREYAL study until now are described in Chapter 8.

Finally, in Chapter 9, a synthesis of all results is provided and the mechanisms of their interaction within the allergic individual are discussed. Special emphasis is put on the significance of multiple integration levels in the study of allergic diseases.

Chapter 1

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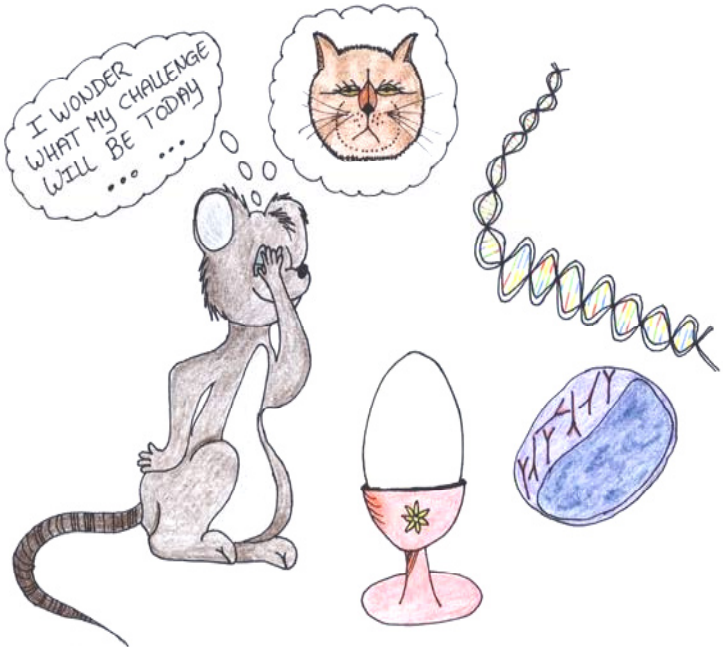
SECTION 1

Murine Allergy Studies



Chapter 2: Genetic influences

Chapter 3: T and B cell interaction

CHAPTER

2

Mouse genetic model for antigen-induced airway manifestations of asthma

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Abstract

Allergic asthma is a genetically complex disease characterized by allergen-specific immunoglobulin (Ig)E, eosinophilic inflammation of the lungs and airway hyperresponsiveness to bronchospasmogenic stimuli. In this study, we compared 13 recombinant congenic (RC) mouse strains in an ovalbumin model of allergic asthma. Different intensities and types of responses are observed throughout the RC strains. Intensities range from resistance to asthma in CcS05, to a very severe bronchoconstrictive reaction upon methacholine challenge for the parental STS strain. All strains show a 'modified' Th2 response except CcS14, which shows a 'true' Th2 response. When data from all strains are pooled, airway reactivity shows significant correlations with the serum Ig levels and the levels of interleukin (IL)-4, IL-5 and IL-13 in the broncho-alveolar lavage (BAL), at low dosage of methacholine (below 25 mg/ml), whereas at high dosage airway reactivity only correlates with BAL neutrophil levels. This indicates that at least two different mechanisms are involved in the airway reactivity to methacholine. None of these correlations can be found in every individual strain, which demonstrates that the asthma traits in this mouse model are genetically dissociated and that the loci can be genetically mapped.

Introduction

Allergic asthma is a heterogeneous disease, which can be characterized by allergen-specific immunoglobulin (Ig)E levels in serum, reversible airway obstruction, chronic eosinophilic inflammation of the airway tissue and hyperresponsiveness of the airways to nonspecific bronchospasmogenic stimuli. Genetic susceptibility to asthma appears to be due to multiple genes that interact with each other and the environment. Genome-wide screens for asthma and atopy have been performed in different ethnic populations and resulted in statistical evidence for linkage to several chromosomal regions including 5q, 11q and 12q [1-6]. Some of these regions contain relevant candidate genes that may regulate IgE responses, Th-cell differentiation, inflammatory processes and airway hyperresponsiveness (AHR). Although an increasing number of linkages for atopic diseases have been confirmed [7], it remains to be demonstrated that all candidate genes are linked to the pathophysiology of asthma.

Fine-mapping and identification of all the genes involved in asthma-related traits has proven to be extremely difficult, not in the least part due to epistatic interactions. Until now, five potential susceptibility genes for atopy or asthma were identified using positional approaches: a desintegrin and metalloproteinase 33 (ADAM33) [8-10], dipeptidyl peptidase 10 [11], plant homeodomain zinc-finger protein 11 [12], G-protein coupled receptor for asthma [13] and serine protease inhibitor Kazal type 5 (SPINK5) [14].

Polymorphisms of both ADAM33 and SPINK5 were not associated with asthma in some of the tested populations [15,16]. This could be due to different phenotyping of asthma or epistatic interactions, but it cannot be excluded that other polymorphisms in close linkage disequilibrium to the polymorphisms of both ADAM33 and SPINK5 are conferring the asthma susceptibility.

Although an animal model of asthma may not exhibit all aspects of the disease, identification of genes involved in certain well-defined characteristics may accelerate further human studies. Given the considerable homology between the human and mouse genome, the mouse has proven to be a useful genetic model for complex human diseases [17,18]. Furthermore, genetic homogeneity and strictly controlled environmental conditions are major advantages of mouse models as well as the fact that the mouse 'toolbox' (gene-targeted mice, genetic and physical map) is very well developed.

Nowadays, a number of genetic tools is available for localization of genes involved in disease development [18]. In several diseases, the recombinant congenic (RC) strains of mice have proven to be a powerful tool to provide the

mapping of genes controlling complex traits [17;19-27]. A series of RC strains comprises approximately 20 homozygous strains, each of which contains on average 87.5% genes of a common background strain and 12.5% of a common donor strain [26,28]. In this way, the RC system transforms a multigenic difference into a set of mono- or oligogenic differences and hence offers higher resolution power in mapping the QTLs (quantitative trait loci) and detecting their mutual interactions than the standard genetic methods. The RC system has been used most successfully to map genes in colon tumor susceptibility [29-32].

One of the RC strains series, CcS/Dem, generated by Demant and colleagues, uses BALB/cHeA as background strain and STS/A as donor strain [33]. The BALB/c mice produce high serum IgE levels, whereas the STS/A shows low serum IgE levels after *Leishmania major* infection [17]. This series of RC strains has been used to dissect the genetics of T cell activation and several models of inflammatory diseases, including *Mycobacterium tuberculosis* and *L. major* infection [17;19-25;27].

In the present study, we used the CcS/Dem series to determine the susceptibility of each strain and both parental strains BALB/cHeA and STS/A for antigen-induced airway manifestations of asthma as described by Deurloo *et al.* [34]. Generated data were used to calculate correlations between the different asthma related characteristics for all individual strains and in the pooled population.

Materials and methods

Mice

Male, 5-9 week-old BALB/cHeA, STS/A and CcS/Dem [22] mice were obtained from the breeding colony of Dr. P. Demant. Seven to eleven mice per strain were phenotyped (see Table 1a and b). Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal experiments. The mice were kept under specified pathogen-free conditions according to the guidelines of the Federation of European Laboratory Animal Science Association [47]. They were housed in enriched macrolon cages under filter top or in a laminar flow cabinet with food and water administered *ad libitum*.

Experimental asthma protocol

On day 0 and 7, the animals were intraperitoneally sensitized with 10 µg OVA in 2 mg Alum adjuvant (Pierce, Rockfort, IL, USA). One week after the second sensitization, blood was taken. Subsequently, the animals were challenged for 20 minutes with 10 mg OVA/ml in saline, starting on day 21 and then once per 3 days for a total of three times. Twenty-four hours after the last challenge, airway responsiveness to methacholine was measured. Consecutively, blood and BAL fluid were collected.

Airway responsiveness

Airway responsiveness was measured in conscious, unrestrained mice before (day 18) and after (day 28) OVA challenges. Airway reactivity was determined by recording respiratory pressure curves in response to inhaled nebulized methacholine (acetyl-β-methylcholine chloride, Sigma, St. Louis, MO, USA) at doses of 1.6, 3.1, 6.3, 12.5, 25 and 50 mg/ml using barometric whole-body plethysmography (BUXCO, Wilmington, NC, USA). Airway responses were expressed in enhanced pause (Penh), an index of airway obstruction as described in detail previously [48].

STS mice were not exposed to doses above 12.5 mg/ml methacholine as higher doses induce a very severe bronchoconstrictive reaction.

OVA-specific Ig levels in serum

After measurement of *in vivo* airway reactivity, mice were killed by intraperitoneal injection of 1 ml 10% urethane in pyrogen-free saline (Sigma, St. Louis, MO, USA). Subsequently, mice were bled by cardiac puncture and serum was collected and stored at -70 °C until further analysis.

Levels of OVA-specific IgE, IgG1 and IgG2a in the serum were measured as described previously [49]. A reference standard was used with arbitrary units of each isotype of 1000 EU/ml. The detection levels of the enzyme-linked immunosorbent assays (ELISA) were 0.05 EU/ml for IgG2a, and 0.5 EU/ml for IgE, and 0.005 EU/ml for IgG1.

Analysis of the cellular composition in the BAL fluid

Immediately after bleeding, the lungs were lavaged through a tracheal cannula with 1 ml saline at 37 °C containing 5% BSA and 2 μg/ml aprotinine (Roche Diagnostics, Basel, Switzerland). Cells were spun down and supernatant was stored at -20 °C until measurement of cytokines by ELISA. Subsequently, lungs were lavaged four times with 1 ml aliquots of saline. The BAL cells were washed with PBS (400 *g*, 4 °C, 5 min) and the pellet was resuspended in 150 μl PBS. Total numbers of BAL cells were counted in a Burker-Türk chamber (Omnilabo, Breda, The Netherlands). For differential BAL cell counts, cytospin preparations were stained with Diff-Quick (Merz & Dade A.G., Dudingen, Switzerland). After they were coded, all cytospin preparations were evaluated by one observer. Cells were identified and differentiated into mononuclear cells, lymphocytes, neutrophils, and eosinophils by standard morphology. At least 200 cells were counted per cytospin preparation.

Cytokine ELISA

Cell-free supernatants of the first milliliter of BAL fluid were analyzed for IL-4, IL-5, IL-10, IL-13, and IFN-γ content by sandwich ELISA using antibody pairs and standards purchased from PharMingen, according to the manufacturer's instructions. The lower detection limits of the ELISAs were 16 pg/ml for IL-4, 32 pg/ml for IL-5, 100 pg/ml for IL-10 and IL-13, and 160 pg/ml for IFN-γ.

Statistical analysis

Results obtained before and after OVA challenge were compared using signed-rank tests. The different strains were compared to BALB/c using Mann-Whitney *U*-test. Correlations were calculated using Spearman's rank correlations. False discovery rate for correlation tests was estimated to be 5% for the 95 and 1% for the 99% confidence intervals by computing correlations in 1000 permuted datasets. Statistical analyses were performed using SAS for Windows version 9.0 (SAS-Institute, Cary, NC, USA).

Results

Airway reactivity

After the ovalbumin (OVA) challenges, the parental STS strain showed a very severe bronchoconstrictive reaction at doses of methacholine above 12.5 mg/ml. Therefore, STS was not exposed to the complete dose-range, and Penh measurements obtained for STS were not used for further statistical analysis.

All tested strains showed an increase in airway reactivity after the OVA challenge compared to before the challenge (data not shown), which was significant at high doses of methacholine (25 and 50 mg/ml) for all strains except CcS03 (only at 50 mg / ml), CcS05, 12, 13 and 15.

After challenge, none of the tested strains, except STS, showed a consistent and significant deviation in Penh compared to BALB/c at doses of methacholine below 25 mg/ml. At 25 and 50 mg/ml, however, significantly lower Penh values were seen in CcS02, 03, 05, 07, 11, 12, 13, 15 and 18. For CcS20, this difference was only significant at 25 mg/ml methacholine. Surprisingly, none of the tested RC strains showed a significant higher extent of airway reactivity, compared to BALB/c, whereas the donor strain, STS, showed a much more severe bronchoconstrictive reaction compared to the background strain BALB/c (see Table Ia and b).

Serum Ig levels

OVA-specific IgE levels were significantly increased after challenge compared to before challenge in BALB/c, CcS02, 07, 14 and 20. IgG1 and IgG2a levels after challenge were increased in all strains, except CcS03 (only IgG2a is increased), 11, 13, 18 and STS (only IgG2a is increased), compared to before challenge.

After challenge, CcS01, 05, 07 and STS had significantly lower OVA-specific IgE levels compared to BALB/c, whereas significantly higher levels were measured in the serum of CcS11, 12 and 14. IgG1 levels after challenge were strongly and significantly lower in CcS05 and CcS13 compared to BALB/c after challenge. Only CcS05 mice showed significant lower IgG2a levels compared to BALB/c (see Table Ia and b).

Cellular composition of the BAL fluid

Owing to the severe bronchoconstriction in the STS mice, broncho-alveolar lavage (BAL) could not be performed reliably. Compared to BALB/c, total cell counts were higher in CcS01, CcS02 and CcS03 and significantly lower in all

Table Ia Mean ± s.e.m. for BALB/c and mean % of BALB/c ± % standard error defined as ((mean for strain ± s.e.m.) / mean for BALB/c) x 100

Parameter	*BALB/c (n = 11)*	*Percentage of BALB/c*					
		CcS 01 (n = 7)	*CcS 02 (n = 9)*	*CcS 03 (n = 7)*	*CcS 05 (n = 13)*	*CcS 07 (n = 10)*	*CcS 10 (n = 11)*
Penh (1.6 mg/ml)	1.2 ± 0.2	175.1 ± 31.6 *	89.3 ± 6.4	74.2 ± 6.9	83.4 ± 6.8	61.7 ± 5.1 *	104.4 ± 21.0
Penh (3.1 mg/ml)	2.8 ± 1.0	105.9 ± 13.6	50.0 ± 4.6	43.0 ± 4.5	49.3 ± 3.7	37.0 ± 3.5	68.2 ± 14.4
Penh (6.2 mg/ml)	3.8 ± 1.2	108.0 ± 20.4	54.5 ± 5.3	42.9 ± 5.4	48.1 ± 3.4	36.2 ± 2.6	84.2 ± 13.0
Penh (12.5 mg/ml)	7.4 ± 1.4	75.4 ± 14.2	40.5 ± 3.2 *	30.8 ± 5.8 **	23.4 ± 3.6 **	23.7 ± 2.3 **	79.9 ± 7.2
Penh (25 mg/ml)	10.7 ± 1.6	60.9 ± 10.5	35.4 ± 2.2 **	29.7 ± 4.5 **	27.7 ± 3.1 **	25.7 ± 3.0 **	72.2 ± 2.7
Penh (50 mg/ml)	11.5 ± 1.7	63.2 ± 7.1 *	37.2 ± 3.4 **	36.9 ± 6.9 **	31.2 ± 4.0 **	32.7 ± 2.9 **	79.1 ± 5.7
Mononuclear cells	1.8 ± 0.2 × 10^6 cells	328.0 ± 56.4 **	133.0 ± 12.8	251.6 ± 44.6 *	23.3 ± 2.4 **	88.2 ± 11.0	43.4 ± 6.1
Eosinophils	2.7 ± 1.0 × 10^6 cells	362.4 ± 70.9 **	201.9 ± 32.0 *	246.7 ± 44.7 *	2.8 ± 1.2 **	106.8 ± 20.1	37.3 ± 7.4
Neutrophils	3.2 ± 0.8 × 10^6 cells	630.8 ± 155.8 *	192.5 ± 29.2 *	271.2 ± 90.2	11.2 ± 4.7 *	169.1 ± 28.9	64.0 ± 11.9
Total cell count	5.4 ± 1.1 × 10^6 cells	366.5 ± 53.1 **	181.0 ± 23.6 *	251.5 ± 41.3 **	9.9 ± 1.3 **	105.6 ± 17.1	40.9 ± 6.1
IgE	1.1 ± 0.3 × 10^5 EU/ml	40.4 ± 8.1	117.8 ± 28.0	96.2 ± 32.2	28.5 ± 11.0 *	30.5 ± 5.6 *	64.9 ± 16.8
IgG1	5.4 ± 1.9 × 10^9 EU/ml	61.4 ± 11.0	66.3 ± 14.5	69.0 ± 18.3	10.1 ± 2.2 *	41.5 ± 12.9	134.6 ± 52.3
IgG2a	5.9 ± 3.7 × 10^5 EU/ml	37.0 ± 14.0	82.4 ± 39.6	62.5 ± 25.5	4.5 ± 1.3	10.6 ± 2.4	34.2 ± 11.8
IL-4	8.8 ± 1.7 pg/ml	150.2 ± 12.4	97.6 ± 13.0	111.6 ± 24.1	95.9 ± 32.4	255.2 ± 100.9	263.0 ± 87.3
IL-5	272.5 ± 86.4 pg/ml	204.0 ± 27.0 *	167.2 ± 28.3	152.3 ± 54.5	5.3 ± 2.9 *	97.6 ± 21.7	59.9 ± 19.5
IL-10	9.0 ± 0.6 pg/ml	1322.1 ± 38.0 **	610.7 ± 46.7 *	1105.8 ± 174.3	112.4 ± 4.8	1109.7 ± 79.4 **	414.4 ± 69.5
IL-13	50.3 ± 15.6 pg/ml	117.4 ± 18.5	104.8 ± 23.6	150.3 ± 39.1	10.8 ± 2.9 *	63.1 ± 8.0	27.7 ± 8.1

*: Significant at 95% confidence level, **: Significant at 99% confidence level.

Table Ib Mean ± s.e.m. for BALB/c and mean % of BALB/c ± % standard error defined as ((mean for strain ± s.e.m.) / mean for BALB/c) x 100

Parameter	Percentage of BALB/c						
	CcS 11 (n = 7)	CcS 12 (n = 11)	CcS 13 (n = 7)	CcS 14 (n = 7)	CcS 15 (n = 7)	CcS 18 (n = 8)	CcS 20 (n = 8)
Penh (1.6 mg/ml)	85.7 ± 13.0	108.6 ± 5.4	69.8 ± 4.3	89.4 ± 21.3	113.4 ± 16.3	169.8 ± 10.2 **	119.2 ± 17.5
Penh (3.1 mg/ml)	67.9 ± 7.0	56.9 ± 3.9	41.7 ± 5.4	56.0 ± 6.7	66.9 ± 11.6	81.0 ± 4.6	68.0 ± 13.1
Penh (6.2 mg/ml)	59.2 ± 5.1	51.4 ± 2.1	49.4 ± 11.8	66.7 ± 3.5	66.6 ± 9.9	76.7 ± 3.9	61.8 ± 12.8
Penh (12.5 mg/ml)	37.8 ± 2.8 **	37.4 ± 3.2 **	39.7 ± 6.4 *	81.1 ± 14.5	38.2 ± 6.0 *	50.5 ± 3.8 *	49.1 ± 12.5
Penh (25 mg/ml)	29.6 ± 3.0 **	29.1 ± 3.5 **	39.0 ± 7.2 **	73.1 ± 9.7	39.8 ± 7.0 **	39.5 ± 6.0 **	54.5 ± 9.0
Penh (50 mg/ml)	31.2 ± 1.9 **	26.1 ± 3.1 **	49.5 ± 8.3 *	84.0 ± 12.3	51.6 ± 8.7 *	48.4 ± 9.3 *	68.8 ± 8.4
Mononuclear cells	28.9 ± 4.9 **	36.0 ± 5.4 **	40.1 ± 9.5 **	52.2 ± 7.1 **	91.1 ± 15.2	13.1 ± 2.5 **	109.3 ± 28.7
Eosinophils	21.9 ± 8.3 *	45.3 ± 10.6	41.4 ± 19.8	43.7 ± 10.1	140.2 ± 22.7	17.5 ± 4.1 *	112.2 ± 33.8
Neutrophils	10.3 ± 7.1 *	4.1 ± 2.1 **	29.5 ± 13.1 *	88.1 ± 32.3	193.7 ± 36.4	29.9 ± 8.4 *	75.5 ± 17.7
Total cell count	23.8 ± 6.7 **	40.7 ± 7.4 *	40.7 ± 16.1	49.1 ± 9.9 *	127.2 ± 18.5	16.6 ± 3.5 **	109.8 ± 30.5
IgE	277.2 ± 52.9 **	257.2 ± 46.0 *	106.6 ± 31.9	841.1 ± 196.8 *	157.1 ± 53.6	232.5 ± 86.0	190.1 ± 38.5
IgG1	94.0 ± 27.0	94.2 ± 17.4	0.1 ± 0.0 *	56.4 ± 16.9	155.7 ± 49.1	68.6 ± 23.8	167.1 ± 38.4
IgG2a	46.0 ± 12.3	16.0 ± 3.3	30.2 ± 21.3	81.7 ± 26.5	208.1 ± 99.3	113.8 ± 95.7	57.9 ± 22.6
IL-4	406.4 ± 115.3 *	461.2 ± 84.1 **	52.2 ± 3.2 *	4013.7 ± * 1119.6	70.4 ± 4.3	693.2 ± 154.0 **	62.8 ± 5.2
IL-5	127.0 ± 50.3	76.6 ± 24.8	61.3 ± 15.4	339.4 ± 74.3 **	75.2 ± 12.7	262.9 ± 52.5 *	58.7 ± 16.3
IL-10	922.6 ± 71.8 *	571.7 ± 33.4 **	178.6 ± 3.3	6477.1 ± 491.2 *	637.9 ± 22.1 **	1902.7 ± 126.2 **	382.1 ± 15.9
IL-13	68.3 ± 81.3	51.2 ± 53.2	26.9 ± 7.0	326.3 ± 47.7 **	49.6 ± 11.0	252.7 ± 27.7	37.9 ± 4.0

*: Significant at 95% confidence level, **: Significant at 99% confidence level.

Chapter 2

Table II Spearman's rank correlation coefficients

Penh 3.2 mg/ml	*Penh 6.3 mg/ml*	*Penh 12.5 mg/ml*	*Penh 25 mg/ml*	*Penh 50 mg/ml*	*Mononuclear cells*	*Eosino -phils*	*Neutro-phils*	*Total cell count*	*IgE*	*IgG1*	*IgG2a*	*IL-4*	*IL-5*	*IL-10*	*IL-13*	*Parameter*
0.81 **	0.65 **	0.46 **	0.30 **	0.26 **	-0.04	0.18 *	0.03	0.12	0.28 **	0.32 **	0.19 *	0.29 **	0.22 *	0.21 *	0.25 *	Penh 1.6 mg / ml
	0.77 **	0.51 **	0.26 **	0.22 *	-0.06	0.11	0.00	0.06	0.29 **	0.23 *	0.17	0.29 **	0.25 **	0.15	030 **	Penh 3.2 mg / ml
		0.68 **	0.49 **	0.45 **	-0.02	0.15	0.06	0.10	0.34 **	0.28 **	0.22 *	0.35 **	0.36 **	0.17	0.38 **	Penh 6.3 mg / ml
			0.78 **	0.70 **	0.08	0.14	0.15	0.13	0.29 **	0.25 **	0.21 *	0.29 **	0.26 **	0.11	0.32 **	Penh 12.5 mg / ml
				0.85 **	0.19 *	0.16	0.23 **	0.17	0.12	0.20 *	0.20 *	0.11	0.13	0.06	0.11	Penh 25 mg / ml
					0.20 *	0.16	0.28 **	0.18 *	0.12	0.23 *	0.26 **	0.07	0.10	0.10	0.11	Penh 50 mg / ml
						0.85 **	0.74 **	0.93 **	0.00	0.30 **	0.41 **	-0.06	0.38 **	0.29 **	0.23 *	Mononuclear cells
							0.73 **	0.98 **	0.23 *	0.45 **	0.50 **	0.09	0.56 **	0.35 **	0.37 **	Eosinophils
								0.76 **	-0.13	0.22 *	0.35 **	-0.01	0.41 **	0.29 **	0.16	Neutrophils
									0.16	0.42 **	0.48 **	0.04	0.52 **	0.34 **	0.33 **	Total cell count
										0.55 **	0.43 **	0.40 **	0.39 **	0.29 **	0.52 **	OVA-IgE
											0.63 **	0.25 **	0.29 **	0.12	0.21	OVA-IgG1
												0.21 *	0.36 **	0.08	0.29 **	OVA-IgG2a
													0.48 **	0.51 **	0.51 **	IL-4
														0.74 **	0.79 **	IL-5
															0.59 **	IL-10

*Significant at 95% confidence level; **significant at 99% confidence level. The Spearman's rank correlation coefficients for positive correlations range from 0 to 1 (-1 to 0 for negative correlations), the higher the absolute value of the coefficient, the tighter the correlation.

other strains except for CcS07, 14, 15 and 20. Despite this significant lower total cell count, the number of eosinophils was not significantly lower in CcS10, 11, 12 and 13 compared to BALB/c.

Cytokine composition of the BAL fluid

No interferon (IFN)-γ could be detected in the BAL fluid of any of the tested strains (detection limit 160 pg/ml).

Compared to BALB/c, higher interleukin (IL)-4 levels were measured in the BAL fluid of CcS01, 07, 11, 12, 14 and 18. None of the tested strains showed significantly lower IL-4 levels compared to BALB/c. IL-5 is higher in the BAL fluid of CcS14 and CcS18, and strongly lower in CcS05 compared to BALB/c. The anti-inflammatory cytokine IL-10 was significantly higher in CcS01, 07, 12, 14, 15 and 18. None of the tested strains showed significantly lower IL-10 content of the BAL fluid, compared to BALB/c. IL-13 was significantly lower in CcS05 and significantly higher in CcS14 and 18.

Correlations between asthma-related parameters

Remarkably, in the pooled population, different correlations between airway reactivity (Penh) and other asthma-related parameters were observed at the high doses of methacholine (25 and 50 mg/ml) compared to low doses of methacholine (12.5 mg/ml and below). At low doses of methacholine, airway reactivity significantly correlated to serum IgE levels, BAL IL-4, IL-5 and IL-13 levels, whereas at high doses of methacholine, airway reactivity correlated to neutrophil content of the BAL (at 25 and 50 mg/ml). At 1 mg/ml methacholine, Penh also correlated with the eosinophil and IL-10 content of the BAL fluid (see Table II). Airway reactivity correlated to both IgG1 and IgG2a (except at 3 mg/ml) at all doses of methacholine.

Strong significant correlations were seen between IgE, IgG1 and IgG2a levels. The IgE levels also significantly correlated with all measured Th2 cytokines and the eosinophil content of the BAL. IgG1 and IgG2a serum levels correlated with all cell types and the IL-4 and IL-5 content of the BAL. Additionally, IgG2a serum levels correlated with the IL-13 BAL levels.

Eosinophil, neutrophil, mononuclear and total cell content of the BAL fluid significantly correlated to each other. A significant correlation was also seen between all cell types and the IL-5, IL-10 and IL-13 levels (no significant correlation between IL-13 and neutrophils), but surprisingly not with IL-4.

Some of these correlations, which were observed in the pooled population, appeared in some of the individual strains, but none was observed in every single strain. Additionally, some strains showed significant correlations that were not detected in the pooled population (data not shown).

Discussion

In this study, we clearly demonstrated the influence of the genetic background on the susceptibility to experimental asthma, as a broad range of susceptibilities is observed throughout the RC CcS/Dem strains. Ranging from a severe methacholine-induced bronchoconstriction in the parental STS strain to resistance observed in the CcS05 strain, which only showed limited airway reactivity at the highest dose of methacholine (50 mg / ml), low IgE, IgG1 and IgG2a levels and low levels of IL-5 and IL-10 in the BAL fluid. The group of Demant already observed that CcS05 mice are also highly resistant to *L. major* infection, another Th2 related disease [23].

Interestingly, not only the susceptibility, but also the type of response varies from one CcS/Dem strain to another. For example, mouse strain CcS14 showed a 'true' Th2 response upon OVA challenge, characterised by high IgE levels, low levels of IgG1 and IgG2a and high levels of Th2 cytokines. These high IgE and low IgG levels can be explained by an IL-4 mediated isotype switch, as high amounts of this cytokine can be found in the BAL fluid. Most other strains showed a 'modified' Th2 response, characterised by lower IgE levels and a higher IgG1/IgE ratio. This 'modified' Th2 response was previously described as a mechanism that induces less severe atopic or asthmatic responses, compared to a 'true' Th2 response [35]. In our study, however, the 'true' Th2 responder, CcS14, did neither show higher airway reactivity, nor allergic inflammation, compared to BALB/c.

These differences in asthma-related phenotypes are in accordance with Whitehead *et al.*, who also show the importance of genetic background on the development of allergen-induced airway diseases, using nine genetically diverse inbred mouse strains in an OVA-model of asthma [36].

Several groups have shown that IL-13 is crucial for the development of asthmatic AHR. IL-13 knock-out mice, for example, show no AHR despite an extensive eosinophilic inflammation of the lungs [37]. The groups of Bleecker and Postma showed an association between a polymorphism in the IL-13 promoter and bronchial hyperreactivity in a Dutch population of asthmatics [38]. Vladich *et al.* recently demonstrated that the IL-13 2044GA polymorphism, which is associated with allergy and asthma, encodes for a form of IL-13, which is more active and less effectively neutralised [39]. In our experiments,

a significant correlation was seen between IL-13 and airway reactivity at low doses of methacholine (below 25 mg/ml), but not at high doses. Interestingly, CcS14 has highly increased and CcS10 highly deceased levels of all measured Th2 cytokines, although no significant difference in airway reactivity was observed, compared to the parental strain BALB/c. This nicely illustrates the complexity of the development of AHR in our mouse model of asthma.

The group of Van Scott demonstrated the importance of IL-10 in the development of asthma using a ragweed sensitized and challenged C57BL/6 IL-10 knock-out mouse. These mice only developed AHR when recombinant IL-10 was administered together with the ragweed challenge, but not when IL-10 was given just prior to airway reactivity measurement using methacholine [40]. Also the group of Gelfand used an IL-10 knock-out mouse, but in an OVA model of asthma. The mice only developed AHR when they were infected with an adenovirus encoding an IL-10 expression cassette prior to challenge [41]. Zhang *et al.* found evidence for linkage between AHR and a locus on mouse chromosome 9, which among other genes also encodes the IL-10R [42]. More recently, Chatterjee *et al.* found genetic association between asthma and a polymorphism in the IL-10 promoter sequence in human [43]. In our experiments, IL-10 is only measured in the BAL at the end of the experiment. A correlation with airway reactivity is only seen at 1.6 mg/ml methacholine.

Also, IgE was investigated for its role in the development of AHR. Wynn *et al.* suggest that the development of AHR is not dependent on the IgE production [44]. This is supported by our results as the parental STS shows the lowest serum IgE levels but shows the strongest increase in Penh. On the other hand, CcS11 has significantly higher serum IgE levels, compared to BALB/c, but has significantly lower airway reactivity at methacholine doses of 12.5 mg/ml and above. Despite these observations, a significant correlation between airway reactivity and IgE levels is observed in our data at low methacholine doses, below 25 mg/ml, but not at higher doses.

These correlations may not reflect causal relationship but they nicely fit in a model in which the response to methacholine is caused by at least two mechanisms. The first one is predominant at low doses of methacholine (below 25 mg/ml) and is related to the Th2-mediated inflammatory state of the lungs and by this correlates to the cytokine and Ig levels and eosinophil count. The second mechanism becomes predominant at high doses of methacholine and is related to the neutrophil content of the BAL. This second mechanism gives rise to much higher airway responses, compared to the first one, and could therefore be considered as the main mechanism involved in AHR in this mouse model of allergic asthma.

Interestingly, none of the correlations described above can be found in every individual strain. This confirms the complex multigenic character of asthma features and highlights the suitability of the CcS/Dem strains to map the genes involved. It also highlights the fact that none of the tested phenotypes are directly linked and that other factors play a role in the development of an asthmatic response in this mouse model.

RC strains are a powerful tool to unravel the genetics of genetically complex processes as it allows mapping of the genes involved. For example, Nicolaides *et al.* identified IL-9 as a quantitative trait locus implicated in the normal airway response to acetylcholine [45]. Also, the CcS/Dem strains, which were also used in our experiments, have already been successfully used by different groups. Lipoldova *et al.*, for example, identified five novel *L. major* resistance loci using CcS05 [17] and Havelkova *et al.* used CcS09 and CcS11 for the identification of genes involved in T cell receptor-induced activation [46].

It can be concluded that the RC system is a useful tool to map and identify asthma related genes, as the same correlations between asthma related characteristics are found in humans and mouse models, and a broad range of intensity and type of 'asthmatic' responses are observed. Moreover, the different asthma characteristics segregated independently, which will make it possible to map loci implicated in these different characteristics. Additionally, it can be hypothesized that at least two mechanisms were involved in the airway reactivity to methacholine in our mouse model of asthma; a first one, predominant at low doses of methacholine, is related to the allergic inflammatory state of the lungs, whereas the second one is related to BAL neutrophils and is predominant at high doses of methacholine.

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CHAPTER

3

IL-5 enhances isotype switching to IgE in splenic cells of CD4 knockout mice, whereas it does not overcome the decrease in the OVA-specific IgE secretion

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Abstract

Since the factors that are responsible for the difference between total versus allergen-specific IgE expression, production and secretion are still unknown, we speculated that the different B cell lineages (B-1a, B-1b and B-2) within different effector organs like spleen and draining lymph nodes could be responsible. Since IL-5 induces B-1 progenitors, we compared mice over-expressing IL-5 in $CD3^+$ T lymphocytes (NJ.1638; IL-5 Tr) to wild type (WT) mice. In addition, the IL-5 transgenic mice were cross-bred with gene knockout mice lacking either $CD4^+$ T lymphocytes ($CD4^{-/-}$IL-5Tr) or $CD8^+$ T lymphocytes ($CD8^{-/-}$IL-5Tr). Subsequently, these mice were ovalbumin (OVA) sensitized and OVA or PBS challenged. We showed a large proliferating $B220^+$ population expressing IL-5Rα, high levels of IgM and low numbers of CD138 in the spleen of IL-5 Tr mice, suggesting they originate from the B-1 lineage. In addition, these B-1 cells undergo massive migration towards the spleen under the influence of the constitutively expressed IL-5 levels. The absence of allergen-specific IgE in the serum of $CD4^{-/-}$ and $CD4^{-/-}$IL-5Tr mice showed that $CD4^+$ T cells are required to obtain allergen-specific and total immuno-globulin isotype switching of B-2 cells. However, IgE expressing B-1 cells do not require $CD4^+$ T cell help to undergo isotype switching, but these cells appear to be incapable of secreting IgE antibodies.

Introduction

IL-5 plays a well established role in the eosinophilopoiesis, and B cell growth and differentiation [1-3], although several mouse models with an over-expression of IL-5 showed contradicting effects on B cells [4]. In an attempt to mimic the IL-5 expression pattern observed in selected disease states, a transgenic mouse line was developed expressing IL-5 in all T cells [4]. This IL-5 transgenesis had a large impact on the cancellous/trabecular volume of the skeletal bones, leading to occupation of the femoral medullary cavity by numerous interconnecting trabeculae [5]. Besides bone metabolism modulation, hematopoietic progenitor migration towards the lungs after allergen challenge, and increased peripheral white blood cell numbers, these IL-5 transgenic mice showed extramedullary hematopoiesis and splenomegaly [4-6].

One of the increased white blood cell populations observed in IL-5 transgenic NJ.1638 mice were B cells [4]. In allergic diseases, the B cell plays an important role as it produces allergen-specific IgE immunoglobulins that are required to trigger the allergen-specific release of inflammatory mediators from mast cells [7]. Appropriately activated B cells can undergo T helper (Th) cell regulated isotype switching through physical contact with CD40 on the B cell and the presence of specific cytokines [8]. Nowadays, it is common knowledge that in mice IL-4 and IL-13 provide isotype switching towards IgE, whereas IL-4, IL-5 and IL-2 synergize to enhance the IgG1 response [8, 9]. However, the difference between the formation of total and allergen-specific IgE is still unknown, although Shang *et al.* suggested that allergen-specific IgE is formed in the draining lymph nodes (DLN), whereas total IgE levels are formed within the spleen [10].

We therefore hypothesized that different B cells that migrate to the different organs could be responsible for the induction of total versus allergen-specific IgE. Although still debated, the existence of different murine B cell lineages has been proposed: B-1a, B-1b and B-2 B cells. Tung *et al.* showed that these distinct B cell lineages can be segregated by their CD19, B220, CD138 and IgM expression. B-1a progenitors are $CD19^{+}/B220^{+}/CD138^{-}/IgM^{hi}$, whereas B-1b progenitors are $CD19^{+}/B220^{+}/CD138^{+}/IgM^{hi}$ and B-2 progenitors are $CD19^{-}/B220^{+}/CD138^{+}/IgM^{lo}$ [11, 12]. B-2 progenitors originate from hematopoietic stem cells in the bone marrow during postnatal life, eventually leading to the generation of surface IgM expressing cells which then migrate to the spleen, where they undergo further maturation into follicular or marginal zone B cells. Following exposure to antigen and signals from T helper cells, follicular B cells can undergo immunoglobulin class switching, somatic hypermutation, and differentiation into plasma and memory B cells [11, 13]. In contrast, both B-1a and B-1b B cells are largely responsible for the innate antibody response and respond readily to a variety of T-independent

antigens; B1a cells being the source of natural antibodies, whereas B1b cells produce immunoglobulins after antigen exposure [11, 13].

Transgenic mice constitutively expressing the IL-5 gene in liver and spleen exhibited an increase in the B-1 B cell numbers [2]. The group of Takatsu *et al.* illuminate the role of IL-5 in the homeostatic proliferation and survival of mature B-1 B cells by the application of a monoclonal anti-IL-5 antibody (Ab) and IL-5Rα knockout mice. They show that the anti-IL-5 Ab treatment decreased the mature B-1 B cell compartment, and the absence of the IL-5Rα resulted in an impaired survival and proliferation of B-1 B cells in the peritoneal cavity [2].

This study investigated the effect of IL-5 transgenesis on the three different B cell lineages in organs known to be affected by the IL-5 transgenesis, bone marrow and spleen, and an presumably unaffected organ, the DLN. In addition, the role of $CD4^+$ or $CD8^+$ T cells with or without the presence of high IL-5 levels by using $CD4^{-/-}$ and $CD8^{-/-}$ mice on a C57Bl/6 background in a backcross with IL-5 transgenic NJ.1638 mice were investigated in a model of allergen induced airway inflammation. Both B and T cell specific surface markers were stained on cells isolated from PBS or ovalbumin (OVA) treated mice. Furthermore, different total and allergen-specific immunoglobulin isotypes were determined in serum to obtain information about the effect of T cell subset absence and IL-5 over-expression in these animals. We showed an important role for T cells and IL-5 in the formation of different B cell lineages and their migration potential. More specifically, the production of OVA-specific IgE is dependent on the present T cell subsets, whereas the presence of IgE^+ B cells in the spleen is independent of the presence of T cell subsets, but largely dependent on the effector functions of IL-5. We provide evidence that the predominant B-2 B cells present in the DLN are responsible for the formation of allergen-specific IgE, whereas the predominant IgE^+ B-1 B cells in the spleen of IL-5 transgenic mice are not contributing to the secretion of IgE.

Materials and Methods

Mice

This study was approved by the Animal Ethical Committee in Gothenburg, Sweden. All mice were provided with ad libitum food and water and housed in specified pathogen-free animal facilities. Wild type C57BL/6 mice were purchased from Mollegaard-Bommice A/S (Ry, Denmark). IL-5 Tr mice (NJ.1638) overexpressing IL-5 specifically in the $CD3^+$ T lymphocytes were obtained from Dr. James J. Lee (Mayo Clinic, Scottsdale, AZ, USA) and maintained in a heterozygous fashion by back-crossing to C57BL/6 mice. IL-5 Tr mice were bred with gene knockout mice lacking either $CD4^+$ T lymphocytes (C57BL/6J $CD4^{tm1Knw}$) or $CD8^+$ T lymphocytes (C57BL/6 $CD8^{atm1Mak}$) (Jackson Laboratories, Bar Harbor, ME) to produce IL-5 Tr knockout mice deficient in $CD4^+$ and $CD8^+$ T lymphocytes, respectively (summarized in Table I).

Table I Used mouse strains

Strain name	$CD4^+$ cells	$CD8^+$ cells	IL-5 transgenic
WT	Normal	Normal	No
IL-5 Tr	Normal	Normal	Yes
$CD4^{-/-}$	Knock-out	Normal	No
$CD4^{-/-}$ IL-5Tr	Knock-out	Normal	Yes
$CD8^{-/-}$	Normal	Knock-out	No
$CD8^{-/-}$ IL-5Tr	Normal	Knock-out	Yes

The genotype of mice produced by these crosses were assessed by the presence of $CD3IL\text{-}5^+$ and loss of either T cell subtype (PCR of tail DNA). Briefly, DNA was isolated from tail biopsies by using the DNeasy Tissue kit according to manufacturer's instructions (Qiagen, Crawley, UK). The PCR reactions of the DNA from WT, $CD4^{-/-}$ or $CD8^{-/-}$ were prepared using the HotStartTaq Master Mix Kit (Qiagen, Crawley, UK) according to the protocol received from The Jackson Laboratory (Jackson Laboratories, Bar Harbor, ME). The PCR reactions of the IL-5 Tr were assessed as previously described with some modifications [4].

Sensitization and allergen exposure

All mice were immunized twice, at an interval of 5 days by intraperitoneal injections of 0.5 ml alum-precipitated antigen containing 8 μg ovalbumin (Sigma-Aldrich, St Louis, Mo) bound to 4 mg Al(OH)3 (Sigma-Aldrich) in PBS. Eight days after the second sensitization, the mice were briefly anesthetized with Isoflurane (Baxter, Deerfield, Ill.) and received an intranasal administration of either 10 μg ovalbumin in 25 μl PBS (OVA group), or 25 μl

PBS (PBS control group), on 5 consecutive days.

BrdU. A thymidine analogue, 5-Bromo-2'-deoxyuridine (BrdU), is incorporated into the DNA during the S-phase of the cell cycle by replacing thymidine [14]. The animals received BrdU (Roche Diagnostics Scandinavia AB, Bromma, Sweden) to pulse-label newly produced inflammatory cells and thereby evaluate their contribution of enhanced inflammation to allergen-induced airway inflammation. The BrdU was injected at a dose of 1 mg in 250 μl PBS intraperitoneal, simultaneously with the allergen challenge.

Cell collection and sample processing

Twenty-four hours after the last ovalbumin exposure, the mice were euthanized with a mixture of xylazin (130 mg/kg, Rompun, Bayer) and ketamin (670 mg/kg, Ketalar, Parke-Davis). First, blood was taken by heart puncture. Serum was obtained after centrifuging the blood at 950*g* for 10 minutes at room temperature. Serum was stored at -80 ºC until further analysis of (allergen-specific) immunoglobulins. Second, the draining lymph nodes, spleen and lungs were isolated. Total cell numbers were determined using standard hematological procedures.

Draining lymph node cells. A single cell suspension of the draining lymph node was made by teasing the cells through a 70 μm nylon mesh (BD Biosciences, Stockholm, Sweden) using 1% FCS in PBS (PBS/1%FCS). Subsequently, the cells were centrifuged at 200*g* for 10 minutes at 4 ºC and the cells were resuspended in PBS/1%FCS.

Spleen cells. The spleen was homogenized in 50 ml PBS/1%FCS and thereafter centrifuged at 200*g* for 10 minutes at 4 ºC. The red blood cells were lysed using a lysing solution containing 0.1% potassium bicarbonate and 0.83% ammonium chloride, and incubated for 20 minutes at room temperature. After centrifugation at 200*g* for 10 minutes at 4 ºC, the cells were resuspended in PBS/1%FCS.

Bone marrow cells. Third, the bone marrow was harvested by excising one femur, which was cut at the epiphyses and flushed with 3 ml of HBBS. Bone marrow cells were centrifuged at 100*g* for 10 minutes at 4 ºC and the cells were subsequently resuspended in PBS/1%FCS.

Flowcytometric analysis

All cells were pre-treated with 2% mouse serum (DAKO) for 15 minutes at 4 ºC to prevent non-specific binding and thereafter stained with the following antibodies: FITC labeled anti-CD3 (clone 145-2C11; BD Biosciences),

Phycoerytrin (PE) labeled anti-CD4 (clone H129.19; BD Biosciences), PerCP labeled anti-CD8 (clone 53-6.7; BD Biosciences), FITC labeled anti-BrdU (clone 3D4; BD Biosciences), PerCP or PE labeled anti-B220 (clone RA3-6B2; BD Biosciences), PE labeled anti-CD125 (clone T21; BD Biosciences), PE labeled anti-IgE (clone 23G3; SouthernBiotech), biotin labeled anti-CD138 (clone 281-2; BD Biosciences), biotin labeled anti-CD19 (clone 1D3; BD Biosciences), biotin labeled anti-IgM (clone 1B4B1; SouthernBiotech) and PerCP labeled streptavidin (BD Biosciences). For each antibody, the appropriate isotype control antibodies were included. The cells were incubated for 30 minutes at 4 °C in the dark with antibodies or isotype controls, followed by a washing step with PBS/1%FCS. The cells incubated with biotin-labeled antibodies were thereafter incubated with PerCP labeled streptavidin for 20 minutes at 4 °C in the dark and washed again with PBS/1%FCS. Subsequently, all cells were incubated with Cytofix/Cytoperm (BD Biosciences) for 15 minutes at room temperature and washed twice with PBS/1%FCS. Cells stained with cell surface markers only were kept at 4 °C until flowcytometric analysis. All cells stained with anti-BrdU or anti-IgE were permeabilized using Cytofix/Cytoperm for 5 minutes at room temperature and resuspended in Perm/Wash buffer (BD Biosciences). To expose the BrdU epitopes, the cells are pre-treated with 300 μg/ml DNAse for 1 hour at 37 °C. Subsequently, the cells are washed with Perm/Wash buffer and incubated for 20 minutes at room temperature with the antibodies or their isotype controls. Cells were again washed with Perm/Wash buffer to remove all unbound antibodies and thereafter washed twice with PBS/1%FCS to reverse the permeabilization. The flowcytometric analysis was carried out using a FACScan flowcytometer (BD Biosciences).

Flowcytometric analysis. Ten thousand cells were computed in list mode and analyzed using the FACSDiva software. Gating was first set on total live lymphocytes based on forward and side scattered characteristics. Subsequently, granulocytes were gated in the FCS/SSC. The non-granulocyte gate was used for all further analyses. $B220^+$ or $CD19^+$ cells were used for further analysis of BrdU, IgM, IgE, IL-5Rα, and CD138. T cells were identified when they were $CD3^+$ within the live non-granulocyte gate.

Absolute cell numbers. Total cell counts differed per organ, per treatment and per strain. To be able to directly compare one specific cell population between organs, treatments and mouse strains, all figures depict absolute cell numbers, calculated from the percentages measured on the flowcytometer and the total cell counts per cell isolation.

Quantification of allergen specific immunoglobulins

OVA-specific antibodies were detected in serum samples using an isotype specific capture ELISA. ELISA plates (Maxi-sorb F96, Nunc A/S, Roskilde, Denmark) were coated with 1 μg/ml anti-IgE (clone R35-72), 4 μg/ml anti-IgG1 (clone A85-3), 1 μg/ml anti-IgG2a (clone R11-89) or 2 μg/ml anti-IgG2b (clone R35-72) in PBS. All antibody preparations were purchased from BD Biosciences, and incubated overnight at 4 °C. The next day, plates were washed and non-specific binding sites were blocked with 1% BSA (fraction V; MP Biomedicals, Illkirch, France) in PBS and incubated for 1 hr at room temperature. After washing with 0.05% Tween-20 in PBS, serum samples were added at 100 μl per well at different dilutions. A standard was added with serial dilutions of pooled serum from OVA-sensitized C57BL/6 mice: 1/10 to 1/10240 for IgE, IgG2a and IgG2b, and $1/5x10^3$ – $1/5x10^6$ for IgG1. The plates were subsequently incubated for 2 hours at room temperature. The plates were washed and 1 μg/ml of OVA labeled to digoxigenin (DIG) by a DIG protein labeling kit (Roche Diagnostics, Basel, Switzerland) was added for 1 hour, followed by incubation with anti-DIG-Fab fragments coupled to horseradish peroxidase (Roche Diagnostics, Basel, Switzerland) for 30 minutes at room temperature. Color development was performed with TMB solution (1-step ultra TMB; Perbio Science, The Netherlands) and assessed with an ELISA reader (type-349; Labsystems, Sweden) at 450 nm and 570 nm. The results were expressed as OD450 units greater than two times background.

Quantification of total immunoglobulins

Total antibody levels in serum samples were assessed similarly as the allergen specific immunoglobulins described above. Adaptations to the protocol are described here. ELISA plates were coated with 1 μg/ml anti-IgE (clone R35-72), 4 μg/ml anti-IgG1 (clone A85-3), 1 μg/ml anti-IgG2a (clone R11-89) or 2 μg/ml anti-IgG2b (clone R9-91), all purchased from BD Biosciences. Used standards ranged from 2 μg/ml to 10 pg/ml for IgE (clone C38-2) and IgG2a (clone HOPC-1), 2 μg/ml to 2 ng/ml for IgG2b (clone MPC-11), and 80 ng/ml to 40 pg/ml for IgG1 (clone S1-68). All biotinylated secondary antibodies were purchased from BD Biosciences and used in the following concentrations: 1 μg/ml for IgE (clone R35-118) and IgG2a (clone R19-15) and 2 μg/ml for IgG2b (clone R12-3) and IgG1 (cloneA85-1). Streptavidin-HRP (Bioscource, Etten-Leur, The Netherlands) was used in a concentration of 167 ng/ml.

Statistical analysis

Normality of distribution was assessed using the Shapiro-Wilk test, and normal distributed data were thereafter compared by using the Student t test. Differences between groups were considered statistically significant at

the $p<0.05$ level. Significant differences are indicated as $ for comparing PBS to OVA challenged animals, * for comparing IL-5 Tr, $CD4^{-/-}$ or $CD8^{-/-}$ to WT mice, # for comparing $CD4^{-/-}$IL-5Tr or $CD8^{-/-}$IL-5Tr to IL-5 Tr and † for comparing $CD4^{-/-}$ to $CD4^{-/-}$IL-5Tr or $CD8^{-/-}$ to $CD8^{-/-}$IL-5Tr mice. Statistical analyses were performed using SPSS for Windows version 12.0.1 (SPSS, Chicago, IL, USA).

Results

Characterization of knock-out and transgenic mice

Genotyping
The used primer sets distinguish between homozygous and heterozygous knockouts or wild type according to the Jackson laboratory [15]. We showed that our $CD4^{-/-}$ and $CD4^{-/-}$IL-5Tr were homozygous knockouts for the CD4 gene and the IL-5 Tr, $CD8^{-/-}$ and $CD8^{-/-}$IL-5Tr displayed a genotype similar to the WT (data not shown). In addition, our $CD8^{-/-}$ and $CD8^{-/-}$IL-5Tr were shown to be homozygous knockouts for the CD8 gene and the IL-5 Tr, $CD4^{-/-}$ and $CD4^{-/-}$IL-5Tr displayed a genotype similar to the WT mice (data not shown). Furthermore, the WT, $CD4^{-/-}$ and $CD8^{-/-}$ were show to be not IL-5 transgenic, whereas the IL-5 transgenesis of the IL-5 Tr, $CD4^{-/-}$IL-5Tr and $CD8^{-/-}$IL-5Tr mice were confirmed (data not shown).

T cell subsets
To verify whether the PCR results corresponded to the actual absence of the CD4 and CD8 proteins expressed on the cell surface of T cells, a triple staining of CD3, CD4 and CD8 was performed. We showed that the number of $CD3^+$ T cells was equal in all mouse strains used and was consistent with previously described numbers on the C57BL/6 background. As expected, the number of $CD4^+$ T cells was significantly lower in the $CD4^{-/-}$ and $CD4^{-/-}$IL-5Tr mice as compared to the WT and IL-5 Tr mice, respectively (data not shown). Also the number of $CD8^+$ T cells was significantly lower in the $CD8^{-/-}$ and $CD8^{-/-}$IL-5Tr mice when compared to the WT and IL-5 Tr mice, respectively (data not shown). When the number of $CD3^+CD4^+$ and the $CD3^+CD8^+$ were added together, the resulting number in both WT and IL-5 Tr approximate the number of $CD3^+$ T cells. This sum of $CD3^+CD4^+$ and the $CD3^+CD8^+$ was also applying to the other four strains, making it unlikely that the knocking out system only resulted in the absence of CD4 or CD8 on the cell surface, but probably (most of) the T helper cells or the cytotoxic T cells were absent in the $CD4^{-/-}$ and $CD8^{-/-}$ mice, respectively.

Cell-surface bound and serum IgE

IgE$^+$ B cells
IgE is the hallmark immunoglobulin isotype in allergic responses. To assess whether isotype switching towards IgE was possible in the absence of CD4$^+$ or CD8$^+$ T cells with and without high levels of IL-5, the presence of CD19$^+$ and CD19$^+$IgE$^+$ cells was assessed. Figure 1 shows both CD19$^+$ and CD19$^+$IgE$^+$ cells in BM, DLN and spleen.

The number of CD19$^+$ cells in the BM (Fig. 1A) of OVA treated IL-5 transgenic mice is significantly reduced compared to WT, whereas both the PBS and the OVA challenged mice showed a significant induction of CD19$^+$ splenic cells compared to WT (Fig. 1C). Although the absence of CD4$^+$ T cells did not alter the number of CD19$^+$ cells in any of the organs, the over-expression of IL-5 resulted in a significant upregulation of splenic CD19$^+$ cells in all IL-5 transgenic mice.

Figure 1 shows numbers of CD19$^+$IgE$^+$ cells in the BM, DLN and spleen of WT mice as well. The presence of a high IL-5 level, significantly reduced the number of CD19$^+$IgE$^+$ cells in the BM (Fig. 1A) compared to WT, whereas the CD19$^+$IgE$^+$ cells in the spleen (Fig. 1C) significantly increased. A significant reduction of CD19$^+$IgE$^+$ cells in the BM (Fig. 1A) and DLN (Fig. 1B) was observed in the absence of CD4$^+$ T cells, which could not be compensated by the presence of high IL-5 levels.

Immunoglobulin levels in serum
Since it is generally accepted that CD4$^+$ Th cells are required for isotype switching and we observed CD19$^+$IgE$^+$ cells in the absence of CD4$^+$ T cells, we assessed secreted IgE antibodies in the serum. To verify whether other immunoglobulin isotypes were present as well, both total and OVA-specific levels of IgE, IgG1, IgG2a and IgG2b were determined using ELISA.

Figure 2A shows the number of total IgE in all tested mouse strains. The mice lacking CD4$^+$ T cells showed significantly less total IgE, whereas the lack of CD8$^+$ T cells did not influence the total IgE levels. Also the over-expression of IL-5 left the IgE levels unaltered. In addition, no differences between PBS and OVA treated animals were observed in any of the mouse strains. In contrast, OVA-specific IgE did show significant differences between PBS and OVA treated mice, as shown in Figure 2B. In addition, the absence of CD4$^+$ T cells resulted in a significant reduction of the allergen-specific IgE levels, whereas the lack of CD8$^+$ T cells did not influence the number of OVA-IgE. The total and allergen-specific IgG1 levels corresponded to the findings obtained for IgE, whereas no differences between mouse strains and treatments were observed for IgG2a and IgG2b (data not shown).

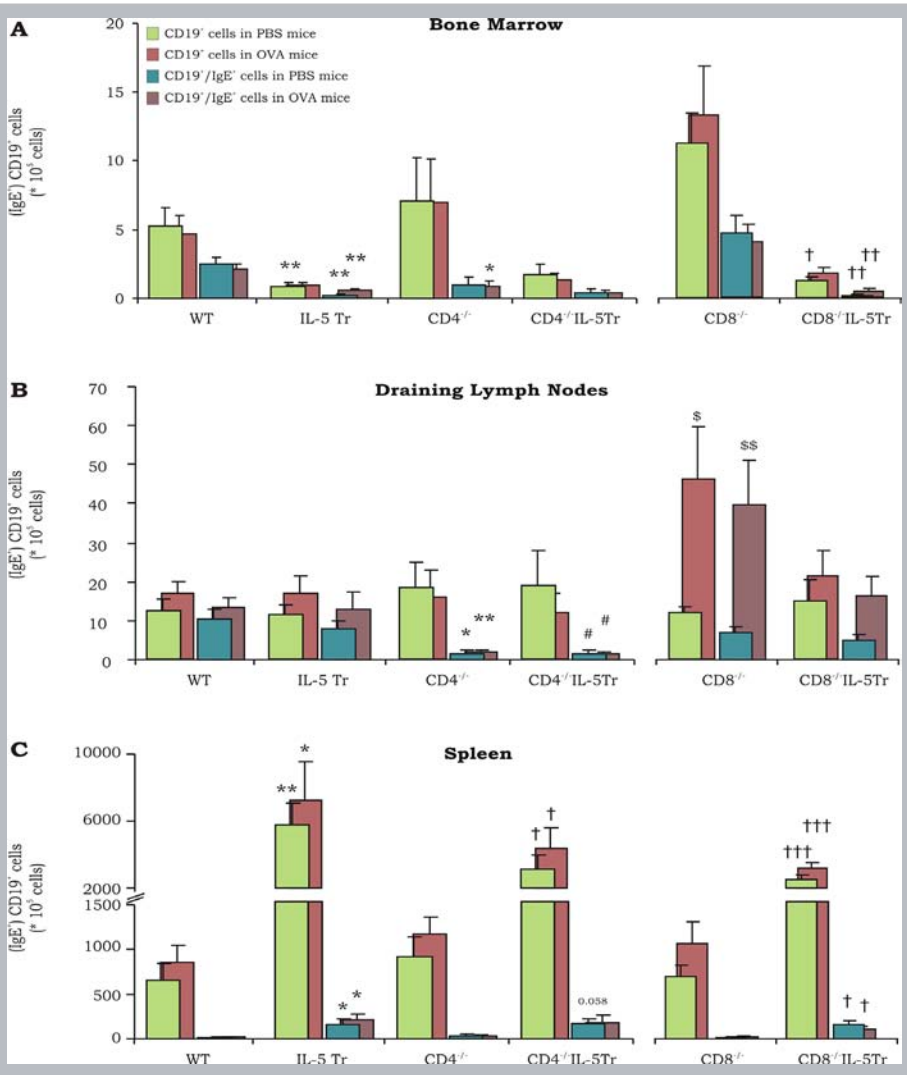

Figure 1. Expression of CD19 on and intracellular IgE in BM, DLN and spleen cells. To assess the expression of CD19 on and intracellular IgE in the different organs, $5*10^5$ cells from PBS and OVA challenged mice were double stained with biotin labeled anti-CD19 (clone 1D3) plus PerCP labeled streptavidin and PE labeled anti-IgE (clone 23G3). The number of cells present in BM (A), DLN (B) and spleen (C) were determined in the non-granulocyte gate displaying cells from WT, IL-5 Tr, $CD4^{-/-}$, $CD4^{-/-}$IL-5Tr, $CD8^{-/-}$, and $CD8^{-/-}$IL-5Tr mice. Data are depicted as the average ± SEM (n=5-7). *P<0.05 and **P<0.01 compared to WT; #P<0.05 $CD4^{-/-}$IL-5Tr compared to IL-5Tr; $P<0.05 and $$P<0.01 PBS compared to OVA treatment; †P<0.05, ††P<0.01 and †††P<0.001 $CD4^{-/-}$ vs. $CD4^{-/-}$IL-5Tr or $CD8^{-/-}$ vs. $CD8^{-/-}$IL-5Tr.

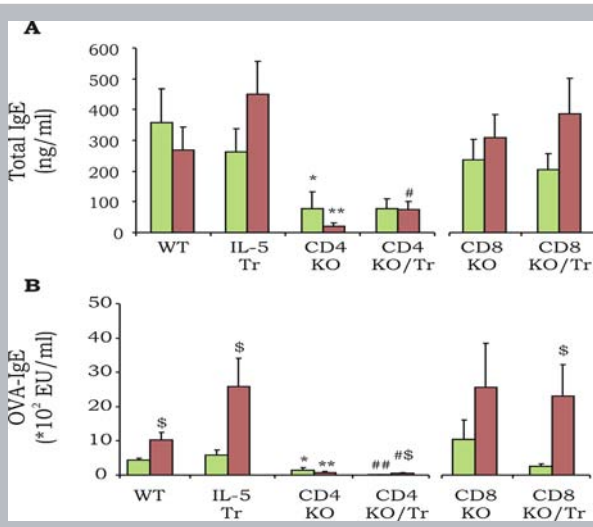

Figure 2. Serum levels of total and OVA-specific IgE. Total (A) and OVA-specific (B) IgE isotype levels in PBS (green bars) and OVA (red bars) challenged mice are depicted as the average ± SEM (n=5-7). $P<0.05 PBS compared to OVA treatment; *P<0.05 and **P<0.01 compared to WT; #P<0.05, and ##P<0.01 $CD4^{-/-}$IL-5Tr compared to IL-5Tr.

Characterization of B cells

To better understand the difference between the isotype switching towards IgE by the $CD19^+$ B cells in the spleen of IL-5 transgenic mice in the absence of $CD4^+$ T cells and the absence of total and allergen-specific IgE in the serum of these mice, we further investigated the present B cell subsets. In addition to the CD19, B220 is commonly used as a pan B cell marker in the periphery [16]. To assess whether the $B220^+$ cells were able to respond to IL-5, we determined the number of IL-5Rα^+ B cells. Thereafter, we aimed to elucidate the progenitor origin by staining for IgM and CD138. Since it has been reported that the intensity of IgM can be used to discriminate between B cell subsets, we finalized our dataset with an IgM intensity plot. All of the above described stainings were performed in BM, DLN and spleen.

Bone marrow

$B220^+$ cells. The number of $B220^+$ cells is significantly reduced in most IL-5 transgenic mice compared to their non-transgenic counterpart (Fig. 3A). This is independent from the absence of either $CD4^+$ T cells or $CD8^+$ T cells, and also from the challenge with PBS or OVA.

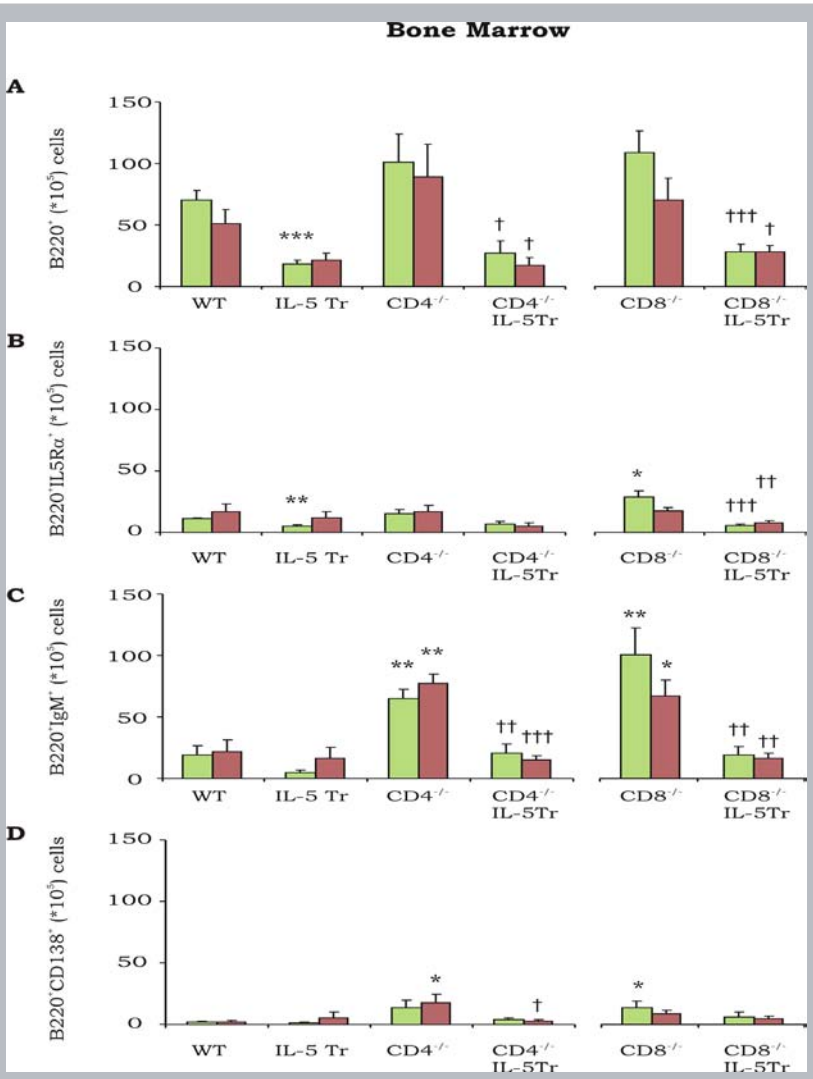

Figure 3. B cell characterization in bone marrow. Five hundred thousand cells from PBS (green bars) and OVA (red bars) challenged mice were stained with PE labeled anti-B220 (clone RA3-6B2), PerCP labeled anti-B220 (clone RA3-6B2) and PE labeled anti-CD125 (IL-5Rα; clone T21), PE labeled anti-B220 (clone RA3-6B2) and biotin labeled anti-IgM (clone 1B4B1) plus PerCP labeled streptavidin, or PE labeled anti-B220 (clone RA3-6B2) and biotin labeled anti-CD138 (clone 281-2) plus PerCP labeled streptavidin. The number of cells present were determined in the non-granulocyte gate in WT, IL-5 Tr, $CD4^{-/-}$, $CD4^{-/-}$IL-5Tr, $CD8^{-/-}$, and $CD8^{-/-}$IL-5Tr mice. Data are depicted as the average ± SEM (n=5-7). *P<0.05, **P<0.01 and ***P<0.001 compared to WT; †P<0.05, ††P<0.01 and †††P<0.001 $CD4^{-/-}$ vs. $CD4^{-/-}$IL-5Tr or $CD8^{-/-}$ vs. $CD8^{-/-}$IL-5Tr.

B220$^+$IL-5Rα$^+$ cells. The number of IL-5 responsive B cells is largely in line with the B220$^+$ cell numbers, since IL-5 transgenesis mostly results in a significant lower number of B220$^+$IL-5Rα$^+$ cells in the bone marrow (Fig. 3B). Interestingly, in CD8$^{-/-}$ mice, the number of B220$^+$IL-5Rα$^+$ cells is significantly higher in the PBS challenged mice compared to the PBS treated WT mice.

B220$^+$IgM$^+$ cells. Although no significant difference was observed between WT and IL-5 Tr mice, the absence of CD4$^+$ Th cells or CD8$^+$ Tc cells both induced a significant increase in the number of B220$^+$IgM$^+$ cells in the bone marrow (Fig. 3C). However, this significant induction by the absence of either T cell subset was again abolished by the presence of high IL-5 levels in their IL-5 transgenic counterparts.

B220$^+$CD138$^+$ cells. The number of B220$^+$CD138$^+$ cells in the bone marrow of the CD4$^{-/-}$ and CD8$^{-/-}$ mice showed a tendency to be increased compared to the WT mice (Fig. 3D). However, only in OVA challenged CD4$^{-/-}$IL-5Tr mice, the IL-5 transgenesis resulted in a significant reduction.

Draining lymph nodes

B220$^+$ cells. Besides the significant increase in B220$^+$ cells in the OVA compared to the PBS challenged CD8$^{-/-}$ mice, no differences between the treatments or mouse strains could be observed (Fig. 4A).

B220$^+$IL-5Rα$^+$ cells. Although the PBS challenged CD4$^{-/-}$ mice displayed significantly more B220$^+$IL-5Rα$^+$ cells than the PBS treated WT mice, no other differences between the B220$^+$IL-5Rα$^+$ cells were observed in the DLN (Fig. 4B).

B220$^+$IgM$^+$ cells. Challenging WT mice with OVA resulted in significantly more B220$^+$IgM$^+$ cells in comparison with the PBS treated WT mice. This significant induction was abolished by the absence of CD4$^+$ T cells, as shown in Fig. 4C.

B220$^+$CD138$^+$ cells. All mice and treatments showed low numbers of B220$^+$CD138$^+$ cells in the DLN. However, the absence of CD4$^+$ T cells resulted in a significant increase of the B220$^+$CD138$^+$ cells in the DLN of PBS treated CD4$^{-/-}$ mice compared to WT (Fig. 4D).

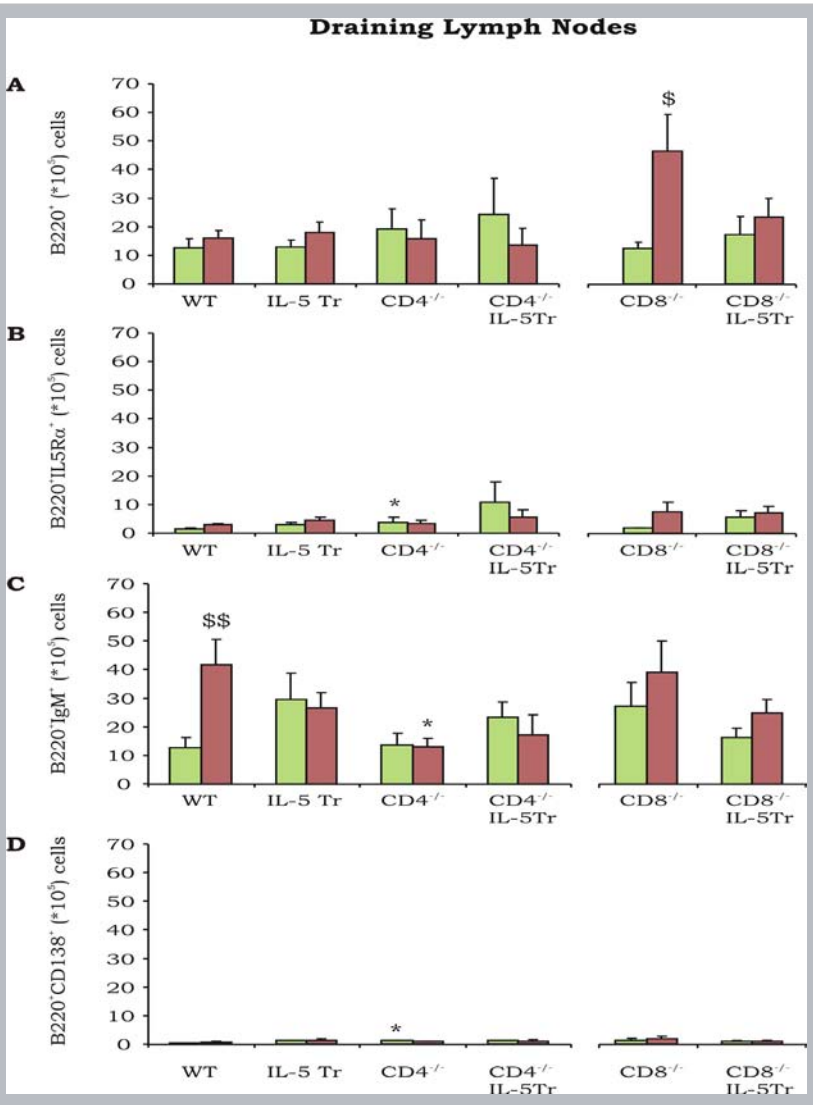

Figure 4. B cell characterization in draining lymph nodes. Five hundred thousand cells from PBS (green bars) and OVA (red bars) challenged mice were stained with PE labeled anti-B220 (clone RA3-6B2), PerCP labeled anti-B220 (clone RA3-6B2) and PE labeled anti-CD125 (IL-5Rα; clone T21), PE labeled anti-B220 (clone RA3-6B2) and biotin labeled anti-IgM (clone 1B4B1) plus PerCP labeled streptavidin, or PE labeled anti-B220 (clone RA3-6B2) and biotin labeled anti-CD138 (clone 281-2) plus PerCP labeled streptavidin. The number of cells present were determined in the non-granulocyte gate in WT, IL-5 Tr, $CD4^{-/-}$, $CD4^{-/-}$IL-5Tr, $CD^{-/-}$, and $CD8^{-/-}$IL-5Tr mice. Data are depicted as the average ± SEM (n=5-7). *P<0.05 compared to WT; $P<0.05 and $$P<0.01 PBS compared to OVA treatment.

Spleen

B220$^+$ cells. The number of B220$^+$ cells in the spleen of all IL-5 transgenic mice significantly increased compared to their non-transgenic counterparts (Fig. 5A).

B220$^+$IL-5Rα$^+$ cells. In line with the observations of the B220$^+$ cells, also the B220$^+$IL-5Rα$^+$ cells significantly increase due to the IL-5 transgenesis compared to their non-transgenic counterparts (Fig. 5B).

B220$^+$IgM$^+$ cells. In contrast to the B220$^+$IL-5Rα$^+$ cells, the B220$^+$IgM$^+$ cells were significantly reduced in the OVA treated IL-5 Tr mice and the CD4$^{-/-}$ mice compared to the OVA treated WT mice (Fig. 5C).

B220$^+$CD138$^+$ cells. Although no significant differences were observed between the mouse strains and the different treatments, the number of B220$^+$CD138$^+$ cells in the spleen of all mouse strains were considerably higher than observed in the BM or DLN.

IgM expression

Since the intensity of the IgM staining on the B cell surface is one of the important indicators of the different B cell lineages, Figure 6 shows the intensity of the IgM expression in both PBS and OVA treated WT and IL-5 Tr mice on cells derived from BM, DLN and spleen. Both PBS and OVA treated IL-5 Tr mice expressed higher levels of IgM on the BM and DLN derived B220$^+$ cells compared to WT, whereas this was not observed in the spleen.

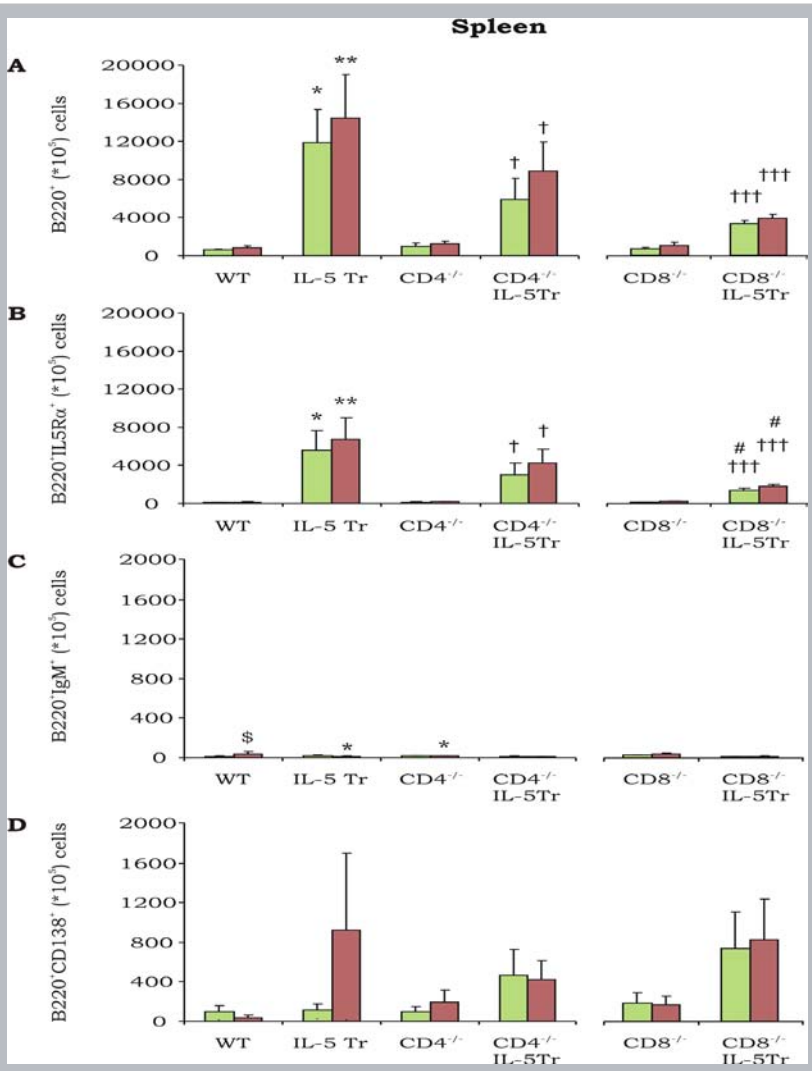

Figure 5. B cell characterization in spleen. Five hundred thousand cells from PBS (green bars) and OVA (red bars) challenged mice were stained with PE labeled anti-B220 (clone RA3-6B2), PerCP labeled anti-B220 (clone RA3-6B2) and PE labeled anti-CD125 (IL-5Rα; clone T21), PE labeled anti-B220 (clone RA3-6B2) and biotin labeled anti-IgM (clone 1B4B1) plus PerCP labeled streptavidin, or PE labeled anti-B220 (clone RA3-6B2) and biotin labeled anti-CD138 (clone 281-2) plus PerCP labeled streptavidin. The number of cells present were determined in the non-granulocyte gate in WT, IL-5 Tr, $CD4^{-/-}$, $CD4^{-/-}$IL-5Tr, $CD8^{-/-}$, and $CD8^{-/-}$IL-5Tr mice. Data are depicted as the average ± SEM (n=5-7). *P<0.05 and **P<0.01 compared to WT; †P<0.05 and †††P<0.001 $CD4^{-/-}$ vs. $CD4^{-/-}$IL-5Tr or $CD8^{-/-}$ vs. $CD8^{-/-}$IL-5Tr; $P<0.05 PBS compared to OVA treatment; #P<0.05 $CD4^{-/-}$IL-5Tr compared to IL-5Tr.

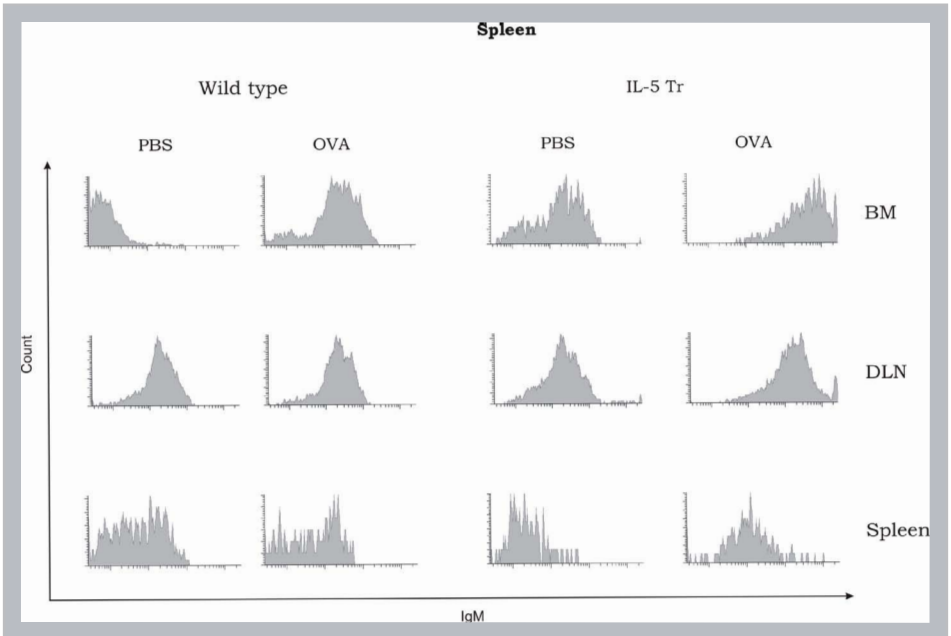

Figure 6. Intensity of the IgM expression. B cell lineages are described to be distinguishable by the intensity of their IgM expression. Five hundred thousand cells were double stained with PE labeled anti-B220 (clone RA3-6B2) and biotin labeled anti-IgM (clone 1B4B1) plus PerCP labeled streptavidin. The intensity of the IgM expression on $B220^+$ cells present in BM, DLN and spleen was determined in the non-granulocyte gate of PBS or OVA treated WT and IL-5 Tr mice. Representative examples of all organs and animal treatments are depicted.

Discussion

This study showed that IL-5 transgenesis leads to massive migration of B cells to the spleen, allowing them to undergo isotype switching towards IgE, even in the absence of $CD4^+$ Th cells. However, the IL-5 transgenesis was not able to rescue the secretion OVA-specific IgE.

IL-5 transgenesis may act at several developmental stages, therefore affecting the normal adult B cell populations since they are formed by an interplay of several processes in a dynamic fashion. Macias *et al.* showed that IL-5 transgenesis results in both extramedullary hematopoiesis and splenomegaly [5].

The spleen of an IL-5 Tr mouse contains small, hard white nodules that are distinct from the splenic parenchymal tissue and the red and white pulp architecture of the normal spleen are lost [5]. The white nodules display morphological characteristics consistent with bone containing osteocytes, which are osteoblasts that are trapped within a bone matrix [17]. This finding is confirmed by transcripts of the osteocalcin gene in the IL-5 Tr splenic tissue [5]. Osteoblasts express Stromal Derived Factor-1 (SDF-1), which in combination with CXCR4 appears to be crucial for the establishment of marrow in the bone [18, 19]. SDF-1 is also a powerful chemoattractant for early and mature hematopoietic cells [18]. In addition, SDF-1 stimulates the proliferation of conventional B2 lymphocytes and their precursors, and is thus a nursing paracrine factor for retention, survival, and expansion of B lymphocytes in body cavities [20]. We found that IL-5 transgenesis strongly increased the total number of $B220^+$ cells in the spleen, whereas the number in the bone marrow (BM) is reduced when compared to WT mice. The presence of the osteocytes expressing SDF-1 in the spleen of these IL-5 Tr mice, can explain why most $B220^+$ cells migrate to the spleen. As mentioned before, SDF-1 stimulates the proliferation of B cells, which is supported by our $BrdU^+$/ $B220^+$ data showing that the WT mice have most newly formed B cells in the BM, whereas the IL-5 Tr mice show equal numbers of newly formed $B220^+$ cells in the BM, but significantly more newly formed $B220^+$ cells in the spleen. These findings are independent from the $CD4^+$ T cells, since similar numbers are observed in $CD4^{-/-}$IL-5Tr compared to $CD4^{-/-}$ mice, implying that $CD4^+$ T cells are not involved in this chemo-attracted migration of $B220^+$ cells by SDF-1 towards the spleen.

The murine B cell population is proposed to exist of three B lymphocyte lineages originating from separate precursors: B1a, B1b and B2 [12]. B-2 progenitors are $CD19^-/B220^+$, whereas both B1 progenitor lineages are $CD19^+/B220^+$. In addition, the B2 cells express high levels of CD138, whereas B1b cells express intermediate levels, and B1a cells show no expression of CD138 [12]. CD138 is expressed on precursor B cells in the bone marrow and

antibody-secreting cells, including plasma cells, but not mature, resting peripheral B cells [12]. In the WT mice, the BM contains lower numbers of $CD19^+$ cells compared to $B220^+$ cells, indicating that these are B-2 progenitors. This is in agreement with the findings of Tung *et al.*, who show that in the adult BM, the frequency of B-2 cells is highest [12]. IL-5 transgenesis decreased the number of BM $CD19^+$ and $B220^+$ cells compared to WT mice. However, previous studies have shown that IL-5 can induce the expansion of B-1 lymphocytes [2, 20, 21] and that B-1 cells have a high self-replenishing activity [20, 22]. In addition, the presence of high IL-5 levels in IL-5 Tr animals compared to WT mice reduced the number of $B220^+$/IL-5Rα^+ cells in the BM. However, the number of $B220^+$/IL-5Rα^+ cells in the spleen were strongly increased. Several studies have shown that B-1 cells are potent in up-regulating their IL-5Rα expression in response to IL-5, leading to their high responsiveness to IL-5 and thereafter IL-10 production and increased SDF-1 sensitivity [20, 22, 23]. Furthermore, it has been shown that IL-5Rα is expressed on all B-1 B cells, but only on a small proportion (2-4%) of resting B-2 cells in the spleen [2]. Taken together, this suggests that significantly induced $B220^+$IL-5Rα^+ cell number in the spleen of IL-5 transgenic compared to WT animals originates from the B-1 lineage.

Half of the B cells migrating into the spleen are $CD19^+/B220^+$ (presumably B-1 cells) and half are $CD19^-/B220^+$ cells (presumably B-2 cells), and therefore CD138 expression could indicate which B cell lineages are present in the spleen. Since the increase in $CD138^+$ cells was not significant in any of the organs comparing IL-5 Tr to WT mice, and the total numbers of $CD138^+$ cells are at most one-tenth of the total $B220^+$ population, expansion of the B-1 cells (low CD138 expression) due to the IL-5 transgenesis is implicated. In addition, B-1 cells have been shown to produce IL-10, which makes them more sensitive to SDF-1 [20]. Furthermore, the relatively high numbers of $B220^+$, $B220^+/CD138^+$ and $B220^+/IgM^+$ cells in the BM of $CD4^{-/-}$ mice compared to the lower number of $B220^+/IgM^+$ cells in the BM of WT mice, implies that the CD4 cells are required to either release the $B220^+$ cells from the BM, and/or let them undergo isotype switching of the antibody subclass. However, the number of these different $B220^+$ subsets in the BM of $CD4^{-/-}$IL-5Tr mice indicates that in the absence of $CD4^+$ T cells, the over-expression of IL-5 is sufficient to reduce the number of $B220^+$ cells in the BM and increase the numbers found in the spleen. Taken together, our data argue for a strong chemokine induced attraction of B-1 cells from the BM towards the spleen, possibly due to the osteocytes within the IL-5 transgenic spleen. The role of the osteocytes in the spleen of these IL-5 transgenic mice will therefore be incorporated in future research.

Since large numbers of B cells are migrating to the spleen of IL-5 transgenic mice, the increase in the number of $CD8^+$ T cells in these mice compared to the WT mice was remarkable. $CD8^+$ T cells are generally considered to produce

Th1-associated cytokines, but they have also been shown to produce IL-4 [24, 25], which in the presence of many naïve B1 cells could induce the isotype switching of the $B220^+$ cells towards $B220^+/IgE^+$ in the $CD4^{-/-}$IL-5Tr spleen.

Shang *et al.* suggested that allergen-specific IgE is formed in the draining lymph nodes, whereas total IgE levels are preferentially formed within the spleen [10]. Furthermore, B-1 cells are known to be present in the peritoneal cavity and spleen, but not in the lymph nodes [26]. We also observed that when detectable numbers of $CD19^+/IgE^+$ cells are present in the DLN, allergen-specific IgE levels could be measured in the serum of WT and IL-5 Tr mice. However, when there was a significant reduction of the $CD19^+/IgE^+$ cells in the DLN compared to WT or IL-5 Tr mice as seen in $CD4^{-/-}$ and $CD4^{-/-}$IL-5Tr, respectively, the amount of OVA-specific IgE is significantly reduced. Therefore, we propose that $CD4^+$ T cells are required for the presence of isotype switched B-2 cells in the DLN that subsequently can respond to the allergen by secreting allergen-specific IgE antibodies. In addition, this effect is independent from the IL-5 transgenesis. The presence of $CD19^+/IgE^+$ cells in the spleen is proposed to contribute to the total IgE level in the serum. However, in the absence of $CD4^+$ T cells, the $CD19^+/IgE^+$ cells appear unable to secrete IgE antibodies resulting in a significant reduction of the total IgE levels in the $CD4^{-/-}$ mice. IL-5 transgenesis resulted in a significant induction of the $CD19^+/IgE^+$ cells in the spleen, whereas they still display a lack in the significant induction of total IgE. Our findings support an altered lineage of $CD19^+/IgE^+$ cells lacking the ability to secrete IgE antibodies and implicate a relative shift from conventional B-2 cell dependent OVA-specific IgE production towards IgE expressing B-1 cells. To indisputably prove this conclusion, elaborate molecular analysis of the used mouse strains should be performed to discriminate the B cell lineage origin of the total and allergen-specific IgE antibodies in serum and compare these to the IgE^+ B cells present in the different effector organs.

Since the number of $CD19^+/IgE^+$ cells in the IL-5 transgenic mice was significantly higher than that observed in the non-transgenic mice, more total IgE antibodies in the serum of these IL-5 transgenic mice were expected. However, the observed total IgE levels in the IL-5 transgenic mice were not increased compared to WT IgE levels. This might be explained by the fact that B-1 cells can downregulate serum IgE levels [26]. In addition, it has been shown that B-1 cells can produce the full range of immunoglobulin isotypes and produce most of the natural antibodies in the serum, whereas these cells participate in relatively few antigen specific responses [10].

In conclusion, IL-5 overexpression leads to a downregulation of B-2 B cells and an upregulation of B-1 B cells in the spleen, in contrast to the bone marrow. Within the spleen, B-1 B cells are capable of switching towards IgE.

However, no allergen-specific and significantly reduced total IgE levels were detectable in the serum of $CD4^{-/-}$ mice, suggesting that the B-1 B cells are not participating in the secretion of the (allergen-specific) IgE. Whether this is due to an active suppression of the B-2 B cells by the B-1 B cells, or whether the B-1 B cells are incapable of secreting IgE without CD4+ T cell help remains to be elucidated by future research.

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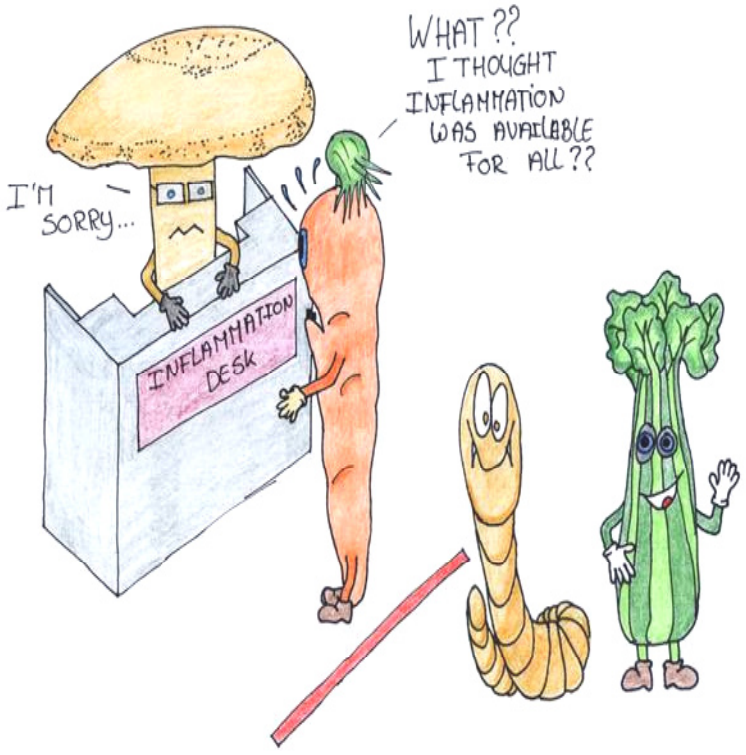
SECTION 2

Human *in vitro* Allergy Studies



CHAPTER

4

T cell responses in fresh and cryopreserved peripheral blood mononuclear cells: kinetics of cell viability, cellular subsets, proliferation, and cytokine production

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Bianca Rappard
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Abstract

Polyclonal stimuli like phorbol myristate acetate (PMA) plus calcium ionophore (Ca-I), concanavalin A (ConA) or anti-CD3 plus anti-CD28 (αCD3/αCD28) are widely used T cell stimuli. All three stimuli act at different sites and in different ways to activate the T cell receptor pathway and are widely used in different concentrations, stimulation durations and read-out systems. This study was designed to establish the most suitable polyclonal stimulus in human peripheral blood mononuclear cells (PBMC) experiments by assessing the kinetics of cell viability, present immunophenotypes, proliferation, and cytokine production of the PBMC. In addition, changes in these read-out parameters due to cryopreservation have been investigated by comparing fresh and cryopreserved PBMC cultures at days 1, 3, 5, and 7. This study showed areduction in the cytokine levels after cryopreservation of PMA/Ca-I stimulated PBMC, whereas no significant differences due to the cryopreservation were observed in ConA or αCD3/αCD28 stimulated PBMC. Cryopreservation did not alter the maximal proliferation capacity of ConA or αCD3/αCD28 stimulated PBMC, whereas it did delay the proliferation. Although cryopreservation had no effect on the $CD3^+CD4^+$ or $CD3^+CD8^+$ T cell subsets, PMA/Ca-I significantly reduced the amount of both T cell subsets over time. In conclusion, PMA/Ca-I is suitable as a positive control in experiments where high cytokine production is expected and only fresh PBMC are used. Proliferation and effects on the T cell subsets in long-term PBMC cultures should use ConA or αCD3/αCD28 as positive control.

Introduction

Polyclonal stimuli are used to stimulate specific peripheral blood mononuclear cell (PBMC) subpopulations (e.g. T cells or monocytes) and thereby assess their maximal immune responsiveness *in vitro*. These polyclonal stimuli can assess possible differences in the intrinsic immune responsiveness of antigen-specific cell cultures. In particular, polyclonal stimuli are used to maximally activate T cells and monocytes towards proliferation or cytokine production.

In the literature, phorbol myristate acetate (PMA) plus calcium ionophore (Ca-I), concanavalin A (ConA) or anti-CD3 plus anti-CD28 (αCD3/αCD28) are widely used T cell stimuli. T cell activation requires at least two signals. The major histocompatibility complex (MHC)-peptide complex associating with the T cell receptor (TCR/CD3) provides signal 1, whereas signal 2 is delivered via the binding of CD28 to B7-1/2 (CD80/CD86) expressed on antigen presenting cells (APC). Subsequently, an immunological synapse is formed, resulting in the activation of several tyrosine kinases and recruitment of adapter proteins and specific downstream signalling leading to T cell activation and specific cytokine production. All three above mentioned stimuli act at different sites and in different ways to activate the T cell receptor pathway [1-3]. Anti-CD3 and αCD28 have been widely used to provide all T cells with the required activation signals [4-7]. ConA is a lectin that binds to glycoproteins expressed on the T cell surface. ConA cross-links the T cell receptor, thereby mimicking the T cell receptor activation which bypasses the requirement of co-stimulatory signals [8]. PMA directly activates cytoplasmic protein kinase C resulting in phosphorylation of the calcium channels and influx of extracellular calcium which is provided by the addition of exogenous calcium ionophore [9].

Literature provides a large array of possible concentrations, stimulation duration and read-out parameters to activate PBMC for these three stimuli. For example, PMA is used separately [1, 2] or in combination with Ca-I [10-13]. The used PMA concentrations range from 50 ng/ml to 2.5 ng/ml, whereas the Ca-I concentration varies between 1 μM and 0.25 μM [1, 2, 10-13]. ConA stimulation is performed with concentrations ranging from 50 to 2 μg/ml [14-20]. Anti-CD3 is sometimes used separately, but is mostly combined with αCD28. Again, large variations in the used concentrations were found, ranging from 10 to 0.1 μg/ml for both αCD3 and αCD28 [21-24]. In addition, all three polyclonal stimuli are used to assess proliferation [13, 15, 23] and cytokine production [10, 20, 21].

Cryopreservation has become a standard procedure that allows phenotypic and functional analysis of large batches of patient samples in cohort studies. These studies require a low operator-dependent inter-assay variability, which are potentially provided by properly optimized cryopreservation. In addition,

logistics might require cryopreservation in order to obtain large numbers of patient materials that can be analyzed at a later time point. However, cryopreservation potentially induces significant changes in the cell viability [25-27], in cytokine production [28-31] or in the surface markers of PBMC [32, 33], which may also alter the efficiency of the polyclonal T cell stimuli. In addition, *in vitro* analysis of immune response-dependent alterations requires that all essential cell types are present in the PBMC cell cultures. If cryopreservation would alter each cell type in a different manner, the interplay between the immune cells could be out of balance leading to misleading interpretations [10, 34, 35].

Antigen-specific stimulations often rely on a small number of responsive T cells. This implicates that relatively small alterations in clinical conditions in which T cells are skewed towards Th1, Th2 or a regulatory T cell subset (e.g. allergy, auto-immune disease or infections) must be detectable. The need to assess relatively small alterations makes it important to determine the basal differences between the individual PBMC samples and the effect of cryopreservation. This is important as differences in the genetic background or the presence of a disease can, for example, influence the possibility of one or more PBMC subsets to become activated [36]. In addition, it is essential to determine the reproducibility of the activation and differentiation capacity of PBMC, before and after cryopreservation, by means of a kinetics study with polyclonal stimuli.

In conclusion, to our knowledge, no literature is available comparing the effect of cryopreservation with different stimuli, at different time points and with different read-out parameters. Therefore, this study was designed to determine which T cell stimulus was the most suitable polyclonal control for establishing the intrinsic differences in our future antigen-specific cultures with either fresh or cryopreserved cells. Since antigen-specific stimulations require the presence of APC, PBMC providing autologous APC, instead of purified T cells were used. Furthermore, the effect of cryopreservation on the different PBMC subsets was determined to rule out selective elimination of a specific PBMC subset possibly required for the antigen-specific stimulations.

We showed profound differences in the functional potential of cryopreserved PBMC relative to fresh PBMC by assessing the kinetics of cytokine expression, proliferation, cell viability and the presence of different immunophenotypes. Therefore, the research question determines which polyclonal stimulus should be used and whether cryopreserved PBMC are suitable for the desired read-out parameters.

Materials and Methods

Blood donors

Five buffy coats from blood donors were obtained from the Sanquin Blood Bank Nijmegen (The Netherlands). An informed consent was obtained before the sample collection and the performed experiments were approved by the local ethical committee.

Isolation of human PBMC

For a whole blood donation, about 500 ml of blood was taken from the donor in 70 ml of citrate-phosphate-dextrose and immediately cooled to about 22 ºC. Within 24 hours after collection, the blood was separated through centrifugation to obtain erythrocytes, a buffy coat and plasma. The buffy coat was subsequently diluted 1:1 with IMDM containing GlutaMAX (IMDM; Gibco-BRL, Paisley, Scotland) before density gradient centrifugation on Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden). The PBMC layer was washed twice with IMDM and the cell concentration was determined. Subsequently, the PBMC were divided into two aliquots: one aliquot was used for immediate testing with fresh PBMC, whilst the other aliquot was cryopreserved as described below.

Cryopreservation and thawing

Three pilot studies were performed to choose the optimal condition for cryo-preservation and suitable thawing of the PBMC. Four different media were tested to cryopreserve the PBMC. In addition, three different cryo-preservation methods and three different methods to thaw the cells were used to compare the viability, cytokine production and immunological phenotype of fresh and cryo-preserved PBMC (data not shown). This permitted the selection of the optimal conditions to cryopreserve the PBMC.

The PBMC were resuspended at $4x10^7$ cells/ml in ice-cold FCS, and subsequently, an equal volume of FCS + 20% Me_2SO (DMSO) was added dropwise while gently shaking the tube. This procedure resulted in a cell suspension of $2x10^7$ cells/ml that was pipetted into 1.8 ml aliquots per pre-cooled cryovial. The vials were transferred into the pre-cooled (4 °C) cryocontainer (Cryo 1 °C freezing container, Nalge Nunc Int., Rochester, NY, USA) with isopropyl alcohol, placed in a -80 °C freezer overnight and thereafter stored in liquid nitrogen. Under these conditions, the isopropyl alcohol decreases the temperature of the cryovials by approximately 1 °C/min.

After 7 days of storage, cryopreserved samples were thawed by rapidly immersing them in a 37 °C water bath until they could be decanted. The cell suspension was transferred to a 50 ml polypropylene tube containing a 10 times larger volume IMDM + 20% FCS. The cells were washed twice with IMDM + 20% FCS.

Cell viability

In this study, cell viability is defined as cells that can be individualy detected by flowcytometric analysis and reflects the number of cells that are negative for staining with Annexin V and propidium iodide (PI). Moreover, these cells are able to be activated resulting in proliferation as detected by individual staining with Ki-67 antibodies. The cell viability of fresh and cryopreserved unstimulated PBMC was determined within 2 hours after isolation or thawing. Half a million PBMC were washed and subsequently incubated with 2 µl Annexin V-APC (BD biosciences, San Diego, USA) in 200 µl Annexin V buffer according to the manufacturer's protocol. After an incubation period of 15 min on ice, the cells were spun down (400*g* for 10 min) and resuspended in 200 µl Annexin V buffer plus 2 µl propidium iodide (PI; 1 mg/ml; Sigma). The cells were thereafter analyzed on a flowcytometer (FACSArray, BD, San Diego, USA). Cells that were negative for both Annexin V and PI have no indications of apoptosis or necrosis since no translocation of the membrane phospholipid phosphatidylserine has occurred and the plasma membrane is still intact. Therefore, Annexin V and PI double negative cells were considered as viable cells [37], whereas both single and double positive cells were regarded as non-viable. The percentage of positive cells was calculated into the absolute number of positive cells by the use of flowcytometric counting beads (BD Pharmingen). The use of these beads permits the presentation of the absolute cell numbers within the table.

The effect of the polyclonal stimuli on the cell viability was determined in an identical way on days 1, 3, 5, and 7, as described below in the paragraph culture conditions.

Immunological phenotype

The immunological phenotype of fresh and cryopreserved unstimulated PBMC was determined within 2 hours after isolation or thawing. Surface antigens were identified with the following two monoclonal antibody mixtures: 1) α-hCD3 (PE-Cy7), α-hCD4 (PE), α-hCD8 (APC) and α-hCD25 (APC-Cy7); 2) α-hCD14 (APC), α-hCD16 (PE), α-hCD19 (APC-Cy7) and α-hCD56 (PE). All antibodies were purchased from BD Biosciences, San Diego, USA.

Per well, $5x10^5$ cells were spun down in a 96-wells U-bottom plate. The cells were incubated with staining buffer (1% FCS and 0.1 M NaN_3 in PBS)

containing the surface markers or the matching isotype controls for 30 min on ice in the dark. The cells were washed once with PBS and resuspended in PBS before flowcytometric analysis. The four-color flowcytometric acquisition was performed on a FACSArray (BD Biosciences), using the BD FACSArray software. An electronic gate was set to exclude debris and at least 10,000 events/sample were acquired. The percentage of positive cells was corrected for the isotype control and thereafter calculated into the absolute number of positive cells by the use of flowcytometric counting beads (BD Biosciences). The use of these beads permits the presentation of absolute cell numbers within the graphs.

The effect of the polyclonal stimuli on the immunological phenotypes was determined in an identical way on days 1, 3, 5, and 7, as described below.

Culture conditions

PBMC were cultured in Yssel's medium at 37 ºC in a humidified atmosphere with 5% CO_2 at a density of $1x10^6$ viable cells/ml. Yssel's medium consisted of IMDM supplemented with 1% Penicillin-Streptomycin (Gibco BRL), extra additions according to Yssel *et al.* [38] and 1% human AB serum (Gibco BRL) for fresh or 2% human AB serum for cryopreserved PBMC.

Cells were plated out in 48-well plates at a concentration of $1x10^6$ cells/ml and cultured at 37 ºC. After five hours of adaptation to the culture conditions, the three different stimuli or a matching volume of medium were added. Cultures were stimulated with 5 µg/ml ConA (Sigma), 150 ng/ml αCD3 (BD Biosciences) plus 100 ng/ml αCD28 (BD Biosciences) or 2 ng/ml PMA (Sigma) plus 1 µg/ml Ca-I (Sigma), or cultured in medium only (untreated PBMC). These optimal concentrations were determined in previous experiments. Tested concentrations of PMA ranged from 1 ng/ml to 0,01 pg/ml, of Ca-I from 500 ng/ml to 5 pg/ml, of ConA from 10 µg/ml to 300 ng/ml, of αCD3 from 450 to 50 ng, and of αCD28 from 300 to 30 ng/ml.

Proliferation capacity

[^{3}H]-thymidine incorporation assay

After 1 to 6 days of stimulation of fresh PBMC, proliferation was measured using [^{3}H]-thymidine incorporation. Cells were plated at $5x10^5$ cells/well in a 96-well plate in Yssel's medium with the different stimulations or medium alone. After addition of [^{3}H]-thymidine (0.4 µCi/well; MP biomedicals), the plates were incubated for 18 h before harvesting. Incorporated [^{3}H]-thymidine was determined in a β-counter (Tri-Carb 2200 CA liquid scintillation analyzer, Canberra Packard). The results are depicted as stimulation index, expressing the ratio of mean counts per minute in stimulated PBMC over unstimulated PBMC.

Ki-67 positive cells
The proliferation capacity of fresh and cryopreserved PBMC was studied after 1 to 7 days of stimulation by intracellular expression of the nuclear Ki-67 antigen (Ki-67; BD Biosciences). The Ki-67 antigen is absent from the nuclei of resting cells, but present in all other phases of the cell division cycle as well as in the mitosis phase [39].

In each well, $5x10^5$ PBMC were incubated in 100 µl cytofix/cytoperm (BD Biosciences) for 15-20 min on ice to fix and permeabilize the cells. Cells were washed twice with perm/wash buffer (BD Biosciences) and incubated with an anti-Ki-67 PE antibody, or the matched isotype control, diluted in perm/wash buffer for 30 min on ice in the dark. Hereafter, the cells were washed with perm/wash buffer, resuspended in PBS and measured on the flowcytometer. The percentage of positive cells was corrected for the isotype and medium control, and thereafter calculated into the absolute number of positive cells by the use of flowcytometric counting beads (BD Biosciences). The use of these beads permits the presentation of absolute cell numbers within the graphs.

Cytokines
Selection of sampling time points by comparing cytokine mRNA and protein
Cells and supernatants were harvested at time points 30 min, 1, 3, 6, 24, and 48 hours. RNA isolation, cDNA synthesis and real-time quantitative polymerase chain reaction (RT-qPCR) were performed as described in detail by Forlenza *et al.* [40]. The primers used for RT-qPCR are listed in Table I. The protein production was measured with Cytometric Bead Array Flex sets as described in more detail below.

Protein detection in culture supernatants
Both fresh and cryopreserved cells were analyzed for their IL-1β, IL-12, IFN-γ, TNF-α, IL-4, IL-5, IL-10 and IL-13 production after 1 to 7 days without stimulation or in the presence of one of the polyclonal stimuli. The cytokine production was measured with Cytometric Bead Array Flex sets (BD Biosciences). This cytometric bead array is a bead-based immunoassay that provides a multiplex determination for soluble proteins like cytokines [41]. All buffers used in this protocol were obtained from the BD CBA Soluble Protein Master Buffer Kit (BD Biosciences). Supernatants of both fresh and cryopreserved cells were collected at 1 to 7 days, stored at -20 °C and tested within 2 weeks.

The procedure was performed according to the manufacturer's protocol. The samples were measured on the FACSArray, using the FCAP software. The sensitivity limits for quantitative determination, according to the manufacturer, were 1.1 pg/ml for IL-1β, 0.3 pg/ml for IL-4 and IFN-γ, 0.5 pg/ml for IL-5,

2.3 pg/ml for IL-10, 2.2 pg/ml for IL-12 , 0.6 pg/ml for IL-13 and 0.7 pg/ml for TNF-α.

Table I *Primers used in RT-qPCR gene expression analysis*

Gene	Primer	Sequence 5'→ 3'	Amplicon size (bp)	Accession number
IL-4	forward	TTGAACAGCCTCACAGAGCA	156	BC070123
	reverse	GCAGCGAGTGTCCTTCTCAT		
IL-5	forward	TCTTTCAGGGAATAGGCACAC	134	NM000879
	reverse	TCTCCGTCTTTCTTCTCCACA		
IL-10	forward	GTGATGCCCCAAGCTGAGA	138	AF043333
	reverse	CACGGCCTTGCTCTTGTTTT		
TNF-α	forward	GGCGTGGAGCTGAGAGATAA	174	X02910
	reverse	GATGGCAGAGAGGAGGTTGA		
IFN-γ	forward	TGGAGACCATCAAGGAAGACA	152	BC070256
	reverse	CAGTTCAGCCATCACTTGGA		
RPLPO	forward	CAGATTGGCTACCCAACTGTT	98	NM053275
	reverse	GGGAAGGTGTAATCCGTCTCC		

RPLPO: Homo sapiens ribosomal protein, large, P0; used housekeeping gene.

Statistical analysis

The General Linear Model was used to determine overall statistical differences between fresh and cryopreserved PBMC responses for each of the stimulation conditions and time-points tested. The analysis design consisted of a repeated measurement for Day, and the equality of error variances were tested using the Levene's test. In the multivariate test, the Wilk's Lambda test was used to assess the significant effects in the overall differences. Whenever the Wilk's lambda test was significant, additional analysis using one way ANOVA was performed. A two-sided-p value of 0.05 or lower was considered to be significant. The statistical analysis was performed by using SPSS Software (version 12.0.1, SPSS Inc., Chicago, USA).

Results

Cell viability

The total number of non-viable cells is shown in Table II. Cryopreserving PBMC resulted in a significant higher number of non-viable cells directly after thawing compared to before the cryopreservation as indicated in the 'Day 0' column. This significant effect due to cryopreservation was retained in the unstimulated PBMC at day 1, 3 and 7 (Table II). In contrast, no significant differences in the cell viability due to cryopreservation were seen in PBMC stimulated with PMA/Ca-I or αCD3/αCD28. In contrast, the ConA cultures showed a significant decrease in non-viable cells after cryopreservation at day 7. The number of non-viable ConA and PMA/Ca-I stimulated fresh and ConA stimulated cryopreserved PBMC was significantly higher than of unstimulated PBMC, on day 1 only (Table II).

Table II Non-viable cells after polyclonal stimulation

Origin	Stimulation	Day 0	Day 1	Day 3	Day 5	Day 7
Fresh PBMC	Medium	6.7 ± 1.5	19.8 ± 4.6	35.8 ± 2.7	43.0 ± 4.5	43.1 ± 3.9
	ConA		39.3 ± 4.5 *	45.4 ± 4.5	52.3 ± 5.9	56.0 ± 6.1
	PMA/Ca-I		39.3 ± 6.2 *	43.5 ± 3.2	55.2 ± 4.0	57.0 ± 4.8
	αCD3/αCD28		27.1 ± 5.5	25.5 ± 3.9	30.0 ± 6.1	36.1 ± 2.0
Frozen PBMC	Medium	14.6 ± 2.1 [a]	39.3 ± 2.8 [aa]	46.4 ± 5.6 [a]	53.3 ± 5.8	53.7 ± 5.5 [a]
	ConA		58.1 ± 4.5 **	56.0 ± 5.5	48.8 ± 5.7	48.5 ± 4.4 [a]
	PMA/Ca-I		50.4 ± 2.9	47.3 ± 4.7	52.6 ± 6.0	59.9 ± 6.3
	αCD3/αCD28		42.1 ± 3.2	43.2 ± 5.1	34.2 ± 4.4	42.9 ± 4.9

Fresh and cryopreserved PBMC were used unstimulated or stimulated with 5 μg/ml ConA, 150 ng/ml CD3 plus 100 ng/ml CD28, 2 ng/ml PMA plus 1 μg/ml Ca-I. PBMC were stained with Annexin V (APC) and just before measurement 2 μg PI was added. Annexin V positive and/or PI positive fresh and cryopreserved cells were analyzed on days 1, 3, 5 and 7 and all single or double positive cells were regarded as non-viable cells. The number of non-viable cells are expressed as average SEM (*10^6 cells/million PBMC) (n=5). Statistical tests were performed comparing the unstimulated PBMC to polyclonal stimulated PBMC: * = $p<0.05$ or ** = $p<0.01$ and the effect of cryopreservation: [a] = $p<0.05$ or [aa] = $p<0.01$.

Immunological phenotype

To determine which PBMC subsets are present in the different culture conditions, the immunological phenotype of fresh and cryopreserved PBMC was determined by using the following monoclonal antibody mixtures: i) α-hCD3, α-hCD4, α-hCD8 and α-hCD25; ii) α-hCD14, α-hCD16, α-hCD19 and α-hCD56.

The effect of cryopreservation on the T cell subsets within PBMC cultures
The absolute number of $CD3^+$ T cells directly after isolation was not significantly altered after cryopreservation (data not shown). Moreover, no significant alterations were observed in fresh compared to cryopreserved $CD3^+4^+$ and $CD3^+8^+$ T cell subsets, as shown in Figure 1. Furthermore, only PMA/Ca-I induced a significant reduction in the absolute number of both fresh and cryopreserved $CD3^+4^+$ and $CD3^+8^+$ cells on day(s) 3 and/or 7.

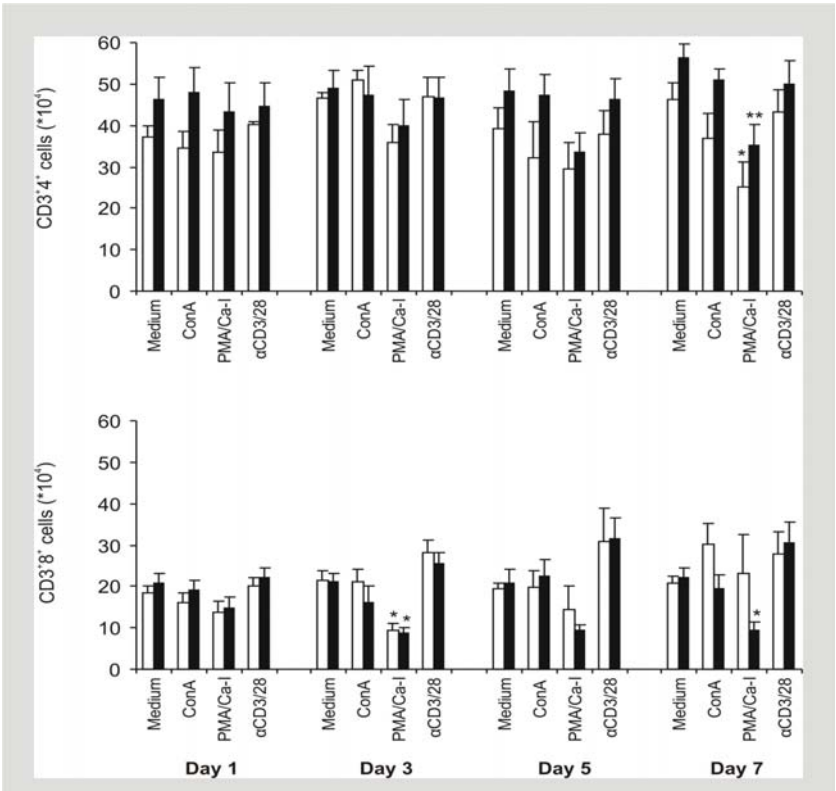

Figure 1. Kinetics and the effect of cryopreservation on T cell subsets. Fresh (open bars) and cryopreserved (closed bars) PBMC were used unstimulated or stimulated with 5 µg/ml ConA, 150 ng/ml CD3 plus 100 ng/ml CD28, or 2 ng/ml PMA plus 1 µg/ml Ca-I. PBMC surface markers were stained with a mixture of α-hCD3 (PE-Cy7), α-hCD4 (PE) and α-hCD8 (APC). $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell subsets were analyzed on days 1, 3, 5 and 7 and cell numbers are expressed as average ± SEM (*10⁴ cells/million PBMC) (n=5). Statistical test were performed comparing the unstimulated PBMC to polyclonal stimulated PBMC: * $p<0.05$ and ** $p<0.01$.

Effect of cryopreservation on the kinetics of CD25 expression
T cells that become activated will upregulate their CD25 expression on their cell surface. Therefore, $CD25^+$ T cells can be considered as activated T cells. The absolute number of $CD3^+4^+25^+$ cells was significantly lowered on days 1 and 3 after ConA stimulation comparing fresh to cryopreserved PBMC (Figure 2). In addition, fresh αCD3/αCD28 stimulated PBMC expressed significantly more CD25 as their cryopreserved counterparts on day 1 only. Except for fresh PMA/Ca-I stimulated PBMC, all stimulation conditions significantly increased the $CD3^+4^+25^+$ cells compared to unstimulated PBMC at all measured time points. In contrast to the $CD3^+4^+25^+$ PBMC, $CD3^+8^+25^+$ cells were not significantly altered by cryopreservation (Figure 2). Only ConA and αCD3/αCD28 significantly increased the number of $CD3^+8^+25^+$ cells compared to unstimulated PBMC at all measured time points.

Effect of cryopreservation on unstimulated B cells, NK cells and monocytes
Besides T cell subsets, also B cells, NK cells and monocytes have been analyzed. Comparing fresh to cryopreserved B cells ($5.9 \pm 0.9x10^4$ vs. $7.6 \pm 0.x10^4$ cells per million), NK cells ($11 \pm 3x10^4$ vs. $14 \pm 3x10^4$ cells per million) and monocytes ($10 \pm 2x10^4$ vs. $12 \pm 2x10^4$ cells per million) showed that cryopreservation had no effect on the composition of these elements of the unstimulated PBMC subsets.

Kinetics of B cells, NK cells and monocytes in fresh and cryopreserved PBMC
The absolute number of B cells and NK cells was not significantly changed due to polyclonal stimulation (data not shown). In addition, no changes in the kinetics were observed. The average number of B cells at all measured time points was $7 \pm 1x10^4$ cells per million, and the absolute number of NK cells was determined at $15 \pm 3x10^4$ cells per million.

Microscopical analysis of the monocyte adhesion over time, showed that in the unstimulated or PMA/Ca-I culture, not all monocytes adhered at day 1, but gradually more monocytes adhered over time. In contrast, both ConA and αCD3/αCD28 stimulated PBMC showed more adhesion of the monocytes already at day 1. These phenomena were seen in both fresh and cryopreserved PBMC cultures. As a result, when isolating the PBMC from the culture plates, adhered monocytes were left in the plates, and were therefore not detectable as $CD14^+$ cells in the flowcytometric analysis. To show how many monocytes adhered to the plastic, the amount of adhered monocytes was calculated from the original amount of $CD14^+$ cells put into culture, minus the amount of $CD14^+$ cells measured at the flowcytometer. As shown in Figure 3, the amount of adhered $CD14^+$ cells in fresh PBMC stimulated with ConA or αCD3/αCD28 was significantly increased at day 1 compared to unstimulated PBMC. At all other determined time points, no significant differences were observed between the polyclonal stimulated and the unstimulated PBMC. In contrast, cryopreserved PBMC showed no significant difference at day 1, 5

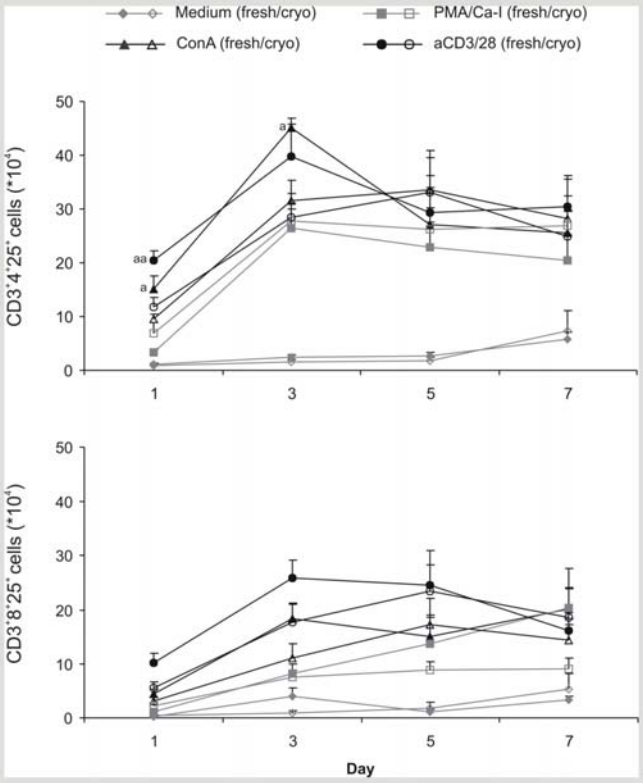

Figure 2. Expression of activation marker CD25 after polyclonal stimulation of T cell subsets. Fresh (closed symbols) and cryopreserved (open symbols) PBMC were used unstimulated (♦/◊) or stimulated with 5 µg/ml ConA (▲/Δ), 150 ng/ml CD3 plus 100 ng/ml CD28 (●/○), 2 ng/ml PMA plus 1 µg/ml Ca-I (■/□). PBMC surface markers were stained with a mixture of α-hCD3 (PE-Cy7), α-hCD4 (PE), α-hCD8 (APC) and α-hCD25 (APC-Cy7). $CD3^+4^+25^+$ (activated $CD4^+$ T cells) and $CD3^+8^+25^+$ cells (activated $CD8^+$ T cells) were analyzed on days 1, 3, 5 and 7 and cell numbers are expressed as average SEM ($*10^4$ cells/million PBMC) (n=5). Statistical tests were performed comparing fresh to cryopreserved PBMC: $^*p<0.05$ and $^{**}p<0.01$.

and 7, but significantly more monocytes adhered at day 3. In addition, cryopreservation significantly increased the number of adhering monocytes at day 1 of PMA/Ca-I stimulated cells.

Proliferation induction

To determine the effect of cryopreservation and polyclonal stimulation on the proliferation of PBMC, two distinct methods to measure proliferation were performed: the [^{3}H]-thymidine incorporation assay and the anti-Ki-67 antibody staining.

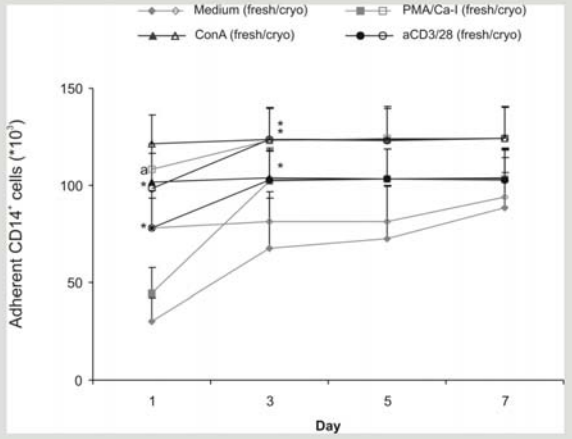

Figure 3. Activation and subsequent adherence of CD14$^+$ cells. Fresh and cryopreserved PBMC were stimulated with 5 µg/ml ConA (▲/Δ), 150 ng/ml CD3 plus 100 ng/ml CD28 (●/○), 2 ng/ml PMA plus 1 µg/ml Ca-I (■/□), or medium only (negative control,♦/◊). PBMC were stained with a mixture of α-hCD14 (APC), α-hCD16 (PE), α- hCD19 (APC-Cy7) and α-hCD56 (PE). Fresh (closed symbols) and cryopreserved (open symbols) CD14$^+$ cells (monocytes) were analyzed on days 1, 3, 5 and 7 and cell numbers are expressed as average SEM (*10^3 cells/million PBMC) (n=5). Statistical tests were performed comparing the medium control to stimulated PBMC: * p<0.001. Fresh PBMC compared to cryopreserved PBMC are indicated as $^+$ p<0.05.

[^{3}H]-thymidine incorporation assay

Anti-Ki-67 antibodies are not frequently used in literature. However, these antibodies are ideally in combination with other flowcytometric analyses. Therefore, the resemblance between the [3H]-thymidine incorporation assay and the αKi-67 Ab proliferation read-out systems was assessed. As shown in Figure 4, ConA showed a maximal stimulation index of 84 at day 3, whereas PMA/Ca-I only induced a maximal SI of 13 at day 2.

Ki-67 expression

As shown in Figure 5, both ConA and αCD3/αCD28 significantly upregulated the number of proliferating cells, with an optimum at days 3 to 5. Cryopreservation did not alter these findings. However, cryopreservation did cause a delay in the proliferation capacity, since ConA already induced 42.4 ± 5.3x10^4 proliferating cells by day 3, whereas after cryopreservation the cells reached an equal number of proliferating PBMC by day 5. A similar delay due to cryopreservation was found in PBMC stimulated with αCD3/αCD28. PBMC cultured with PMA/Ca-I showed low numbers of proliferating cells on all time points. In addition, unstimulated PBMC did not proliferate on any of the analyzed time points (Figure 5).

Both the [^{3}H]-thymidine incorporation assay and the αKi-67 antibody staining showed a maximum induction of proliferation after ConA stimulation at day 3, and a steady, but low number of proliferating cells after PMA/Ca-I stimulation. Although the [^{3}H]-thymidine incorporation assay indicates the

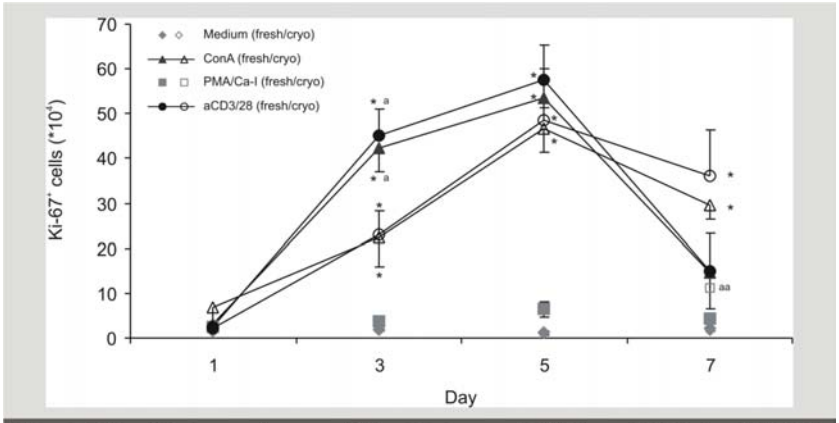

Figure 4. [^{3}H]-thymidine incorporation assay. [^{3}H]-thymidine incorporated PBMC were detected after 0.5, 1, 2, 3, 4 and 5 days stimulation with 5 µg/ml ConA, 2 ng/ml PMA plus 1 µg/ml Ca-I, or medium only.

number of proliferating cells accumulated over a particular period of time, and the αKi-67 antibody staining indicates the number of proliferating cells at a specific time point, these results support the use of the αKi-67 antibodies to reliably measure proliferation.

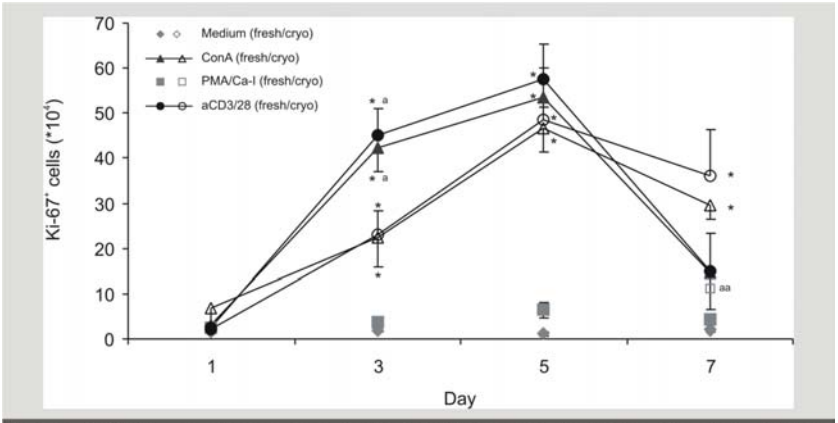

Figure 5. Proliferation capacity of polyclonal stimulated PBMC on alternate days. Ki-67 positive cells (proliferating cells) were detected on days 1, 3, 5 and 7 in fresh and cryopreserved PBMC stimulated with 5 µg/ml ConA, 150 ng/ml CD3 plus 100 ng/ml CD28, 2 ng/ml PMA plus 1 µg/ml Ca-I, or medium only. In the graph, average ± SEM of the absolute number of Ki-67$^+$ proliferating cells *10^4 /million PBMC (n=5) are depicted. Statistical tests were performed comparing unstimulated to stimulated PBMC: $^{\#}$ p<0.001. Fresh PBMC compared to cryopreserved PBMC are indicated as: * p<0.05, ** p<0.01.

Comparison of mRNA and protein levels

Activation and differentiation of PBMC is partly regulated by cytokines. To determine which cytokines are produced in our cultures, we used two methods to determine the cytokine production: 1) the detection of mRNA expression and 2) the secretion of proteins in the culture supernatant. Since mRNA is not necessarily directly translated into protein, we also determined the time point at which the proteins of interest are detectable in the culture supernatants.

The used housekeeping gene, RPLPO, was constant until 6 h after stimulation, but thereafter unreliable, probably due to ribosomal activation. Figure 6A and B are therefore lacking data at 24 or 48 h after stimulation. IL-4 mRNA expression is maximal after 3 hours of PMA/Ca-I stimulation, while αCD3/αCD28 and ConA induced IL-4 mRNA expression is still increasing at 6 h after stimulation (Fig. 6A). The IL-4 protein production is initiated at 3 to 6 h after polyclonal stimulation (Fig. 6C). IL-10 mRNA expression showed a ongoing induction from 3 h onwards in the presence of ConA or αCD3/αCD28 (Fig. 6B), leading to protein expression after 6 h of stimulation (Fig. 6D). Based on these results, cytokine protein levels of fresh and cryopreserved PBMC were determined from day 1 onwards. A similar conclusion was drawn for IL-5, TNF-α and IFN-γ (data not shown).

Cytokine protein levels

The effects of polyclonal stimulation on IL-1β, IL-10, IL-12, and TNF-α.

The comparison between mRNA and protein showed that all cytokines of interest are detectable in the culture supernatant from day 1 onwards. Therefore, we determined the presence of cytokines in the culture supernatant at days 1, 3, 5 and 7. To show the effect of cryopreservation on monocyte derived cytokines and T cell derived cytokines more specifically, these two sources are separately depicted in Figure 7 and 8, respectively.

As shown in Figure 7, no significant differences between unstimulated and PMA/Ca-I stimulated PBMC could be observed. IL-1β levels after stimulation with PMA/Ca-I were significantly lowered in the culture supernatant of cryopreserved PMBC compared to fresh PBMC. No significant differences in the cytokine production after αCD3/αCD28 stimulation were observed due to the cryopreservation procedure. Therefore, Table III only reports the values obtained with fresh PBMC. ConA stimulated PBMC showed no significant alterations of the IL-10, IL-12, and TNF-α levels by the cryopreservation procedure. As shown in Table III, the only significant difference due to cryopreservation was found in the IL-1β levels produced by ConA stimulated PBMC on day 1 (fresh: 19931 ± 4740 pg/ml; cryopreserved: 2857 ± 1645 pg/ml).

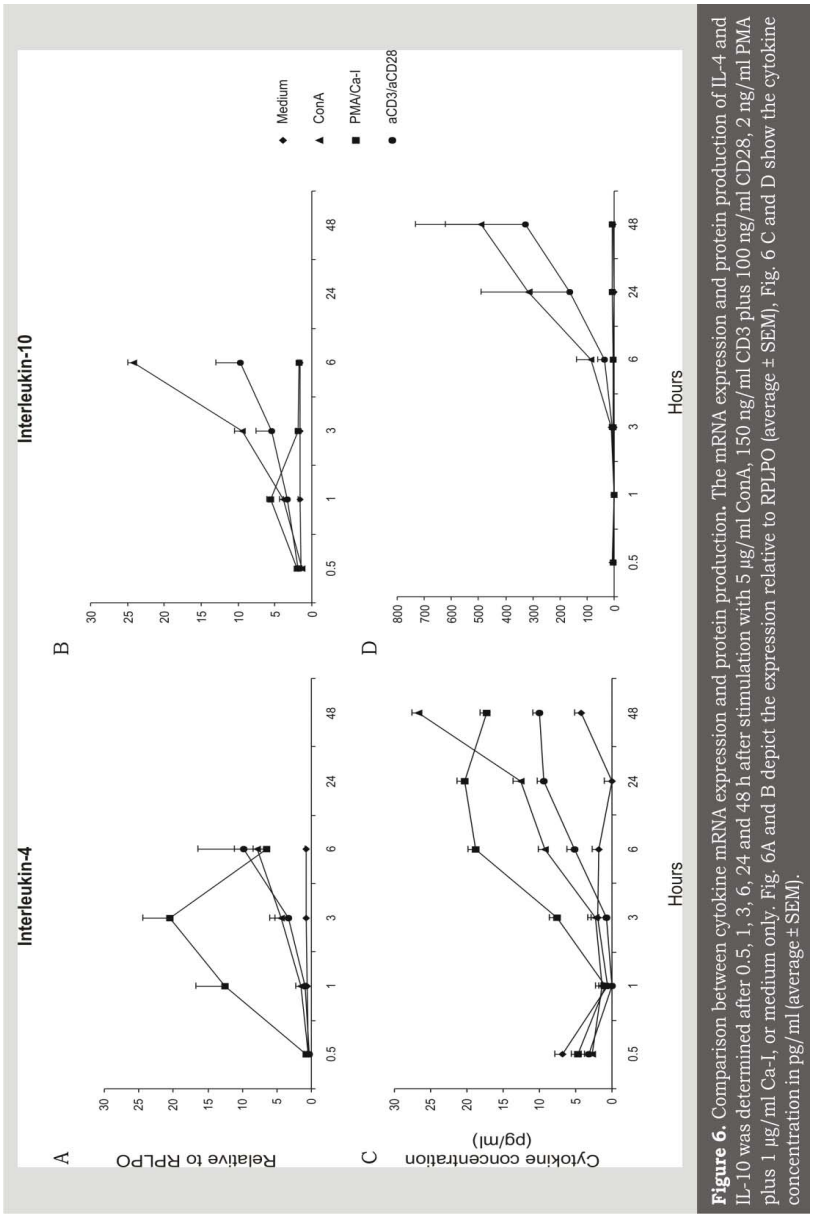

Figure 6. Comparison between cytokine mRNA expression and protein production. The mRNA expression and protein production of IL-4 and IL-10 was determined after 0.5, 1, 3, 6, 24 and 48 h after stimulation with 5 µg/ml ConA, 150 ng/ml CD3 plus 100 ng/ml CD28, 2 ng/ml PMA plus 1 µg/ml Ca-I, or medium only. Fig. 6A and B depict the expression relative to RPLPO (average ± SEM), Fig. 6 C and D show the cytokine concentration in pg/ml (average ± SEM).

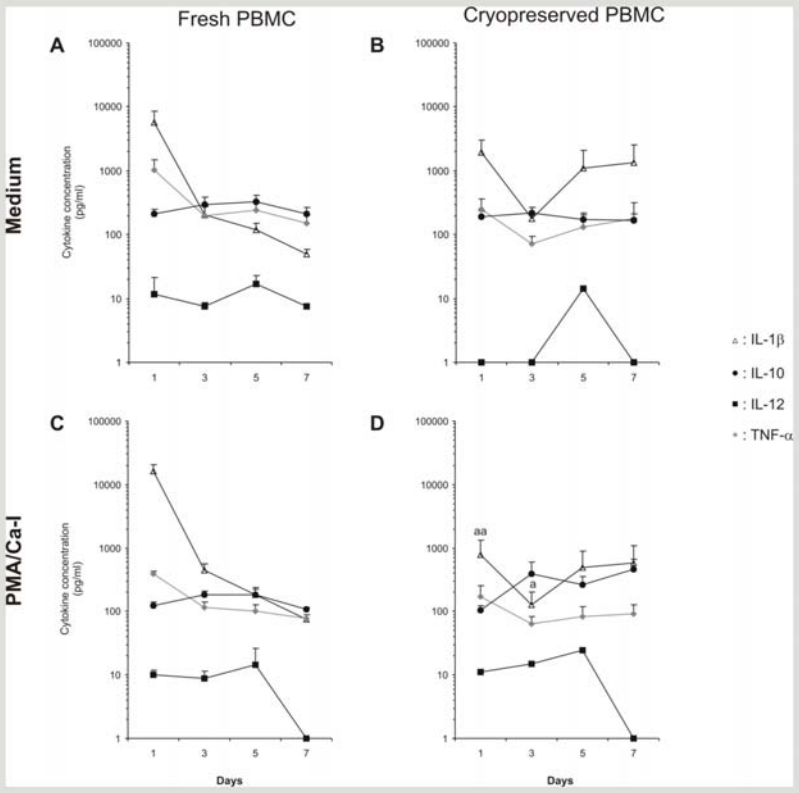

Figure 7. Effect of cryopreservation on the monokine profile of unstimulated and PMA/Ca-I stimulated PBMC. The production of IL-1β (Δ), IL-12p70 (■), TNF-α (♦) and IL-10 (●) was determined with a CBA assay. Fresh (A and C) and cryopreserved (B and D) PBMC were cultured in medium alone (A and B) or medium containing 2 ng/ml PMA plus 1 µg/ml Ca-I (C and D). Cytokine production was determined on days 1, 3, 5 and 7 and cytokine levels are expressed as average ± SEM per million PBMC (n=5). Fresh PBMC compared to cryopreserved PBMC are indicated as: ^{a} $p<0.05$, ^{aa} $p<0.01$.

The effects of polyclonal stimulation on IL-4, IL-5, IL-13 and IFN-γ.
As shown in Figure 8, all measured T cell cytokines significantly increased in PMA/Ca-I stimulated PBMC compared to unstimulated cells. Although cryopreservation of unstimulated PBMC did not significantly alter the cytokine profile, cryopreservation did significantly alter the Th2 cytokines IL-4 and IL-13 after PMA/Ca-I stimulation. Again, Table III shows the data obtained with fresh PBMC, as no significant differences in the cytokine production after αCD3/αCD28 or ConA stimulation due to the cryopreservation procedure were observed.

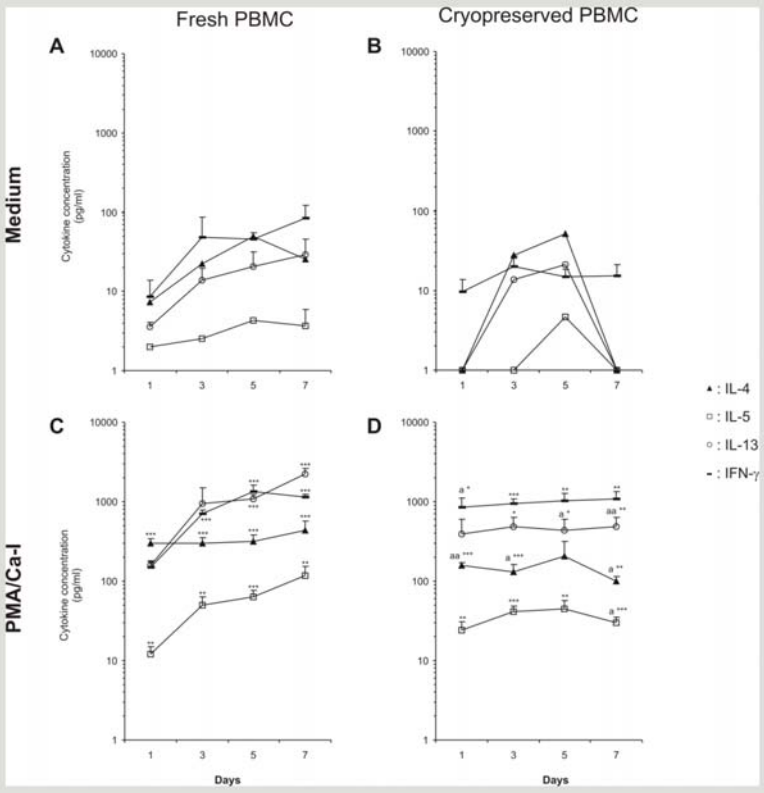

Figure 8. Effect of cryopreservation on T cell cytokines from unstimulated and PMA/Ca-I stimulated PBMC**.** The production of IL-4 (▲), IL-5 (□), IL-13 (○) and IFN-γ (-) was determined with a CBA assay. Fresh (A and C) and cryopreserved (B and D) PBMC were cultured in medium alone (A and B) or medium containing 2 ng/ml PMA plus 1 µg/ml Ca-I (C and D). Cytokine production was determined on days 1, 3, 5 and 7 and cytokine levels are expressed as average ± SEM per million PBMC (n=5). Statistical test were performed comparing the unstimulated PBMC to polyclonal stimulated PBMC: * p<0.05, ** p<0.01 and *** p<0.001. The significant effect of cryopreservation is indicated with ^{a} p<0.05 or ^{aa} p<0.01.

Table III Cytokine production of αCD3/αCD28 or ConA stimulated fresh PBMC

Stimulation	Cytokine	Day 1	Day 3	Day 5	Day 7
αCD3/αCD28	IL-1β	10485 ± 2811	805 ± 340	485 ± 244	208 ± 108
	IL-12p70	44 ± 37	53 ± 46	19 ± 9	*bd*
	TNF-α	3311 ± 1746	1734 ± 851	506 ± 137	225 ± 67
	IL-10	349 ± 80	358 ± 100	182 ± 47	88 ± 20
	IL-4	25 ± 6	9 ± 4	21 ± 1	*bd*
	IL-5	3 ± 1	4 ± 1	7 ± 3	*bd*
	IL-13	45 ± 6 *	89 ± 7	121 ± 26	85 ± 8
	IFN-γ	320 ± 95	474 ± 58	413 ± 42	256 ± 76
ConA	IL-1β	19931 ± 4740 aa	1034 ± 359	700 ± 352	324 ± 155
	IL-12p70	21 ± 17	13 ± 10	8 ± 2	*bd*
	TNF-α	3631 ± 1983	1732 ± 1330	1096 ± 795	286 ± 127
	IL-10	453 ± 82 *	420 ± 105	364 ± 121	166 ± 34
	IL-4	41 ± 3	20 ± 5	*bd*	*bd*
	IL-5	5 ± 2	11 ± 3	8 ± 2	6 ± 2
	IL-13	60 ± 15	96 ± 13	68 ± 6	50 ± 7
	IFN-γ	169 ± 51	450 ± 71	432 ± 80	320 ± 31

Fresh and cryopreserved PBMC were stimulated with 5 μg/ml ConA or 150 ng/ml CD3 plus 100 ng/ml CD28. Culture supernatants were analyzed on days 1, 3, 5 and 7 and the cytokine levels are expressed as average ± SEM (pg/ml) (n=5). Statistical tests were performed comparing the unstimulated PBMC to polyclonal stimulated PBMC (* $p<0.05$) and fresh versus cryopreserved PBMC (^{aa} $p<0.01$).

Discussion

In this study, we determined the effects of cryopreservation on PBMC samples isolated from five different blood donors, and tested individually. To assess the effects of cryopreservation, we used three different polyclonal T cell stimulations, i.e. ConA, PMA/Ca-I and αCD3/αCD28, on different time points and determined the cell viability, proliferation capacity, cytokine production and composition of the PBMC population.

T cell stimuli can, but do not necessarily, alter the number of viable cells. For example, we observed that αCD3/αCD28 did not result in significantly more non-viable cells than seen in unstimulated PBMC, whereas both ConA and PMA/Ca-I resulted in significantly higher numbers of non-viable cells. Macian *et al.* showed that activation induced cell death is one of the mechanisms for eliminating effector T cells that are no longer in use [42]. This activation induced cell death might explain the high numbers of non-viable cells in ConA cultures, since those cultures also displayed one of the highest number of proliferating cells. Disis *et al.* showed that both fresh and cryopreserved PBMC contained about 95% viable cells. This percentage is equal to the amount of viable cells we obtained after isolation, but is somewhat higher than the viability found after our cryopreservation procedure [43]. Similar to this, Venkatamaran showed that cryopreserved samples contained greater than 98% viable recovered cells [29, 30]. This impairment can not be explained by the cryopreservation medium, since we also used FCS in our cryopreservation medium. Disis *et al.* showed that FCS presence in the cryopreservation medium leads to a low amount of cell death [43]. The discrepancy might be explained by the way the cell viability was determined. Both Disis *et al.* and Venkatamaran used the trypan blue exclusion technique, whereas we determined the cell viability by means of Annexin V plus propidium iodide. In this way, not only the late apoptotic or necrotic cells identified with trypan blue or propidium iodide, but also the early apoptotic cells will be visualized, likely resulting in higher cell death numbers [29, 43].

Focusing on the T cell subsets, no significant alterations were observed in the unstimulated fresh compared to cryopreserved $CD3^+4^+$ cells, activated $CD3^+4^+$ cells, $CD3^+8^+$ cells and activated $CD3^+8^+$ cells. This is in agreement with Sleasman et al., who also reported that the CD4 expression did not change due to cryopreservation [44]. In addition, the ratio of CD4:CD8 is about 2:1 in our study, concomitant to the ratio reported by Chaplin *et al.*, who showed that 60-70% of T lymphocytes are $CD4^+$ and 30-40% are $CD8^+$ cells [45]. Activation of the T cell subsets with the different polyclonal stimulations all resulted in an upregulation of the activation marker CD25. Also other groups show that lectins, PMA, PMA/Ca-I and αCD3/αCD28 are all able to induce CD25 upregulation [1, 46]. The kinetics of this CD25 upregulation is delayed in cryopreserved cells compared to fresh PBMC, but both PBMC conditions

result in more than 30% of activated $CD4^+$ T cells within the total PBMC population after αCD3/αCD28 stimulation.

Besides T cells, also B cells, NK cells and monocytes were assessed. Comparing fresh to cryopreserved B cells, NK cells and monocytes, it is shown that cryopreservation had no effect on the composition of these PBMC subsets. In addition, the absolute amount and kinetics of B and NK cells was not significantly changed due to polyclonal stimulation. The relative amount of B and NK cells in this study were consistent with literature, which reports that peripheral blood leukocytes consist of 15% B cells and a small fraction of NK cells [45]. In addition, Sleasman *et al.* showed that monocytes in fresh and cryopreserved PBMC range from 8-10%, which was in agreement with our $CD14^+$ values at day 0 [44]. In contrast, the absolute number of adherent $CD14^+$ monocytes was affected over time by all three polyclonal stimuli. The number of adhering $CD14^+$ cells in the medium control gradually increased over time, whereas PMA/Ca-I on day 1 still showed non-adherent $CD14^+$ cells, but thereafter the number of adherent cells increased significantly. Interestingly, ConA and αCD3/αCD28 stimulated PBMC showed only adherent $CD14^+$ cells from day 1 onwards. A number of T cell mediated contact-dependent interactions, like CD40-CD40L, CD23, CD31 and CD69, might play an important role in the differences observed between the different polyclonal stimuli and the number of directly adhering monocytes [47]. Such interactions have been described for T cells activated with αCD3 plus αCD28 [48] or ConA [49].

Besides cell viability as a read-out system, also proliferation is often determined. In literature, [^{3}H]-thymidine, BrdU and Ki-67 detecting antibodies are used to determine cell proliferation [3, 23, 50]. [^{3}H]-thymidine and BrdU give comparable numbers of proliferating cells, since BrdU can be incorporated into newly synthesized DNA of replicating cells during the S phase of the cell cycle. It then substitutes thymidine during the DNA replication. Antibodies specific for BrdU can then be used to detect the incorporated BrdU, thus indicating the cells that were actively replicating their DNA during the whole culture duration [51]. Detection of Ki-67 on the other hand, only shows which cells are not in the G0 phase at the time of examination [50], therefore leading to much lower numbers of proliferated cells compared to [^{3}H]-thymidine and BrdU positive cells. Therefore, only an indirect comparison between our proliferation data and literature is possible. Both ConA and αCD3/αCD28 significantly upregulated the amount of proliferating cells, with an optimum at days 3 to 5. These findings are in agreement with literature which shows that ConA leads to a considerable amount of proliferation at day 3 [15] and day 7 [16]. Our findings did not differ when comparing fresh to the cryopreserved PBMC, which is in agreement with Disis *et al.* who showed that cryopreservation did not alter lectin or allergen induced proliferation [43].

In addition to proliferation, cells can also be activated to differentiation and cytokine production. We compared the mRNA expression and protein production of IL-4, IL-10, IFN-γ and TNF-α to get a better insight in the appropriate sampling time points. Although the relative numbers differ due to the use of a different housekeeping gene, the trends in the kinetics of IL-4 and IL-10 mRNA expression after αCD3/αCD28 stimulation are similar when comparing our data to the data of Sareneva *et al.* [52]. And although some papers determine the cytokine protein production at earlier time points, several studies determined the protein production after 24, 48 and 72 hours of polyclonal stimulation [11, 20, 46, 52]. We measured IL-1β, IL-4, IL-5, IL-10, IL-12, IL-13, IFN-γ, and TNF-α protein production after 1 to 7 days. All measured cytokines showed different responses and kinetics to the chosen polyclonal stimuli. When comparing our data with the study of Poulsen *et al.*, the IL-4, IL-5 and IFN-γ levels produced by unstimulated PBMC at day 1 are similar [11]. However, after stimulation with PMA/Ca-I, our PBMC produced higher amounts of IL-4 and IL-5, but less IFN-γ, IL-12 and TNF-α than observed in literature [46]. Similar results were found for αCD3/αCD28 and ConA, which in our study are equal or in higher amounts produced than found in literature. More specific, IL-4 and IL-5 levels in our study were equal to literature, whereas our PBMC produced more IL-12p70 and IFN-γ after ConA stimulation, and more TNF-α and IL-10 after ConA or αCD3/αCD28 stimulation compared to literature [11, 20, 46]. Interestingly, our results are most comparable with the study of Poulsen *et al.*, which also uses Yssel medium for their PBMC cultures. All other studies showed lower cytokine levels [11, 20, 46, 52]. Therefore, also the culture medium might influence the activation capacity. Laan *et al.* showed that proliferation was optimal when using RPMI as culture medium, whereas PBMC cultured in Yssel medium showed better cytokine responses [53]. Yssel medium was first used as a serum-free medium based on IMDM that induced proliferative responses in mixed lymphocyte cultures that are comparable to those obtained in medium containing serum [38]. IMDM contains many energy sources and in combination with the glucose in the human AB serum and the sodium pyruvate in IMDM, the cultured cells are provided with a much larger energy source than RPMI-1640 medium alone.

Besides the influence of the polyclonal stimuli, the cryopreservation procedure can alter the maximal protein production in two ways: by a small delay in the activation of cytokine production, or by lowering the maximal protein production level. In our study, the cytokine levels of PMA/Ca-I stimulated PBMC were negatively influenced by cryopreservation. Alterations of the cytokine levels after cryopreservation have also been shown by other groups [28-30, 54]. However, cryopreserved PBMC were equally capable of proliferation as seen in fresh PBMC cultures. This is also observed in cultures with other stimulation conditions [43]. Although the cryopreservation procedure did not result in alterations of the maximal activation activity, there was a small

delay in the activation of our cryopreserved PBMC. Therefore, we believe that it is important to know at which time points the desired cellular analyses should be carried out.

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CHAPTER

5

Thermal stability in relation to allergenicity of natural and recombinant Bet v 1, Api g 1 and Dau c 1

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Abstract

The major birch pollen allergen is the PR-10 allergen Bet v 1. Bet v 1-related food allergens Api g 1 and Dau c 1 are present in celery and carrot, respectively. These allergens are generally unstable to thermal processing, as observed for whole foods and recombinant isoforms, but little is known about allergen isoforms purified from their natural source. Recombinant variants of these three allergens are increasingly used for diagnostic applications, notwithstanding their possible changed physico-chemical and immunomodulatory properties. Natural allergens, unlike recombinant preparations, usually are mixtures of isoforms. Therefore, recombinant and purified natural Bet v 1, Api g 1 and Dau c 1 were subjected to heat treatment to correlate structural changes as measured by circular dichroism with immune reactivity determined by lymphocyte proliferation and cytokine production in PBMC cultures and ELISA. All allergens refolded after heating to 95 °C, but changes in secondary structure were observed after cooling the allergen samples, especially for the natural Bet v 1 preparation. Although Api g 1 and Bet v 1 showed a similar midpoint of thermal denaturation, *Tm*, natural and in particular recombinant Dau c 1 were less stable to heating. Prolonged heating for 2 hours at 100 °C destroyed allergenicity for all allergens, whereas heating for 30 minutes at 95 °C resulted in only minor effects. Although this allergenic potential could not be measured in PBMC cultures, it was apparent from differential activity in ELISA assays.

Introduction

In Northern European countries, birch pollen allergy is the primary cause for cross-reactive allergies towards other trees (Betulaceae), fruits (Rosaceae), vegetables (Apiacaea) and legumes (Fabaceae). The major allergen in birch pollen is Bet v 1, which belongs to the PR-10 class of pathogenesis-related proteins [1-3]. Members of this protein family in foods show a high amino acid identity with Bet v 1 and are also similarly folded as shown with X-ray diffraction and NMR studies [4-7]. The PR-10 protein structure consists of a seven-stranded anti-parallel β-sheet folded around a C-terminal α-helix. The β-sheet is separated from the C-terminal α-helix by two small α-helices. A hydrophobic cavity in the Bet v 1 structure has the ability to bind a broad range of ligands such as fatty acids, cytokinins, flavonoids and sterols. This phenomenon may be of importance for the biological function [4, 8-10].

To evoke an allergic reaction, the allergen needs to pass epithelial barriers either as intact protein or epitope-bearing peptides, and subsequently needs to be taken up by an antigen presenting cell (dendritic cell or monocyte) that processes the allergen into (smaller) peptides. These peptides, containing T cell epitopes, are presented within the major histocompatibility class II (MHC-II) complex to be recognized by the T cell receptor on allergen-specific naïve $CD4^+$ T cells. This recognition leads to differentiation of T helper 2 cells (Th2). Under the influence of the cytokines produced by Th2 cells (e.g. IL-4 and IL-13) the allergen-specific B cells will induce antibody isotype switching leading to the production of IgE antibodies that can bind to type I Fcε receptors on mast cells. These events together are called the sensitization phase. A second exposure to the same allergen will lead to the cross-linking of the IgE antibodies on the surface of the mast cell, resulting in the subsequent degranulation of the mast cell releasing inflammatory compounds like histamine, which cause the typical allergic complaints of e.g. hay fever or food allergy [11].

The major symptom of IgE-mediated allergic reactions to Bet v 1 is a rapid and local inflammatory reaction in the upper and lower respiratory organs. Birch pollen-related food allergy is characterized by local reactions in and around the oral cavity (oral allergy syndrome) [12, 13]. In contrast to many other food allergens in which the IgE-reactivity is preserved after some physical-chemical treatment, Bet v 1 allergens have been characterized as structurally labile as they are unstable to heating, denaturation and proteolysis [14-19]. Upon heating, the PR-10 allergen changes conformation and shows decreased IgE binding capacity after it has refolded upon cooling down [20]. The effect of the matrix on protein thermal stability may also play an important role. It was shown that heat treatment of Pru av 1, the cherry PR-10 allergen in the presence of reduced sugars leads to a reduced IgE-binding capacity due to Maillard reactions [16]. IgE-binding to Pru av 1 and

Mal d 1 from apple was also reduced by enzymatic browning by polyphenol oxidase and peroxidase activities [16, 21]. After purification, PR-10 allergens appear much more stable [16].

For clinical and diagnostic applications, purified recombinant allergens are increasingly used. These proteins have been thoroughly characterized, although it is unknown whether these proteins have the same conformation as their natural counterparts. The recombinant Bet v 1.0101 and Bet v 1.0401 or Bet v 1.1001 isoforms display differences in immune reactivity. Bet v 1.0101 is the most commonly used allergen in diagnostic applications, because it strongly binds to specific IgE. The other isoforms Bet v 1.0401 or Bet v 1.1001 differ in 7 or 9 amino acids from Bet v 1.0101, respectively, and have been characterized as hypoallergenic making them useful as allergy vaccines [22, 23]. Hitherto, it remains unclear why these isoforms have a decreased IgE binding capacity and whether this is due to structural differences in protein folding of the recombinant proteins. Differences in circular dichroism (CD) spectra have been observed between the hypoallergenic and hyperallergenic variant, whereas X-ray diffraction showed no clear difference between the crystal structure of the different variants [8, 23]. In contrast to the recombinant PR-10 proteins, purification of the natural allergens Bet v 1, Api g 1 and Dau c 1 from birch, celery and carrot, respectively, resulted in a mixture of isoforms [24].

In the present study, the heat-stability of recombinant PR-10 allergens Bet v 1 from birch, Api g 1 from celery and Dau c 1 from carrot was compared with the heat-stability of the same allergens after purification from their natural source. The aim was to study possible molecular differences of PR-10 proteins by linking differences in thermal stability to immune reactivity. By assessing both T cell responses and allergenicity (IgE binding), we were able to show that our natural and recombinant PR-10 proteins had comparable secondary structure and IgE binding capacity, whereas neither were capable of inducing a T cell response. However, heat treatment affected the natural and recombinant PR-10 proteins in different manners.

Materials and Methods

Allergens

From each of the natural allergens nBet v 1, nApi g 1 and nDau c 1, isoform mixtures were purified according to Bollen *et al.* [24]. Briefly, ammonium sulphate precipitation was followed by hydrophobic interaction and size exclusion chromatography. The single recombinant allergen isoforms rBet v 1a, rApi g 1 and rDau c 1.2 were purchased from Biomay (Vienna, Austria). All allergens were dissolved in 10 mM potassium phosphate buffer, pH 7.0, buffer exchanged and concentrated on a Microsep 3K centrifugal device (Pall Life Sciences, Ann Arbor, MI, USA). Protein concentrations were determined using the MicroBCA™ Protein Assay (Pierce, Rockford, IL, USA) with BSA as a standard.

Prior to the human peripheral blood mononuclear cell (PBMC) cultures and ELISA, five different allergen solutions were prepared at 0.1 mg/ml in potassium phosphate buffer, pH 7.0. One of the samples was kept at room temperature and three other samples were heated in a GeneAmp PCR 9700 apparatus at 1 °C/min, which was comparable to heating in the CD spectropolarimeter. The fifth sample was heated in the GeneAmp apparatus for 2 hours at 100 °C to destroy all IgE binding capacity. The different heat treatments have been described in Table I. Explanation of the given sample labels (Untreated, TG, MG, MQ, and HL) are given in the legend of Table I. All samples were allowed to equilibrate at room temperature for at least 1 hour before continuing the experiment.

Table I. Heat treatment of all allergen samples prior to immune experiments

Sample	Heating rate (°C/min)	Final Temp.	Time at final Temp.	Cooling rate (°C/min)
Untreated	-	24 °C	-	-
TG*	1.0	*Tm*	30 min	1.0
MG†	1.0	95 °C	30 min	1.0
MQ‡	1.0	95 °C	30 min	Quick
HL§	Quick	100 °C	120 min	Quick

*TG: Heated to Tm, Gradually cooled. Tm was determined as described in section 3.1
†MG: Heated to Maximum temperature reached with CD, Gradual cooling
‡MQ: Heated to Maximum temperature reached with CD, Quick cooling
§HL: Heated at High temperature for a Long time period

Circular Dichroism (CD)

CD spectra of natural and recombinant Bet v 1, Dau c 1 and Api g 1 were recorded at 20 °C on a Jasco J-715 spectropolarimeter (Jasco Corporation, Tokyo, Japan) at a protein concentration of 10 μM in 10 mM potassium phosphate buffer of pH 7.0. Prior to use, the buffer was passed through a 0.2 μm syringe filter (Schleicher & Schuell, Dassel, Germany). Far UV spectra were recorded from 190-260 nm with a quartz cuvette of 1 mm path length, by accumulating 20 scans at a scanning speed of 50 nm/min, using a 0.2 nm step width and 2.0 nm bandwidth. Spectra were corrected for buffer background. The mean residue weight ellipticity $[\theta]_{MRW}$ (units in deg·cm^{2}·dmol^{-1}) was calculated from the following equation:

$$[\theta]_{MRW} = \frac{100 \times \theta_{obs}}{C \times l \times n} \qquad \text{Equation 1}$$

Here, θ_{obs} is the observed signal in degrees, *C* is the concentration in mol/L, *l* is the path length of the cuvette in cm and *n* is the number of amino acids. The program CDNN was used to deconvolute the secondary structure [25].

Thermal denaturation spectra were recorded for all allergens by heating to 95 °C and cooling to room temperature at a rate of 1 °C/min at 222 nm with a bandwidth of 1.0 nm. After cooling, full spectra were measured from 195-260 nm under the same conditions as stated in the previous paragraph. The thermal denaturation curve data were fitted for both the heating and cooling steps, according to a non-linear least square fit method [26] using the program TableCurve (Jandel Scientific, Erkrath, Germany). With this method, six parameters can be estimated corresponding to equation 2, which includes the slopes and intercepts of the baselines of the folded/native (β_N) and unfolded/denatured (β_D) states with the ellipticity values (intercepts) for the folded (α_N) and unfolded (α_D) state. The other derived parameters are the temperature at the midpoint of denaturation, *Tm*, and the enthalpy of unfolding at *Tm*, ΔH_{Tm}.

$$\theta = \frac{\alpha_N + \beta_N T + (\alpha_D + \beta_D T) e^{-\left[\Delta H_{Tm}\left(1-\frac{T}{Tm}\right)\right]/RT}}{1 + e^{-\left[\Delta H_{Tm}\left(1-\frac{T}{Tm}\right)\right]/RT}} \qquad \text{Equation 2}$$

Blood donors for human allergen specific PBMC culture
Blood withdrawal was performed at the Hospital Gelderse Vallei (Ede, The Netherlands). Birch pollen-specific IgE was determined for all three donors. Their sera contained 66.5, 58.5 and 19.7 kU/L Bet v 1-specific IgE, whereas the specific IgE levels were < 0.1 kU/L for Bet v 2, Bet v 4 and Bet v 6. An informed consent was obtained before sample collection and experiments were approved by the local ethical committee.

Isolation of human PBMC
From each individual, 30 ml of blood was collected in EDTA coated Vacutainers (BD Biosciences, San Diego, USA), diluted 1:1 with IMDM containing GlutaMAX (IMDM, Gibco-BRL, Paisley, Scotland) and then centrifuged on a Ficoll-Paque PLUS gradient (Amersham Biosciences, Uppsala, Sweden). The PBMC layer was washed twice with IMDM. Cell viability and concentration were determined by the trypan blue exclusion test (Sigma-Aldrich, St.Louis, MO, USA).

Culture conditions
Human PBMC were cultured at a density of 10^6 viable cells/ml in Yssel's medium (27), supplemented with 1% Penicillin-Streptomycin (Gibco BRL) and 1% human AB serum (Gibco BRL), at 37 ºC in a humidified atmosphere with 5% CO_2 as described in more detail by Jeurink *et al.* [28]. Cells were plated out in 48-well plates at a concentration of 10^6 cells/ml and cultured at 37 ºC. After five hours of adaptation, cultures were stimulated with either 150 ng/ml anti-CD3 (BD Pharmingen) plus 100 ng/ml anti-CD28 (BD Pharmingen), 10 µg/ml untreated Bet v 1, 10 µg/ml heat-treated Bet v 1 or cultured in medium only.

Cell viability
Half a million human PBMC were washed with PBS/0.25% BSA and subsequently incubated with 2 µl Annexin V-APC (BD biosciences, San Diego, USA) in 200 µl Annexin V buffer according to the manufacturer's protocol. After an incubation period of 15 min on ice, cells were centrifuged (10 min 400 g), resuspended in 200 µl Annexin V buffer plus 2 µl Propidium Iodide (PI) (1 mg/ml, Sigma) and analyzed on a flowcytometer (FACSArray, BD, San Diego, USA) In this study, cell viability is defined as cells that can be individually detected by flowcytometric analysis and reflects the number of cells that are negative for both staining with Annexin V and PI. Moreover, these cells are able to be activated resulting in proliferation as detected by individual staining with Ki-67 antibodies.

Immunological phenotype
The immunological phenotype of the human PBMC subsets was determined by exposing the surface antigens to either of the following two monoclonal antibody mixtures: 1) anti-hCD3 (PE-Cy7), anti-hCD4 (PE), anti-hCD8 (APC) and anti-hCD25 (APC-Cy7), or 2) anti-hCD3 (PE-Cy7), anti-hCD14 (APC), anti-hCD16 (PE), anti-hCD19 (APC-Cy7) and anti-hCD56 (PE). All antibodies were purchased from BD Biosciences, San Diego, USA.

Per well, $5 \cdot 10^5$ cells were centrifuged in a 96-wells U-bottom plate. The cells were incubated with staining buffer (1% FCS and 0.1 M NaN_3 in PBS), containing the surface markers or the matching isotype controls, for 30 min on ice in the dark. The cells were washed once with PBS and resuspended in PBS for flowcytometry. The four-color flowcytometric acquisition was performed on a FACSArray (BD, San Diego, USA), using the BD FACSArray software. An electronic gate was set to exclude debris and at least 10,000 events/sample were acquired. The percentage positive cells was corrected for the isotype control.

Proliferation capacity
Proliferation capacity of the human PBMC was studied by intracellular expression of the nuclear Ki-67 antigen (Ki-67, BD Pharmingen, San Diego, USA). The Ki-67 antigen is absent in the nuclei of resting cells, but present in all other phases of the cell division cycle as well as in the mitosis phase [29]. In each well, $5 \cdot 10^5$ PBMC were incubated with 100 µl cytofix/cytoperm (BD Pharmingen) for 15-20 min on ice to fix and permeabilize the cells. Cells were washed twice with perm/wash buffer (BD Pharmingen) and incubated with anti-Ki-67 PE antibody, or the matched isotype control, diluted in perm/wash buffer for 30 min on ice in the dark. Hereafter, the cells were washed with perm/wash buffer, resuspended in PBS and measured on the flowcytometer. The percentage of cells positive for the Ki-67 mAb were corrected for the isotype control, and thereafter the absolute number was calculated by the use of flowcytometric counting beads (BD Pharmingen).

Cytokines
Human PBMC culture supernatants were analyzed for their IL-1β, IL-12, IFN-γ, TNF-α, IL-4, IL-5, IL-10 and IL-13 content. The cytokine production was measured with Cytometric Bead Array Flex sets (BD Pharmingen, San Diego, USA), according to the manufacturers' protocol. All buffers used in this protocol were obtained from the BD CBA Soluble Protein Master Buffer Kit (BD Pharmingen). Supernatants were collected, stored at -20 ºC and tested within 2 weeks. The samples were measured on the FACSArray, using the FCAP software (BD Biosciences). Detection limits for quantitative determinations, according to the manufacturer, were 1.1 pg/ml for IL-1β,

0.3 pg/ml for IL-4 and IFN-γ, 0.5 pg/ml for IL-5, 2.3 pg/ml for IL-10, 2.2 pg/ml for IL-12 , 0.6 pg/ml for IL-13 and 0.7 pg/ml for TNF-α.

Patient sera for ELISA

Sera were obtained from the Hospital Gelderse Vallei (Ede, The Netherlands) and the Laboratory for Primary Health Care (SHO, Velp, The Netherlands). The sera of birch pollen allergic individuals with IgE levels >100 kU/L for birch were initially screened for their response to Bet v 1 and cross-reactivity to Api g 1 and Dau c 1. Based on these results, three serum pools containing 14 sera for the Bet v 1 serum pool, 14 sera for the Api g 1 serum pool and 7 sera for the Dau c 1 serum pool were prepared. Serum of two patients within the Dau c 1 serum pool contained carrot-specific IgE levels of 4.59 and 13 kU/L. As a negative control, a healthy serum pool of three volunteers was included with birch pollen-specific IgE levels of <0.35 kU/L.

Indirect ELISA

In an indirect ELISA, 300 ng of the heat-treated or untreated allergens in 100 µl PBS pH 7.4 were coated on 96-well MaxiSorp microplates (Nunc, Wiesbaden, Germany) by an overnight incubation at 4 °C. For the indirect ELISA, a coat with 300 ng untreated rBet v 1 was included as a positive control. The negative control, a healthy control serum pool, and blanks for non-specific binding of the secondary antibody were included for all sample treatments. All values obtained in the indirect ELISA experiments were corrected for the blank values and were expressed as percentage of the positive control (untreated rBet v 1).

The subsequent procedure was carried out at room temperature by incubation on an orbital shaker. Plates were blocked with 200 µl/well blocking buffer (4% (w/v) BSA in PBS, pH 7.4) for 1 h. After each incubation step, plates were washed 4 times with 400 µl/well washing buffer (0.05% (w/v) BSA and 0.05% Tween-20 in PBS). The 1:1 serum dilutions in PBS containing 0.1% BSA were added in duplicate at 100 µl/well and incubated for 1.5 h. After washing, 100 µl/well of an 1 µg/ml biotin-labeled mouse anti-human IgE (BD Biosciences) was incubated for 1 h, followed by a 30 min incubation of 100 µl/well streptavidin poly-HRP (1:10,000, Sanquin, Amsterdam, The Netherlands). The enzymatic color development started by the addition of 100 µl/well TMB substrate solution (KPL, Gaithersburg, MD, USA) and the reaction was stopped by adding 100 µl/well 1 M H3PO4. The developed color was measured with a microplate spectrophotometer at 450 nm using 690 nm as a reference wavelength.

Inhibition ELISA

Untreated natural or recombinant allergens of rBet v 1a, nBet v 1, rApi g 1.0101 and nApi g 1 were coated on the plate by an overnight incubation of 300 ng/well allergen solution in PBS, pH 7.4 at 4 ºC. Untreated and heat-treated samples of natural and recombinant Bet v 1 and Api g 1 were pre-incubated overnight at 4 ºC with the appropriate serum pools as mentioned in the section patient sera. This pre-incubation required a 1:1 dilution of the serum pool with the allergen solution in PBS containing 0.1% (w/v) BSA, at a final allergen concentration of 0.3, 0.06, 0.012 or 0.0024 μg/ml for rBet v 1 and nBet v 1, complemented with 3 and 30 μg/ml for rApi g 1 and nApi g 1. The allergen pre-incubation concentration, determined in pilot experiments, covered an inhibition range from approximately 100 to 0%.

In a cross-reactive inhibition ELISA, untreated rBet v 1 was coated to the plates at 300 ng/well as mentioned above. The serum pools responding to Api g 1 and Dau c 1 were pre-incubated with heat-treated or untreated rApi g 1 or nApi g 1 and rDau c 1 or nDau c 1, respectively, at a concentration of 30, 3, 0.3, 0.06, 0.012 or 0.0024 μg/ml.

For both inhibition experiments, the following controls were included without serum pre-incubation: 1) Coating with the respective untreated allergen that accounted for the calculation of 0% inhibition with the positive serum pool and as negative control with the healthy control serum pool, 2) Coating with rBet v 1 with the positive serum pool as the positive control and with the healthy serum pool as negative control. An extra negative control sample was included by pre-incubating the healthy control serum with the different concentrations of the untreated allergens. For all different coatings, blanks were used to determine non-specific binding, which were extracted from all detected values. The rest of the procedure and detection of IgE binding was performed as described in the section indirect ELISA. Finally, the blank-corrected OD-values of untreated allergen coated to plate, without allergen-serum pre-incubation, were used to express the 0% inhibition values relative to the inhibition values of the untreated and heat-treated samples.

Results

Circular Dichroism (CD)

Previous research showed that purification of the natural allergens Bet v 1, Api g 1 and Dau c 1 from birch, celery and carrot, respectively, resulted in mixtures of isoforms [24]. The CD spectra of these allergen mixtures were very similar to other PR-10 spectra described in literature [18, 30, 31]. Also, the immune reactivity of these allergen mixtures was similar to their recombinant forms as determined by Western blotting and ELISA [24].

For the present study, new CD spectra were recorded for a more detailed comparison between the purified natural allergen isoform mixtures and commercially available recombinant allergens. The effect of heating on the protein conformation was determined by heating the protein to 95 °C followed by gradual cooling down to 20 °C. The effect of heat treatment on the secondary structure of all measured allergens is shown in Table II. Differences in secondary structure elements are not only observed between the three allergens from different plant origin, but also as a result of heating and cooling down. The latter effect is best described as a small but consistent decrease in the proportion of α-helix, which is accompanied not only by an increase in anti-parallel and β-turn, but also in random coil.

Table II. Secondary structure of recombinant and natural Bet v 1, Api g 1 and Dau c 1 before and after heating to 95 °C followed by cooling to 20°C.

	nBet v 1		rBet v 1		nApi g 1		rApi g 1		nDau c 1		rDau c 1	
Before/After heating	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
α-Helix	20.9	18.0	21.7	19.5	30.2	24.9	29.8	24.3	33.3	28.9	33.4	29.5
Anti-parallel	21.7	22.3	19.8	22.0	17.1	19.2	16.6	20.6	14.0	16.2	14.1	15.8
Parallel	6.8	6.1	6.8	6.5	8.0	7.2	7.7	7.3	7.8	7.4	7.7	7.3
β-Turn	15.2	17.9	14.9	16.0	12.6	14.8	13.0	14.6	12.8	14.3	13.1	14.6
Random Coil	33.7	34.4	34.6	34.2	29.8	31.8	30.2	31.3	29.5	30.9	29.2	30.4
Total Sum*	98.3%	98.6%	97.7%	98.3%	97.7%	97.9%	97.3%	98.1%	97.3%	97.6%	97.4%	97.6%

The secondary structure composition was deconvoluted from the Circular dichroism spectra between 195-260 nm with CDNN. *Total sum is the sum of all secondary structure elements and shows that the prediction is near 100% and in the same range for all allergens for direct comparison.

The CD spectra of nBet v 1 and rBet v 1 clearly showed differences between the natural and recombinant allergens before and after the heat treatment (Figure 1), while no such spectral differences were observed between rApi g 1 and nApi g 1 or r Dau c1 and nDau c 1. In general, when nBet v 1 and rBet v 1 unfold, the spectra as a whole shift to a lower wavelength, whereas a decrease in amplitude for $[\theta]_{MRW}$ is observed for the maximum at 196 nm and the minimum at 222 nm. The cooled allergens nBet v 1, nApi g 1 and nDau c 1 intersected the x-axis at a lower wavelength of an approximate difference of 2.2, 1.0 and 0.5 nm, respectively, and all three showed a decrease in the

amplitudes for $[\theta]_{MRW}$ at 196 nm and 222 nm. For the three recombinant allergens, a smaller shift of about 0.5 nm was observed.

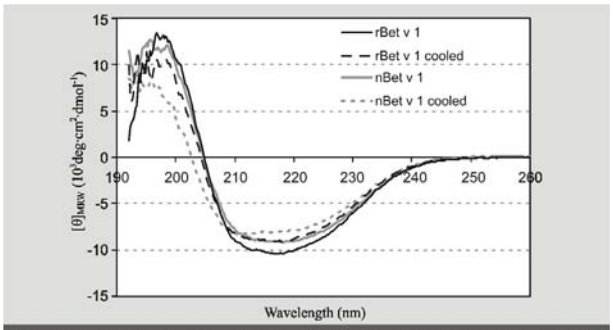

Figure 1. CD Spectra of recombinant and natural Bet v 1 before and after heating. Spectra are shown of nBet v 1 and rBet v 1 measured at 20 °C and after heating to 95 °C and cooling down to 20 °C (cooled).

Thermal denaturation was also followed by measuring changes in the CD signal at 222 nm to obtain thermal denaturation curves (Figure 2). The raw data showed typical s-shaped curves, which were fitted by using a non-linear least square fit method by assuming a two-state unfolding process. The cooling curves were corrected for the part of protein that was not able to refold due to precipitation or aggregation. From the fits of the heating data, two parameters were derived as a measure for the thermal stability, *Tm*, the temperature indicating the midpoint of thermal unfolding, and ΔH_{Tm}, the enthalpy of unfolding at *Tm* (Table III). Compared to all allergens, unfolding of rDau c 1 started at a low temperature of 30 °C (Figure 2C) with a *Tm* of 45.1 °C, whereas nDau c 1 appeared to be more stable given its *Tm* of 55.7 °C.

Table III. Thermodynamic parameters from CD thermal denaturation curves.

Heating		
Natural	***Tm*** (°C)	ΔH_{Tm} (kJ/mol)
nBet v 1	67.8 ± 0.5	204.2 ± 12.0
nApi g 1	66.5 ± 0.1	265.1 ± 7.2
nDau c 1	55.7 ± 0.1	247.0 ± 5.3
Recombinant	***Tm*** (°C)	ΔH_{Tm} (kJ/mol)
rBet v 1	63.7 ± 0.1	371.4 ± 13.0
rApi g 1	69.7 ± 0.1	368.0 ± 12.8
rDau c 1	45.1 ± 0.1	238.1 ± 6.5

Tm and ΔH_{Tm} are given with the standard deviation from the fits of Figure 2.

Recombinant and natural Api g 1 and Bet v 1 showed higher and similar *Tm*-values of approximately 65 °C. Values of ΔH_{Tm} were lower for the natural allergens compared to rBet v 1 and rApi g 1, due to a longer trajectory of unfolding, probably related to the fact that the natural allergens contained multiple isoforms each of which had a slightly different *Tm*-value. The ΔH_{Tm}-value of rDau c 1 was lower than the other recombinant allergens and was in the same range as the natural allergens.

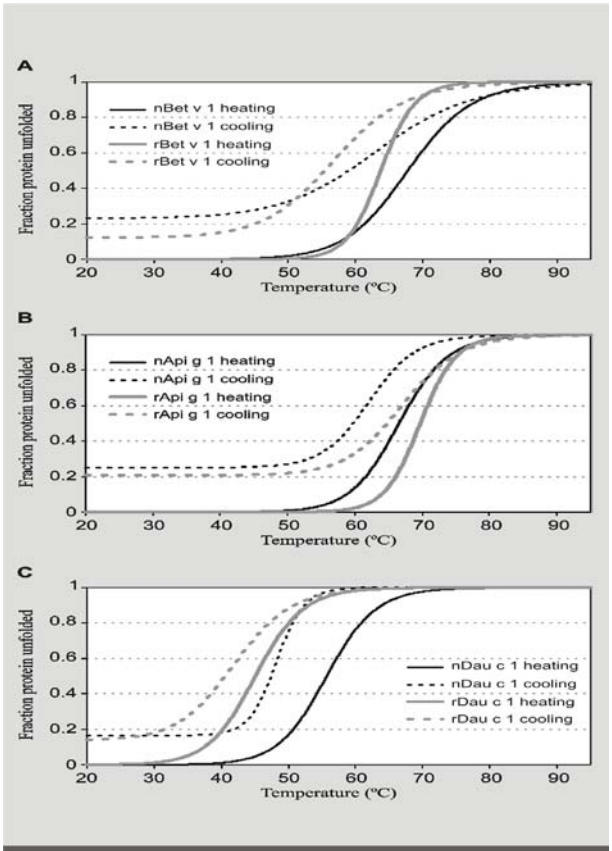

Figure 2. Thermal denaturation of natural and recombinant Bet v 1 (A), Api g 1 (B) and Dau c 1 (C) followed by CD at 222nm. The curves represent the best fits of the data to a two-state unfolding transition and are shown as the fraction of unfolded protein.

The effect of thermal treatment of rBet v 1 on the T cell response.
Heat-treated rBet v 1 was exposed to the PBMC of three birch pollen allergic individuals to this allergen. Viability of cells is a prerequisite for cell activation. The viability of the PBMC was assessed by using Annexin V in combination with propidium iodide. The viability of the Bet v 1 stimulated cells did not change compared to the unstimulated medium control, whereas the anti-CD3/anti-CD28 stimulated cells showed an induction of viable cells (data not shown).

Activation of PBMC can lead to different outcomes like proliferation, differentiation and cytokine production. To assess which of these activation parameters were affected by the addition of anti-CD3/anti-CD28 or Bet v 1, several flowcytometric analyses were performed. Isolated human PBMC, cultured without stimulus, should display a low proliferation rate. As shown in Figure 3A, the number of proliferating Ki-67$^+$ cells was low in the medium control, but strongly upregulated in the anti-CD3/anti-CD28 stimulated cells. However, this induced proliferation could not be observed in the untreated Bet v 1 stimulated control (Figure 3A) or heat-treated Bet v 1 stimulated control (data not shown).

To assess which cells were mainly proliferating within the PBMC cultures, a human PBMC subset staining was performed. The assessed subsets comprised T cells (CD3$^+$), B cells (CD19$^+$), NK cells (CD16$^+$/CD56$^+$) and monocytes (CD14$^+$). As depicted in Figure 3B, mainly the T cells, B cells and NK cells were induced in the anti-CD3/anti-CD28 stimulated human PBMC culture compared to the medium control, whereas stimulation by untreated Bet v 1 (Figure 3B) or heat-treated Bet v 1 (data not shown) showed no effect on the PBMC subsets.

T helper cells are required to obtain a proper immune response to an allergen. To assess whether these important cell types were present within the used PBMC culture, T cell subsets (cytotoxic T cells, Tc, T helper cells, Th) were determined simultaneously with their activation status. Figure 3C shows that in the medium control both Th and Tc cells were present, although very low amounts were activated. However, addition of anti-CD3/anti-CD28 to the culture resulted in increased numbers of Th and Tc cells, and also induced activation of both T cell subsets. As for PBMC subsets addition of untreated (Figure 3C) or heat-treated Bet v 1 did not alter the number or the activation status of the T cell subsets.

As mentioned before, besides proliferation and differentiation, also cytokine production is a measure for cell activation. Therefore, cytokine production of the (un-)stimulated PBMC cultures was measured by using the Flexsets (Table 4). Anti-CD3/anti-CD28 was able to induce an upregulation of the monocyte-derived IL-1β and TNF-α, whereas IL-12 was not altered compared

to the medium control. Also the Th1 cytokine IFN-γ, both Th2 cytokines IL-5 and IL-13 and the regulatory cytokine IL-10 were upregulated by anti-CD3/anti-CD28 when compared to the medium control. However, none of the measured cytokines were altered when comparing the Bet v 1 stimulated cells to the medium control, as shown in the lower part of Table IV.

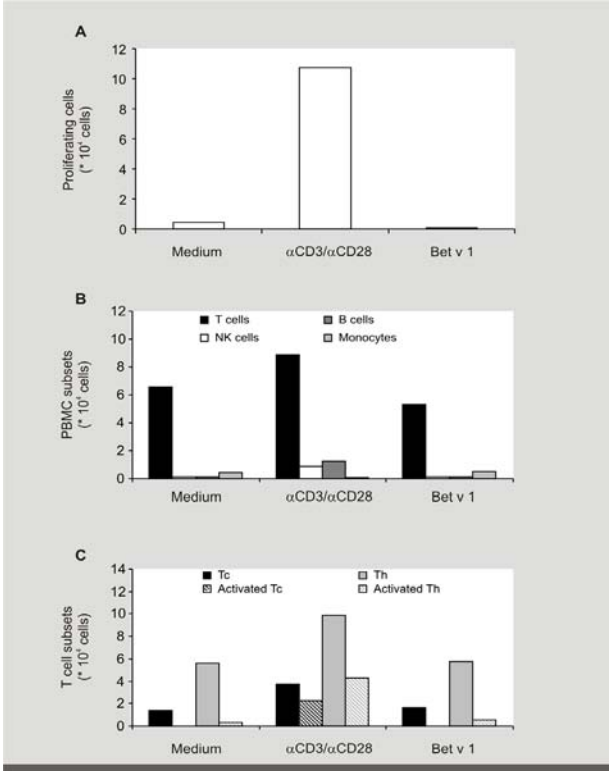

Figure 3. Human PBMC cultures stimulated with untreated Bet v 1 (Bet v 1) were cultured for 7 days. PBMC stimulated with anti-CD3 plus anti-CD28 were cultured for 3 days (αCD3/αCD28). At both time points, Ki-67 positive cells were detected and the relative number of Ki-67+ cells is depicted in Figure 3A. Figure 3B shows the PBMC surface markers to analyze $Cd3^+$ cells (T cells), $CD16^+CD56^+$ (NK cells), $CD19^+$ cells (B cells) and $CD14^+$ cells (monocytes). Figure 3C shows the PBMC surface markers to analyze $CD3^+CD4^+$ cells (Th), $CD3^+CD4^+CD25^+$ (activated Th), $CD3^+CD8^+$ cells (Tc) and $CD3^+CD8^+CD25^+$ cells (activated Tc).

Effect of heating on the IgE binding capacity.

The IgE binding capacity of heat-treated and untreated allergens was tested by means of indirect ELISA and inhibition ELISA. Allergens were heated and labeled as described in Table I. By including a sample that was heated to *Tm* (Table III), a situation was created, in which 50% of the allergen population

was unfolded. The allergens nDau c 1 and nApi g 1 did not completely refold after heating to *Tm* as assessed by the CD measurements (data not shown).

Table IV. Cytokine production by human PBMC.

Stimulation	IL-1β	IL-12	TNF-α	IFN-γ	IL-5	IL-13	IL-10
Medium	12.2	2.9	2.8	6.3	1.6	4.2	3.5
αCD3/28	819	1.3	1762	59939	14.3	287	51
Bet v1	b.d.	b.d.	b.d.	47.7	1.8	2.2	b.d.
Bet v 1 MQ	b.d.	b.d.	1.5	12.4	2.7	4.0	3.8
Bet v 1 MG	b.d.	b.d.	b.d.	17.2	3.7	4.9	4.4
Bet v 1 TG	1.5	2.3	1.6	36.2	2.6	3.9	3.1

B.d.: Below detection limit

Heat-treated allergens were coated directly onto the microtiter plates for the indirect ELISA and the results are shown relative to the positive control Bet v 1 (Figure 4). For the measurements of IgE binding to Bet v 1, Api g 1 and Dau c 1, different serum pools were used. Differences between recombinant and natural Bet v 1 were observed for the MG and MQ treatments without affecting IgE binding to nBet v 1, but decreasing IgE binding for 50% to rBet v 1. A clear decrease was observed in antibody binding capacity of the sera to the Bet v 1 cross-reactive allergens Api g 1 and Dau c 1. Dau c 1 did not show any IgE binding for either recombinant or natural Dau c 1, corresponding to the individually tested samples with a low carrot-specific IgE level. Recombinant and natural Api g 1 showed a response of 30% relative to rBet v 1 and no difference for the sample heated to *Tm*. Both treatments leading to MG and MQ samples reduced the response by 50%, whereas the HL treatment showed an equal response to the healthy control value.

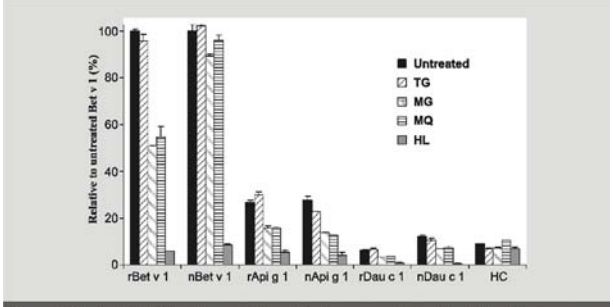

Figure 4. Indirect ELISA of thermally treated allergen coated to the plate. All values were compared relatively to the positive control by using untreated Bet v 1. A representative Healthy Control (HC) was included, which did not differ among the other used coatings. All values are means of duplicate measurements. Samples included are: Untreated; TG, heated to Tm and gradually cooled down; MG, heated to 95 °C and gradually cooled down; MQ, heated to 95 °C and quickly cooled down; HL, heated for 2 hours at 100 °C.

Differences between the inhibition of 300 ng/well rBet v 1a and nBet v 1 were observed in the inhibition ELISA, when the serum was pre-incubated with the heat-treated allergens (Figure 5A and B). Heat treatment to *Tm* or 95 °C did not alter the inhibition of rBet v 1a (Figure 5A). For nBet v 1, the MQ- and MG-treated allergen decreased the inhibition by 50% when pre-incubated with 6 ng of the treated allergen (Figure 5B).

The inhibition ELISA with Api g 1 showed similarly shaped inhibition curves for rApi g 1 and nApi g 1 (Figure 5C and D) for the untreated samples and the samples heated to *Tm*. The MG and MQ heated samples decreased IgE binding to nApi g 1 more than to rApi g 1, as shown by a larger decrease in the inhibition curve of nApi g 1 (Figure 5C). An inhibition ELISA for Dau c 1, by coating untreated Dau c 1 to the plate could not be performed, due to the low binding capacity of Dau c 1 or low concentration of specific IgE in the serum.

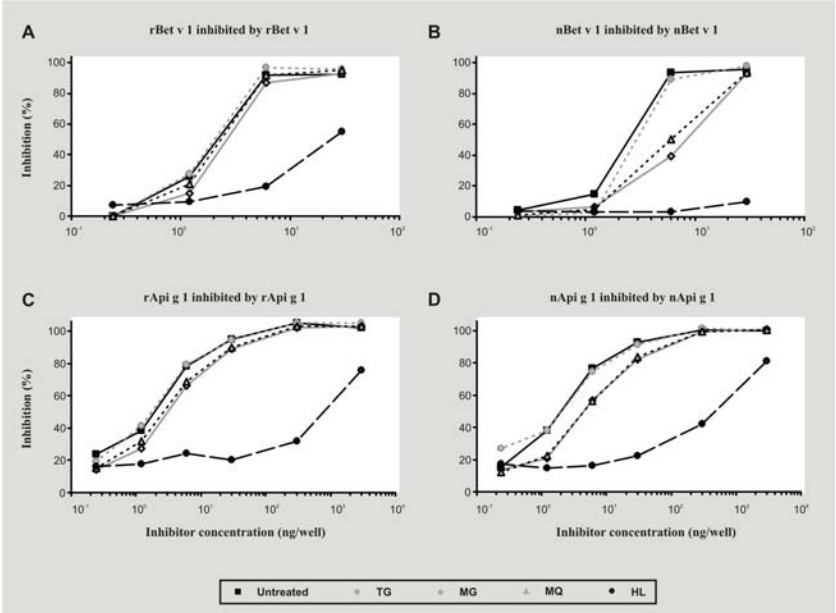

Figure 5. Inhibition ELISA with heat-treated samples of rBet v 1 (A), nBet v 1 (B), rApi g 1 (C) and nApi g 1 (D) by coating with the respective untreated allergen. All values are means of duplicate measurements. Included samples are: Untreated; TG, heated to *Tm* and gradually cooled down; MG, heated to 95 °C and gradually cooled down; MQ, heated to 95 °C and quickly cooled down; HL, heated for 2 hours at 100 °C.

A cross-reactivity inhibition ELISA was performed by coating rBet v 1 to the plate and pre-incubating the treated and untreated samples of natural and recombinant Api g 1 and Dau c 1 with serum (Figure 6). The inhibition curves of rApi g 1 showed 20% inhibition, which decreased to the 10% inhibition level of the HL treatment (Figure 6A). Natural Api g 1 showed 40% inhibition, which decreased to 20% at a lower inhibitor concentration (Figure 6B). The rDau c 1 did not cause any inhibition (Figure 6C), whereas nDau c 1 was only able to completely inhibit IgE binding by pre-incubating serum with 3000 ng of untreated nDau c 1 (Figure 6D). The curves of nDau c 1 showed that the samples heated to 95 ºC exhibited a 60% inhibition capacity, which was similar to the MQ and MG of nBet v 1 sample at a lower inhibitor concentration.

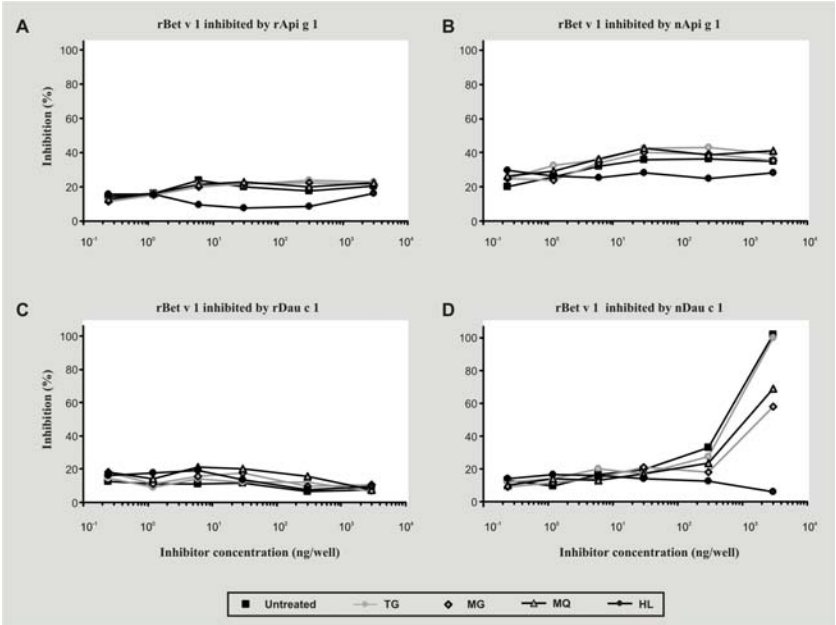

Figure 6. Cross-reactivity inhibition ELISA with rBet v 1 coated to the plate and serum pre-incubated with heat-treated samples of rApi g 1 (A), n Api g 1 (B), rDau c 1 (C) and nDau c 1 (D). All values are means of duplicate measurements. Included samples are: Untreated; TG, heated to *Tm* and gradually cooled down; MG, heated to 95 °C and gradually cooled down; MQ, heated to 95 °C and quickly cooled down; HL, heated for 2 hours at 100 °C.

Discussion

In the present study, we report small differences in thermal stability of Bet v 1 and Api g 1 for the recombinant forms, and for corresponding allergens, purified from their natural source. Striking differences were observed for recombinant and natural Dau c 1, showing a lower thermal stability than the other allergens. More profound conformational changes were observed for all the natural allergens after heating compared to the untreated samples. Particularly for the natural allergens, these conformational changes resulted in differences in allergenic potential as measured by comparing the IgE binding ability in the indirect and inhibition type ELISA.

To investigate the T cell activation by Bet v 1, human PBMC cultures from allergic individuals, with a proven birch pollen allergy, were investigated. Besides a possible increase in cytokine production, also proliferation of the PBMC would indicate that the cells were activated by the allergen-derived peptides. However, in our study, Bet v 1 was not potent enough to induce either cytokine production or T cell proliferation. This could be explained by the low frequency of allergen-specific T cells present in the PBMC isolated from blood of selected birch pollen-allergic individuals. Also literature is scarce on Bet v 1-specific stimulation of human PBMC where often T cell clones are used [17]. Due to the fact that each T cell clone resembles the response of one single Bet v 1-specific T cell, a large range of T cell clones of one person should be obtained in order to establish the allergen-specific response of one individual. Bet v 1 changes conformation when heated, but is presented to the T cell in the form of linear peptides of approximately 12 to 18 amino acids by the antigen-presenting cell eventually leading to a predicted similar T cell response as shown with T cell clones in the study of Bohle *et al.* [17]. For this reason, the PBMC cultures in this study were discontinued for the other allergens.

Besides T cell responses, B cell responses and resulting formation of IgE antibodies are important in an allergic reaction. Therefore, a number of studies were undertaken to assess the effects of heat treatment on conformational B cell epitopes, as reflected by CD and ELISA with both natural and recombinant Bet v 1, Dau c 1 and Api g 1. First, CD spectra were determined. Bohle *et al.* [17] also measured thermal denaturation curves for recombinant allergens and found *Tm*-values similar to ours. However, unlike that study, we report the ability of rDau c 1 to refold, which might be due to the nine times higher protein concentration leading to protein aggregation, or the step-scan procedure these authors used for the temperature scan [32]. The increased time requirement for measuring at higher temperatures in this step-scan procedure might have affected the refolding ability of rDau c 1. We also observed differences in conformation after cooling down with a step-scan method on nDau c 1 and nApi g 1 (data not shown). Although both proteins

were able to refold, a larger conformational change was observed compared to protein only heated and cooled down with a continuous scan.

Changes in $[\theta]_{MRW}$, as measured with CD, best represent the changes in α-helical content [33]. After heating, a conformational change was observed for all allergens resulting in the decreased α-helical content, as shown in Table 2. The structural changes were visible from the spectrum of nBet v 1 after heating (Figure 1) and also from the cooling curves which showed partial folding (Figure 2). These results did not directly imply that proteins were not able to refold completely in solution, because an undefined amount of the protein precipitated at higher temperatures and therefore no longer contributed to the CD signal. This finding was strengthened by the extrapolation of the spectra of $[\theta]_{MRW}$ at 222 nm of unheated and cooled allergens as it showed identically shaped spectra for the recombinant allergens, but less so for the natural allergens. As secondary structure predictions from CD required the accurate protein concentration (33), this suggested that conformational changes due to heating were smaller for recombinant proteins than for the natural allergens. However, the natural allergens were present as isoform mixtures and CD measurements were incapable of distinguishing between structural changes of one specific isoform within the mixture.

Recombinant allergens contained only a single isoform, whereas natural allergens were composed of a mixture of isoforms. The mixture presumably induced broadening of the transition area of the thermal denaturation curve resulting in a less steep slope and a lower ΔH_{Tm}. By mass spectrometric analyses, Schenk *et al.* [34] showed that the isoforms Bet v 1m, Bet v 1a and Bet v 1d were predominantly present in the natural sample of *Betula pendula* '*Youngii*' in a ratio of about 30:45:25. In our study, the most striking differences between the recombinant and natural samples were observed for Bet v 1. In the indirect ELISA (Figure 4), heating to 95 °C did not affect nBet v 1, whereas the IgE binding to rBet v 1 was reduced by 50%. This can be explained by the isoform mixture of nBet v 1 containing a particular isoform that is more stable to heating, which is supported by the higher *Tm*-value and the decreased slope of the CD thermal denaturation curve (Figure 2A). Alternatively, the higher IgE binding affinity of a single isoform other than Bet v 1a, can become apparent after heating. Furthermore, heating to *Tm* did not affect the total IgE binding capacity. Heating for 2 hours at 100 °C increased the amount of in insoluble precipitate and abolished all IgE binding to all assessed PR-10 proteins.

The thermal stability of Dau c 1 was different from the other allergens. The *Tm*-values of recombinant and natural Bet v 1 and Api g 1 were around 65 °C, whereas 56 °C and 45 °C was determined for nDau c 1 and rDau c 1, respectively. The difference in *Tm*-values between nDau c 1 and rDau c 1

might be explained by the presence of a remaining HIS-tag attached to the N-terminus of rDau c1 which caused a decreased thermal stability of the N-terminal β-strand that is possibly responsible for the stabilization of the C-terminal loop region and α-helix through hydrogen bonding. Another possibility is the lability of the isoform Dau c 1.0103 (Accession no. CAB03715), which was the isoform that constituted the commercially available rDau c 1. The nDau c 1 mixture contained at least two isoforms, Dau c 1.0104 (Accession no.CAB03716) and a homologue of the parsley protein PcPR1-3 (Accession no.CAA31085) [24].

Furthermore, the *Tm*-value for rApi g 1 resembled the *Tm*-value of Api g 1.01, as reported by Wangorsch *et al.* [35]. Besides the thermal denaturation curves of the recombinant allergen Api g 1.01, these authors also analyzed Api g 1.02. Interestingly, the thermal denaturation curve of Api g 1.02 showed a transition around 55 °C, which resembled the value of nDau c 1. Api g 1.02 has a 63% identity with the parsley homologue PcPR1-3 found in the mixture of nDau c 1, but only a 50% identity with Dau c 1.0103. Therefore, the PcPR1-3 homologue could influence the thermal stability of the nDau c 1 sample, resulting in a lower *Tm*-value than nApi g 1 that did not contain this isoform.

For all ELISA experiments, samples were exposed to heat treatment, comparable to heating performed in the CD measurements, to link the results of conformational changes directly to IgE binding capacity. However, binding of an allergen to a polystyrene microtiter plate might induce a conformational change [36], which subsequently affects the binding capacity to the plate as well as the IgE binding capacity to treated and non-treated immobilized allergens. The inhibition results of recombinant and natural Bet v 1 (Figure 5A and B) were different from the results observed in the indirect ELISA. For the indirect ELISA, rBet v 1 showed a 50% decrease in IgE binding capacity for the treated samples, but for the inhibition ELISA no decrease was observed. However, natural Bet v 1 showed the opposite effect. Again, this difference suggests differential IgE binding potentials and thermal stability by the presence of multiple isoforms. Heat treatment of this natural isoform mixture can result in an altered IgE binding potential, which was visible in the inhibition ELISA, as this inhibition occurred in solution. However, in the indirect this difference could not be observed, as all isoforms are immobilized at the polystyrene surface, in a more or less altered conformational configuration. This observation can be complemented with the conformational differences between rBet v 1 and nBet v 1 (Figure 1 and 2A), since nBet v 1 showed a more permanent heat-induced structural loss than rBet v 1. This structural loss was increased by prolonged heating for the ELISA experiment and caused the reduced IgE binding capacity of nBet v 1. The higher affinity of heat-treated nBet v1 compared to rBet v 1, resulted in an unchanged signal for the indirect ELISA, whereas a decreased signal in the inhibition

ELISA was due to the higher affinity of the untreated nBet v 1 bound to the polystyrene plate.

No effects were observed for the cross-reactivity inhibition ELISAs with rBet v 1 coated on the polystyrene plate and serum pre-incubated with rApi g 1, nApi g 1 and rDau c 1. This was probably due to the low levels of allergen-specific IgE for Dau c 1 and Api g 1. Only nDau c 1 was able to completely inhibit IgE binding to rBet v 1 at a high concentration. In contrast to the Dau c 1 response in this study, our previous ELISA experiments showed similar reactivity of Dau c 1 and Api g 1 [24]. However, our current study used a phosphate containing coating buffer of pH 7.4 instead of a carbonate buffer of pH 9.6, which possibly induced conformational changes of the protein [37]. To overcome the lack of Dau c 1 plate-binding, a cross-reactive inhibition ELISA was performed by binding untreated rBet v 1 to the polystyrene plate which enabled comparison of the natural isoform mixtures with the recombinant single isoforms. The low thermal stability of Dau c 1 was reflected in the ELISA experiments as both natural and recombinant Dau c 1 did not show IgE binding in an indirect or inhibition ELISA. In the cross-reactive inhibition ELISA, natural, but not recombinant Dau c 1, was able to inhibit rBet v 1 in solution, which emphasizes the difference between natural and recombinant Dau c 1.

The inhibition curve of rBet v 1 in this study was similar to the one published by Wagner *et al.* [23] who performed cross-reactivity ELISAs with hypoallergenic variants of Bet v 1 in which Bet v 1a (1.0101) was the strongest inhibitor. The other allergens, rBet v 1d (1.0401) and rBet v 1l (1.1001), were still able to inhibit the IgE binding to rBet v1a, although a higher inhibitor concentration was needed. For these inhibition experiments, Wagner *et al.* used patient sera with a very low response to the hypoallergenic variant coated to the plate. The experiments performed in the present study showed even less reactivity of Api g 1 and Dau c 1 to IgE. It is tempting to assume that the hypoallergenic variants of Bet v 1 have a higher affinity for IgE than Dau c 1 and Api g 1. Therefore, care should be taken in adopting such recombinant isoforms as hypoallergenic proteins.

Moneo *et al.* described sensitization of 4 patients for carrot, who produced IgE-type antibodies that were able to recognize Dau c 1, but not Bet v 1 [38]. This observation is not in line with the common perception of pollen-fruit syndrome, starting with sensitization for Bet v 1 and later occurring cross-reactions to PR-10 containing vegetables and fruits, and suggests that Dau c 1 can be a primary sensitizer [38, 39]. As in our study, Dau c 1 was found to be less heat-stable than other PR-10 proteins and larger physico-chemical stability of Dau c 1 apparently cannot account for such observations. Likely, other (food) components play a role in the sensitization process.

Conclusions

Heat treatment affects the allergenic structure leading to changes in IgE binding capacity of an allergen in solution in comparison to the binding capacity of the polystyrene-bound allergen. However, no changes in the IgE binding capacity were observed for allergens heated to *Tm* and also no differences were observed for the thermal treatments MG and MQ considering the different cooling rates. In contrast, IgE binding capacity is largely destroyed by heating the protein for 2 hours at 100 °C. In addition, differences in heat-induced structural changes and allergenicity between natural and recombinant allergens are based on natural isoform mixtures and their differences in affinity of binding to specific IgE antibodies. Furthermore, allergen-specific IgE binding to free and polystyrene-bound recombinant Bet v 1 showed opposite results when compared to the natural isoform mixtures.

Both natural and recombinant Dau c 1 were found to be significantly less thermo-stable than Bet v 1- and Api g 1-forms, respectively. Therefore, future studies will be needed to elucidate the allergenicity of the isoforms present in the natural isoform mixtures, as these might resolve the discrepancy between negative IgE levels determined with recombinant proteins and these 'non-allergic' individuals that actually do display allergic symptoms.

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CHAPTER

6

Immunomodulatory capacity of fungal proteins on the cytokine production by human peripheral blood mononuclear cells

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Abstract

Immunomodulation by fungal compounds can be determined by the capacity of the compounds to influence the cytokine production by human peripheral blood mononuclear cells (hPBMC). These activities include mitogenicity, stimulation and activation of immune effector cells. Eight mushroom strains (*Agaricus blazei, Coprinus comatus, Flammulina velutipes, Ganoderma lucidum, Grifola frondosa, Volvariella volvacea, Lentinus edodes,* and *Pleurotus ostreatus*) were tested for the immunomodulating activity of the isolated protein fractions and polysaccharides fractions present in mycelia and culture liquid. The fungal proteins and polysaccharides have been investigated for their *in vitro* effect on the cytokine profile (IFN-γ, IL-4, IL-10, IL-12 and TNF-α) of unstimulated or hPBMC stimulated with the polyclonal stimulations PMA/Ca-I, ConA or LPS. In addition to their influence on the cytokine profile, the hemagglutination activity of the fungal proteins on rabbit red blood cells was determined. Proteins from *V. volvacea* and *G. lucidum* showed immunomodulating activity without the presence of any mitogen, however, neither of them decreased the production of IL-4 and IFN-γ in combination with a stimulus. All used stimuli resulted in a induction of IL-12 in the presence of the protein extracts, suggesting a direct effect on monocytes. This effect might lead to the indirect immunomodulation of T cell activation and cytokine production. In addition, both protein extracts showed more hemagglutination activity after trypsin treatment of the rabbit red blood cells, indicating the presence of carbohydrate binding proteins, like lectins and FIPs. In conclusion, the protein extracts of *V. volvacea* and *G. lucidum* contain immunomodulating activity by acting directly on monocytes and thereby modulating T cell activation. Further purification of the fungal extracts is needed to clarify whether there are FIPs or lectins present that are responsible for this immunomodulating activity.

Introduction

For centuries, mushrooms have been abundant sources of bioactive compounds for treatment of various diseases [1-5]. Mushrooms display in vivo and *in vitro* immunomodulatory activity, in particular since fungal compounds exhibit anti-tumour activity, based on modulation of the immune system. Immunomodulatory activity is demonstrated for crude fungal extracts and isolated compounds like polysaccharides, polysaccharopeptides, polysaccharide-proteins and proteins from the fruiting body, spores, mycelia, and culture medium of various mushrooms (Reviewed in [6]). The major immunomodulating activity of these bioactive compounds include their mitogenicity, potential stimulation of hematopoietic stem cells and activation capacity of immune effector cells like hPBMC [6-9].

hPBMC represent a heterogeneous population of immune cells including B cells, T cells, monocytes, NK cells and various granulocytes [10]. In a close interplay, these cells orchestrate innate and acquired immune responses that might either be enhanced or reduced by addition of mushroom compounds to these hPBMC cultures. When addition of fungal compounds results in altered differentiation or functional responses of hPBMC cultures *in vitro*, this would signify the ability of these fungal compounds to induce systemic immune responses that modulate disease resistance.

One approach to evaluate immunomodulating activity is to determine the capacity of fungal compounds to influence the cytokine production by hPBMC. Cytokines are soluble glycoproteins that are crucial in the induction and regulation of immune responses. Changes in cytokine levels can result in various pathological conditions and cause disturbances in the cytokine-mediated interplay between innate and acquired immune responses [11]. Although unstimulated hPBMC do not produce much cytokines *in vitro*, various stimuli cause preferential stimulation of T cells or monocytes in hPBMC. Phorbol Myristate Acetate plus calcium ionophore (PMA/Ca-I), and Concanavalin A (ConA) are widely used to activate T cells, however, both result in a different effector action. Lectins, carbohydrate-binding (glycol-) proteins which potentially link to cell surface glycoproteins, such as ConA, are T cell activators by binding to particular sugar residues on the TCR and CD3 proteins in the absence of antigen-presenting cells [12]. In contrast, PMA mimics diacylglycerol and activates protein kinase C and thus eventually T cells [13,14]. Lipopolysaccharide (LPS) strongly activates macrophages and monocytes, and induces cytokines such as IL-12 and TNF-α both *in vivo* and *in vitro* [15,16].

The ability of fungal compounds, like fungal immunomodulatory proteins (FIPs), to alter the cytokine response has been described earlier [17]. Hsu *et al.* reported the purification of FIP-Vvo and suggested that the immuno-

modulatory effects might be due to cytokine regulation of hPBMC. In addition, the proteins were capable of agglutinating rat red blood cells and were able to stimulate proliferation [18]. FIPs are classified into a distinct family since they are a group of fungal proteins defined by amino acid sequence similarity and their actions on immunological responses [19-21]. FIPs have been isolated and purified from *G. lucidum* [19], *F. velutipes* [20], *V. volvacea* [18], *Ganoderma tsugae* [17], and *V. volvacea* [22] and are designated as LZ-8, *Fve*, *Vvo*, *Gts*, and *Vvl*, respectively. All of these studies were either performed with mice, or assessed the immunomodulating activity as effects on the proliferation of hPBMC.

The aim of this study was to assess the bioactivity of fungal proteins and polysaccharides by determining which of the yielded proteins or polysaccharides displayed immunomodulating activity. As proposed in literature, the immunomodulating activity was assessed by measuring the cytokines IFN-γ, IL-4, IL-10, IL-12 and TNF-α in the culture supernatants of freshly isolated hPBMC cultures. hPBMC were used unstimulated or were stimulated with PMA/Ca-I, ConA or LPS. Another assessment of the bioactivity of the fungal proteins was done by determining the carbohydrate-binding specificity in a hemagglutination test using rabbit red blood cells (RRBC). In addition, the difference in hemagglutination activity of untreated and trypsin treated RRBC gave an indication of which type of proteins are present in the different protein fractions.

Materials and Methods

Micro-organisms and media

Mother spawn of the mushroom strains *Agaricus blazei* M7700, *Coprinus comatus* M8102, *Flammulina velutipes* M4600, *Flammulina velutipes* M4622, *Ganoderma lucidum* M9720, *Grifola frondosa* M9821, and *Volvariella volvacea* M6100 were commercially obtained from Mycelia (SacO2, Combiness, Belgium). *Lentinus edodes* S-9-2 was commercially obtained from Fungisem S.A. (Autol, Spain) and *Pleurotus ostreatus* 2222 was a kind gift of the Instituto Nacional de Engenharia Tecnologia e Inovação (INETI, Lisboa, Portugal). All stock cultures were maintained on a potato dextrose agar (PDA) slant and subcultured every 1.5 months. Slants were incubated at 25 ºC for 7-15 days, depending on the strain. Slants of *V. volvacea* and *A. blazei* were stored at room temperature (RT), all other slants at 4 ºC.

Culture liquid

A. blazei, *C. comatus*, *F. velutipes*, *G. lucidum*, *G. frondosa*, *V. volvacea*, *L. edodes* and *P. ostreatus* were grown on PDA medium in a Petri dish, and subsequently transferred to the culture media. Table I shows the media on which the mushrooms were grown, the days of growth, the yield of the dry mycelia mass and the proteins yield from these mycelia. The culture was grown at 25 °C in three litres of medium in Erlenmeyer flasks until the mycelium had covered the liquid surface. The potato malt peptone (PMP) medium contained 10 g/l malt extract, 1 g/l peptone and 24 g/l potato dextrose broth. The mushroom complete medium (MCM) contained 20 g/l glucose, 0.46 g/l KH_2PO_4, 1 g/l K_2HPO_4, 0.5 g/l $MgSO_4.7H_2O$, 2 g/l peptone and 2 g/l yeast extract. The yeast malt extract medium (YM) consisted of 10 g/l glucose, 3 g/l malt extract, 5 g/l peptone and 3 g/l yeast extract.

Table I. Culture medium, days of growth, yield of studied mushroom mycelia and extracted proteins.

Mushroom	Medium	Days of growth	Dry mycelia (g/l)	Protein yield (mg/g dry mycelia)
Agaricus blazei	PMP	109	2.78	2.1
Coprinus comatus	MCM	18	1.18	2.4
Flammulina velutipes 4600	MCM	30	1.80	0.2
Flammulina velutipes 4622	MCM	15	3.89	0.9
Ganoderma lucidum	MCM	23	1.70	1.4
Grifola frondosa	PMP	17	0.95	2.1
Lentinus edodes	YM	81	3.05	0.6
Volvariella volvacea	MCM	22	1.27	6.4

PMP: potato malt peptone
MCM: mushroom complete medium
YM: yeast malt extract medium

Isolation of intracellular polysaccharides
After cultivation, the mycelia were separated from the culture broth by vacuum filtration. The mycelia were washed three times with distilled water to remove contaminating extracellular polysaccharides. The mycelia were ground in a mortar with liquid nitrogen, lyophilized, and subsequently stored at -20 °C. Intracellular compounds were extracted from the lyophilized mycelia with distilled water of 120 °C (1 g/100 ml) for 20 min and cooled down to 4 °C. Polysaccharides were precipitated by adding two volumes of cold ethanol, vigorous stirring, and allowing polysaccharide precipitation overnight at 4 °C. The precipitated polysaccharides were collected by centrifugation (10,000*g* for 20 min at 4 °C), redissolved in distilled water and the whole precipitation procedure was repeated once. The precipitated polysaccharides were dialyzed with Spectra/Por3 molecular-porous membrane tubing (MWCO:3500; Spectrum Medical Industries Inc.) against distilled water to remove small compounds during at least 24 hours with three or four changes of the distilled water. After the dialysis, the polysaccharides were lyophilized and the sample weight estimated. The lyophilized polysaccharides were stored at -20 °C until further use.

Before use in the cell culture, the polysaccharides were redissolved in phosphate buffered saline (PBS: 136 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4, pH 7.2) at a concentration of 1 mg/ml and sterilized by filtration through a non-binding Millex-GV 0.22 μm membrane filter (Millipore, Carrigwohill, Ireland).

Isolation of extracellular polysaccharides
Filtered cultivation medium was concentrated by evaporation at 75 °C in a Rotavapor system or by freeze-drying. The secreted extracellular polysaccharides were precipitated from the concentrate or the water dissolved powder in the same way as described above for the intracellular polysaccharides.

Isolation of fungal proteins
All steps were carried out at 4 °C. Lyophilized mycelia were homogenized (Waring blender) and extracted with ice-cold 5% (v/v) acetic acid in the presence of 0.1% (v/v) 2-mercaptoethanol (1 g/30 ml) for 3 hr. The homogenates were centrifuged at 10,000*g* for 20 min and, subsequently, the supernatant was filtered through glass wool. Soluble proteins in the supernatant were precipitated by addition of ammonium sulphate up to 95% saturation for the acetic acid extraction. After stirring for 1 h, the precipitates were collected by centrifugation at 20,000*g* for 30 min. The protein pellets were redissolved and dialysed against distilled water for more than 40 h with at least four changes of the dialysis solution. Before the lyophilisation, the

dialysate was spun down (20,000*g* for 10 min) to remove denaturated proteins. Before use in the cell culture, the protein extracts were redissolved in 10 mM PBS at a concentration of 1 mg/ml and sterilized by filtration through a non-binding 0.22 μm membrane filter.

Determination of the total carbohydrate content of the polysaccharide extracts

The polysaccharide extracts were analyzed for their total carbohydrate content with the modified phenol-sulphuric acid method described by Fox and Robyt (1991). Twenty-five μl test solution and 25 μl of 5% (w/v) phenol were pipetted into a 96-well assay plate in triplicate. Test samples and standards of known glucose concentration were placed in triplicate wells of each plate. The used standards were 0 (distilled water), 10, 30, 50, 70, and 90 μg/ml glucose. After loading all samples and standards, the plate was vortexed for 30 s and placed on crushed ice. Subsequently, 125 μl of concentrated H_2SO_4 was added to each well. The plate was mixed for 30 s, sealed in a plastic zipper bag and heated in a water bath at 80 °C for 30 min. The optical density was determined using a Multiskan Spectrum (Thermolab Systems, Vantaa, Finland) at 490 nm.

Determination of the protein content of the polysaccharide and protein extracts

Protein content was determined by both the standard and micro bicinchoninic acid (BCA) assay (Smith *et al.*, 1985).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Crude protein extracts were studied by performing a SDS-PAGE analysis. SDS-PAGE (15% w/v) was performed on a mini-protean II gel apparatus (Bio-Rad) according to Laemmli and Favre (1973). The gels were stained with Coomassie Brilliant Blue R-250 and the used molecular weight standard (Amersham Biosciences) contained phosphorylase b (97 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Isolation and culture of human peripheral blood mononuclear cells (hPBMC)

hPBMC from a blood donor were obtained from a buffy coat supplied by the Sanquin Blood bank of Nijmegen (The Netherlands) and isolated from sodium citrate treated blood (diluted 1:1 with Iscove's Modified Dulbecco's Medium

(IMDM) containing GlutaMAX (Gibco-BRL, Paisley, Scotland)) by density gradient centrifugation on Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden). Briefly, the hPBMC layer was washed twice with IMDM and subsequently tested for its viability (as determined by trypan blue exclusion). One million hPBMC per ml were cultured in Yssel's medium at 37 °C in a humidified atmosphere with 5% CO_2. The medium consisted of IMDM supplemented with 1% Penicillin-Streptomycin, 1% human AB serum and additions according to Yssel *et al.* [23]. Cultures were stimulated with *Escherichia coli* derived LPS (10 ng/ml; Sigma-Aldrich, Stockholm, Sweden), ConA (2.5 and 5 μg/ml; Sigma), PMA (2 ng/ml; Sigma) plus Ca-I (1 μg/ml; Sigma) or medium only (control) for 62 h. The protein or polysaccharide extracts, at a final concentration of 25 μg/ml, and the stimuli were added to the culture simultaneously. The optimal dose of 25 μg/ml extract has been determined in preliminary dose-response experiments. To rule out possible endotoxin contamination of the protein or polysaccharide extracts, 20 μg/ml polymyxin B (PMB, Sigma) was added to the cultures. PMB can bind to endotoxins, and therefore prevent the activation through the LPS pathway. As internal control, the stimulation with LPS was therefore performed with and without polymyxin B [24].

Cytokine activity determination with a colorimetric sandwich ELISA

The production of IL-4, IL-10, IL-12, IFN-γ, and TNF-α was detected with Enzyme-Linked Immunosorbent Assay (ELISA), using commercially available ELISA kits from Biosource (Cytosets, Biosource Europe SA, Nivelles, Belgium). Supernatants were collected at 62 h of culture, stored at -20 °C and tested within 2 weeks. Nunc Maxisorb 96-wells plates (Sanbio, Uden, The Netherlands) were coated overnight at 4 °C. The concentrations of the used coating antibodies were 1 μg/ml for IL-4, IL-10, IL-12, and IFN-γ, and 2 μg/ml for TNF-α. The coating antibodies were diluted in PBS. After the coating, each well was incubated for 2 h at RT on a plate shaker with 200 μl blocking solution (5% (w/v) BSA in PBS, pH 7.4). Plates were washed five times with 250 μl PBS-Tween (0.1% Tween-20 in PBS, pH 7.4) and, subsequently 100 μl sample or standard was added to duplicate wells. Samples and standards were diluted in assay buffer (blocking buffer with 0.1% Tween-20). Biotinylated detection antibodies were diluted in assay buffer to the final concentration of 0.2 μg/ml for IL-4 and IL-12, 0.4 μg/ml for IL-10 and IFN-γ and 0.8 μg/ml for TNF-α. Further, 50 μl biotinylated mAb was added to the samples and standards. The plates were incubated for 2 h and washed as before. Then, 100 μl streptavidin poly-horse radish peroxidase (Sanquin, 1 mg/ml), diluted 1:10,000 in assay buffer, was added to each well and the plates were incubated for 30 min at RT while shaking. After washing as described before, 100 μl/well chromogen tetramethylbenzidine (TMB) was added and incubated for 30 min in the dark (RT). To stop the reaction, 100 μl 1M H_3PO_4 was added per

well. The optical densities (OD) at 450 nm and 690 nm were measured in an ELISA Multiskan MS reader (Merlin Diagnostic systems BV, Breda, The Netherlands).

Values are expressed as pg/ml (average of duplicate wells ± SD) deduced from the OD of the standard curve after subtracting the blanks, reference values and the spontaneous secretion of unstimulated cells. The experimentally established sensitivity limits for quantitative determinations were 5 pg/ml for IL-4 and IL-10, 2 pg/ml for IL-12, 4 pg/ml for TNF-α, and 1.5 pg/ml for IFN-γ.

Hemagglutination assay

Rabbit blood in Alsever´s medium (Harlan, Horst, The Netherlands) was washed four times with cold PBS (500*g* for 10 min) and suspended at a concentration of 10% in PBS plus 0.1% (w/v) sodium azide. Half of the resulting erythrocyte suspension was treated with 0.1% (w/v) trypsin for 1 hour at 37 °C and washed four times with PBS as described above. Finally, the erythrocytes were resuspended at a concentration of 2% in PBS. The hemagglutination test was carried out in V-bottom microtiter plates (Corning Inc.). A 50 µl aliquot of the protein extracts was serially diluted twofold with PBS, followed by the addition of 50 µl of 2% (v/v) rabbit erythrocyte suspension (untreated or treated with trypsin). The plates were kept at RT for 1 hour and the hemagglutination activity was determined visually. The hemagglutination titre was expressed as the reciprocal of the highest dilution of the protein solution showing visible agglutination. Thereafter, the specific hemagglutination activity was calculated as the hemagglutination titer per mg protein per ml and expressed as units [25]. ConA served as a positive control, while PBS was used as a negative control.

Statistical analysis

All cytokine data are expressed as mean ± standard deviation (SD). The results were statistical analyzed using a Student's *t* test and considered statistically significant at the $p<0.05$ level.

Results

Analysis of the protein crude extracts by SDS-PAGE gels

The protein extracts were analyzed by SDS-PAGE (15% w/v). As shown in Figure 1, all extracts contain a diversity of proteins. The majority of extracts contain 12 - 17 kDa proteins, which is in the molecular weight range of known FIPs [18, 20, 26]. In addition, most extracts contain higher molecular weight proteins which is in line with higher molecular weights of identified lectins [27].

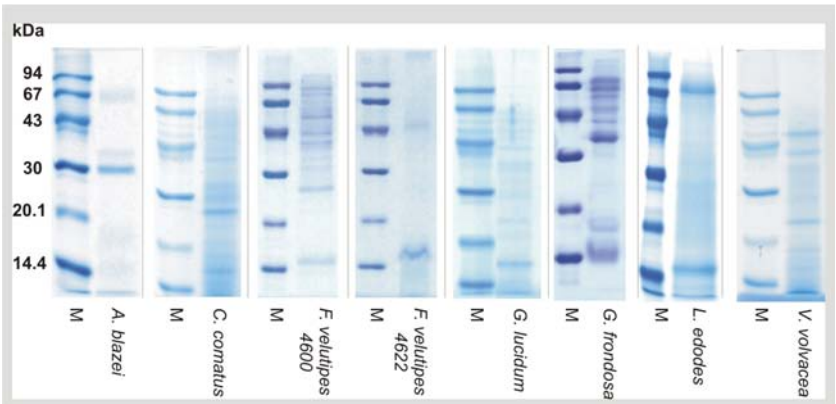
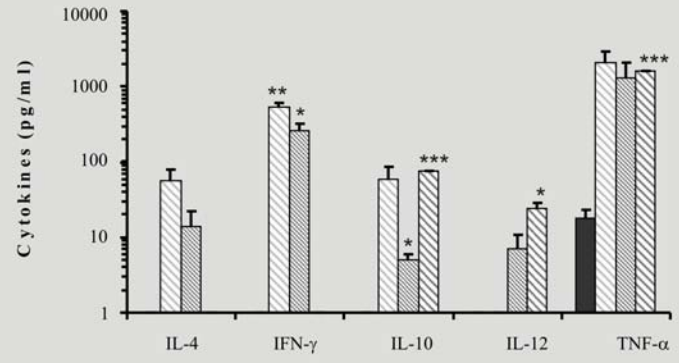


Figure 1. Protein crude extracts were analyzed by SDS-PAGE (15% w/v) stained with Coomassie Brilliant Blue. 'M' indicates the molecular weight marker.

Cytokine production in absence of mushroom extracts

Figure 2 shows the cytokine production of the negative control (hPBMC culture with medium only) and the positive controls (stimulation with PMA/Ca-I, ConA and LPS). The ability of cytokine production emphasizes the viability of the cells and the sustainable culturing conditions. The untreated hPBMC produced only low levels of TNF-α (18 ± 5 pg/ml), whereas IFN-γ, IL-4, IL-10 and IL-12 were not detectable. When the hPBMC were stimulated with ConA, a significantly elevated production of IFN-γ and upregulated TNF-α, IL-4 and IL-10 were observed. However, the other T cell stimulus, PMA/Ca-I, resulted in a significant upregulation of IL-10 and IFN-γ compared to the medium control. The difference between these two T cell stimuli might reflex the difference in the site of activation; ConA mimics TCR stimulation by binding to the receptor, whereas PMA/Ca-I activates the T cells at a more downstream intracellular part. LPS stimulation resulted in a significant increase in TNF-α, IL-10 and IL-12 levels compared to medium.



	IL-4	IFN-γ	IL-10	IL-12	TNF-α
■ Medium	*bd*	*bd*	*bd*	*bd*	18 ± 5
□ ConA	56 ± 23	536 ± 74	60 ± 25	*bd*	2100 ± 816
▨ PMA/Ca-I	14 ± 8	255 ± 65	5 ± 1	7 ± 4	1298 ± 788
▧ LPS	*bd*	*bd*	75 ± 2	24 ± 5	1620 ± 10

Figure 2. Capacity of hPBMC to produce cytokines in response to the used stimuli without addition of mushroom proteins. Each value represents the mean ± SD of duplicates. *p<0.05, **p<0.01, ***p<0.001 compared to the medium control. *bd*: below detection.

Effects of the protein extracts on the cytokine production of unstimulated hPBMC

One way to evaluate the immunomodulatory activity of the protein extracts is to determine their ability to directly stimulate hPBMC without the presence of a stimulus, thereby signifying the presence of autologous mitogenic activity. Proteins extracted from *V. volvacea* were able to induce a low, but significantly increased production of IFN-γ (24 ± 2 pg/ml) and TNF-α (149 ± 5 pg/ml) compared to the medium control. *G. lucidum* proteins induced an higher response than *V. volvacea* with a low, but significant, induction of IL-4, IL-10 and IL-12, and strongly elevated IFN-γ (125 ± 3 pg/ml) and TNF-α (472 ± 26 pg/ml) levels compared to the medium control. The other protein extracts only resulted in significantly reduced amounts of IL-12 (data not shown).

Effects of the protein extracts on the cytokine production of ConA stimulated hPBMC

When hPBMC were stimulated with ConA only, the IL-4 production was upregulated to 56 ± 23 pg/ml compared to no detectable IL-4 levels in hPBMC cultured in medium only. In addition, IFN-γ production was enhanced; from undetectable in medium to 536 ± 74 pg/ml in ConA stimulated hPBMC.

Incubating the hPBMC with the proteins extracts plus ConA resulted in immunomodulation of some measured cytokines (Figure 3A and C). The protein extract from *V. volvacea* significantly reduced the IFN-γ production of ConA stimulated hPBMC, while a stronger reduction was caused by the *G. lucidum* protein extract. Although *F. velutipes* 4600, *G. frondosa* and *A. blazei* largely reduced the IL-4 and IFN-γ production, only protein extracts from *F. velutipes* 4622, *C. comatus* and *L. edodes* were able to completely abolish this cytokine production to medium control levels (Figure 3A).

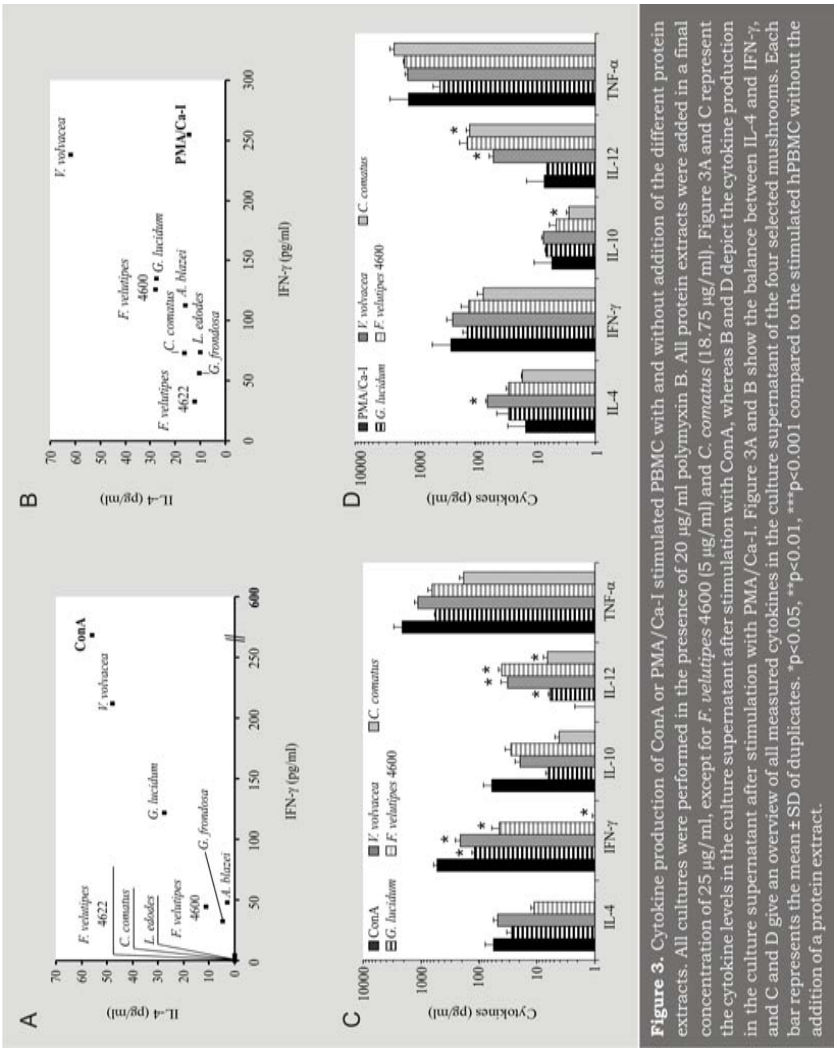


Figure 3. Cytokine production of ConA or PMA/Ca-I stimulated PBMC with and without addition of the different protein extracts. All cultures were performed in the presence of 20 µg/ml polymyxin B. All protein extracts were added in a final concentration of 25 µg/ml, except for *F. velutipes* 4600 (5 µg/ml) and *C. comatus* (18.75 µg/ml). Figure 3A and C represent the cytokine levels in the culture supernatant after stimulation with ConA, whereas B and D depict the cytokine production in the culture supernatant after stimulation with PMA/Ca-I. Figure 3A and B show the balance between IL-4 and IFN-γ, and C and D give an overview of all measured cytokines in the culture supernatant of the four selected mushrooms. Each bar represents the mean ± SD of duplicates. *p<0.05, **p<0.01, ***p<0.001 compared to the stimulated hPBMC without the addition of a protein extract.

As shown in Table II, hPBMC stimulated with ConA alone showed undetectable IL-12 levels. Discarding *F. velutipes* 4622 and *A. blazei*, addition of all protein extracts resulted in an increase in IL-12 production. In contrast to the IL-12 production, none of the extracts resulted in a significant alteration of the IL-10 levels.

Table II. Effect of fungal protein extracts on the cytokine production by T cell specific and monocytes specific stimuli.

Sample	IFN-γ ± SD (pg/ml)	IL-4 ± SD (pg/ml)	IL-10 ± SD (pg/ml)	IL-12 ± SD (pg/ml)	TNF-α ± SD (pg/ml)
ConA without extract	536 ± 74	56 ± 23	60 ± 25	*bd*	2100 ± 816
A. blazei	48 ± 2*	*bd*	11 ± 3	6 ± 3	339 ± 71
C. comatus	*Bd*	*bd*	*bd*	7 ± 1*	182 ± 32
F. velutipes 4600	45 ± 15*	11 ± 2	28 ± 8	40 ± 6*	639 ± 120
F. velutipes 4622	*Bd*	*bd*	*bd*	4 ± 0.3	25 ± 6
G. lucidum	122 ± 12*	28 ± 6	6 ± 1	6 ± 0.3*	562 ± 14
G. frondosa	33 ± 17*	*bd*	17 ± 3	13 ± 1*	164 ± 90
L. edodes	*Bd*	*bd*	5 ± 0	6 ± 0.5*	135 ± 2
V. volvacea	212 ± 48*	48 ± 8	20 ± 4	32 ± 10*	1128 ± 160
PMA/Ca-I without extract	255 ± 65	14 ± 8	5 ± 1	7 ± 4	1298 ± 788
A. blazei	113 ± 77	16 ± 6	*bd*	67 ± 33	1071 ± 153
C. comatus	73 ± 18	16 ± 0.3	*bd* *	123 ± 18*	2255 ± 406
F. velutipes 4600	126 ± 44	28 ± 2	4 ± 1	136 ± 49	1511 ± 55
F. velutipes 4622	33 ± 1*	13 ± 0.1	4 ± 1	155 ± 53	936 ± 111
G. lucidum	135 ± 24	28 ± 16	7 ± 0.2	6 ± 0.1	387 ± 110
G. frondosa	57 ± 4	11 ± 0.4	4 ± 2	52 ± 11*	282 ± 19
L. edodes	73 ± 57	10 ± 4	*bd*	70 ± 33	981 ± 203
V. volvacea	238 ± 56	62 ± 5*	7 ± 0.4	49 ± 9*	1325 ± 129
LPS without extract	*bd*	*bd*	75 ± 2	24 ± 5	1620 ± 10
A. blazei	*bd*	*bd*	26 ± 11*	71 ± 9	1043 ± 47**
C. comatus	*bd*	*bd*	8 ± 1***	43 ± 1	955 ± 84**
F. velutipes 4600	*bd*	*bd*	41 ± 6*	114 ± 18	841 ± 29**
F. velutipes 4622	*bd*	*bd*	15 ± 1**	46 ± 11	545 ± 95**
G. lucidum	19 ± 10	*bd*	10 ± 4**	15 ± 5	755 ± 99**
G. frondosa	*bd*	*bd*	51 ± 4*	84 ± 13	321 ± 17***
L. edodes	*bd*	*bd*	34 ± 20	110 ± 4	1117 ± 32**
V. volvacea	*bd*	*bd*	22 ± 0.3**	30 ± 7	626 ± 5***

The added protein extract concentration was 25 μg/ml for all except *F. velutipes* 4600 (5 μg/ml) and *C. comatus* (18.75 μg/ml). *bd*: below detection limit. Each value represents the mean ± SD of duplicates. *p<0.05, **p<0.01, ***p<0.001 compared to the stimulated hPBMC without the addition of a protein extract.

Figure 3A shows that proteins of *V. volvacea*, *G. lucidum*, *F. velutipes* 4600, and *C. comatus* result in a similar correlation between the IL-4 an IFN-γ production as seen in ConA stimulated hPBMC. This suggests that these proteins do not influence the T cell subsets, since no skewing towards Th1 (more IFN-γ) or Th2 (more IL-4) cells could be observed. However, unaltered T cell subsets might be accompanied by altered monocyte derived cytokine production. Therefore, a comparison of all measured cytokines after addition of these four fungal protein extracts is given in Figure 3C. It is shown that the IFN-γ and TNF-α levels produced by ConA stimulated hPBMC with or without protein extracts were higher compared to IL-4, IL-10 and IL-12. IFN-γ was significantly reduced in all four cultures, in contrast to an overall significant increase of IL-12.

Effects of the protein extracts on the cytokine production of PMA/Ca-I stimulated hPBMC

hPBMC stimulated with PMA/Ca-I alone, slightly upregulated the IL-4 production to 14 ± 8 pg/ml compared to undetectable IL-4 in PBMC cultured in medium only. The IFN-γ production was significantly upregulated; from undetectable in medium to 255 ± 65 pg/ml in PMA/Ca-I stimulated hPBMC. However, the upregulation of IL-4 and IFN-γ by ConA stimulation was consistently higher than found in PMA/Ca-I stimulated cells.

Incubation of the hPBMC with the protein extracts plus PMA/Ca-I resulted in immunomodulation of some or all measured cytokines (Figure 3 B and D). Proteins from *V. volvacea*, unlike in the ConA stimulated cells, significantly increased the IL-4 production. In contrast, *F. velutipes* 4600 proteins significantly reduced the IFN-γ production in combination with an unaltered IL-4 level (Figure 3B).

As shown in Table II, hPBMC stimulated with PMA/Ca-I produce a low amount of IL-12 and IL-10. Addition of protein extracts from *C. comatus*, *G. frondosa* and *V. volvacea* proteins significantly increased the IL-12 production. In contrast to the IL-12 production, IL-10 was significantly downregulated by *C. comatus*.

The difference in activation potential between ConA and PMA/Ca-I is shown in Figure 3A and B. As mentioned before, the proteins of *V. volvacea*, *G. lucidum*, *F. velutipes* 4600, and *C. comatus* in combination with ConA showed a correlation between IL-4 and IFN-γ. Figure 3B depicts the discrepancy between the protein extracts in combination with PMA/Ca-I compared to ConA stimulated hPBMC in Figure 3A. A correlation between IL-4 and IFN-γ seen in hPBMC stimulated with ConA alone, is not observed in cells stimulated with PMA/Ca-I alone. However, the proteins of *V. volvacea*, *G. lucidum*, *F. velutipes* 4600, and *C. comatus* do show the correlation between these two

cytokines. Again, unaltered T cell subsets do not exclude altered monocyte derived cytokine production and a comparison of all measured cytokines is given in Figure 3D. It is shown that the IFN-γ and TNF-α levels produced by PMA/Ca-I stimulated hPBMC with or without protein extracts were much higher compared to IL-4, IL-10 and IL-12. The IL-4 production was significantly increased by proteins from *V. volvacea*. In contrast, IFN-γ production was only significantly downregulated by *F. velutipes* 4600. *V. volvacea* and *C. comatus* significantly increased the IL-12 levels, whereas the IL-10 production was only significantly decreased by *C. comatus* proteins.

Effects of the protein extracts on the cytokine production of LPS stimulated hPBMC

As shown in Table II, LPS (alone or in combination with the fungal proteins) was not able to induce a significant upregulation of IL-4 or IFN-γ. Beside *L. edodes* (for IL-10), all protein extracts induced a significant downregulation of IL-10 and TNF-α levels.

Effects of the polysaccharide extracts on the cytokine production

Fungal polysaccharides were examined for their ability to stimulate or suppress the production of IFN-γ, IL-4, IL-10, IL-12 and TNF-α in unstimulated and ConA-, PMA/Ca-I- and LPS-stimulated hPBMC.

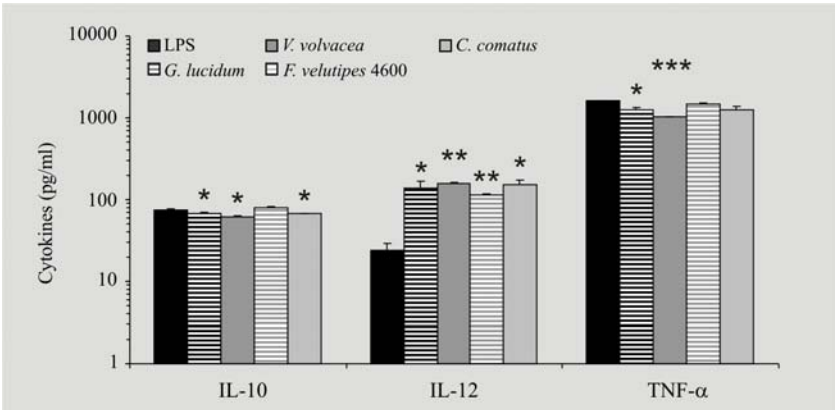


Figure 4. Cytokine production of LPS stimulated hPBMC with and without addition of the different polysaccharide extracts. All cultures are stimulated with 10 ng/ml LPS, in the absence of polymyxin B. All polysaccharide extracts were added in a final concentration of 25 µg/ml, except for *F. velutipes* 4600 (5 µg/ml) and *C. comatus* (18.75 µg/ml). An overview of all measurable cytokines in the culture supernatant (IL-4 and IFN-γ levels were below the detection limit) is given. Each bar represents the mean ± SD of duplicates. *p<0.05, **p<0.01, ***p<0.001 compared to the stimulated hPBMC without the addition of a polysaccharide extract.

Some polysaccharide extracts showed immunomodulatory activity of unstimulated hPBMC (data not shown). In addition, some polysaccharide extracts regulated the production of IFN-γ, IL-4, IL-10, and TNF-α in PMA/Ca-I-stimulated hPBMC, whereas all polysaccharide extracts stimulated the production of IL-12 (data not shown). The polysaccharide extracts from *C. comatus* and *L. edodes* downregulated all measured cytokines (data not shown).

In cultures stimulated with LPS, both *F. velutipes* 4600 and 4622 were unable to significantly increase the IL-10 production, whereas all other polysaccharide extracts did significantly elevate the IL-10 levels (Table III). In addition, all polysaccharide extracts significantly stimulated the production of IL-12. Only the polysaccharide extracts of *C. comatus* and *F. velutipes* 4600 were unable to significantly inhibit the TNF-α levels as shown in Figure 4.

Table III. Specification of the fungal intracellular polysaccharide extracts and their effect on the cytokine production of LPS stimulated hPBMC.

				Cytokine production after stimulation with LPS		
Fungus	Weight (mg/g mycelia)	Composition $[CHO]_n$ (%)	P (%)	IL-10 ± SD (pg/ml)	IL-12 ± SD (pg/ml)	TNF-α ± SD (pg/ml)
LPS without extract				75 ± 1.8	24 ± 5	1620 ± 10
A. blazei	1.4	46	*bd*	60 ± 0.2**	143 ± 2**	899 ± 27**
C. comatus	10.9	65	14	67 ± 1*	154 ± 21*	1268 ± 134
F. velutipes 4600	80.1	67	8	79 ± 3	115 ± 1**	1488 ± 51
F. velutipes 4622	159.6	62	11	75 ± 4	191 ± 10**	1153 ± 10***
G. lucidum	129.7	73	8	69 ± 0.2*	141 ± 28*	1262 ± 90*
G. frondosa	25.8	57	8	43 ± 1**	148 ± 15**	1133 ± 43**
L. edodes	25.2	19	43	44 ± 2**	124 ± 2**	879 ± 9***
V. volvacea	92.3	76	8	63 ± 2*	159 ± 3**	1020 ± 16***

The polysaccharide extract concentration used was 25 μg/ml for all extracts except for F. velutipes 4600 (5 μg/ml) and C. comatus (18.75 μg/ml). Each cytokine value represents the mean ± SD of duplicates. *p<0.05, **p<0.01, ***p<0.001 compared to the stimulated hPBMC without the addition of a polysaccharide extract. Bd: below detection limit, $[CHO]_n$: Carbohydrates, P: Proteins.

Protein contamination of the polysaccharide extracts was analysed by determining the carbohydrate content using the phenol-sulphuric acid method and the protein content by means of the micro BCA method. For nearly all polysaccharide extracts the protein contamination was low, with exception of *L. edodes* extract as shown in Table III.

Hemagglutination assay

The hemagglutination test was conducted with rabbit red blood cells (RRBC) with or without pre-treatment with trypsin. Trypsin can increase the binding of lectins to red blood cells by unmasking lectin-binding proteins [28]. The proteins of *G. lucidum*, *V. volvacea* and *C. comatus* have been tested for their hemagglutination capacity. Table IV shows that *G. lucidum* resulted in specific agglutination activity of 12 units when using RRBC without trypsin treatment, while after treatment with trypsin the agglutination activity increased 64-fold (752 units). The same was seen in the hemagglutination assay with proteins from *V. volvacea*: without trypsin treatment, the specific agglutination activity was 2 units, whereas after trypsinization this agglutination activity increased 254-fold (508 units).

Table IV. Hemagglutination assay of protein fractions with RRBC

Mushroom	Pre-treatment	Protein (mg/ml)	Specific HA activity (units)
G. lucidum	No trypsin	0.681	11.7
	Trypsin		751.8
G. frondosa	No trypsin	0.425	-
	Trypsin		-
V. volvacea	No trypsin	2.017	1.9
	Trypsin		507.7

The specific HA activity is expressed as units, calculated as titre per mg protein per ml. HA: Hemagglutination, -: No HA activity detected.

Discussion

This study showed that mushroom mycelia can be cultured efficiently in selected media and, by using specific isolation procedures, high yield production of polysaccharides and proteins can be obtained. These polysaccharides and proteins display defined immunomodulatory activity as shown by modulation of cytokine production in *in vitro* hPBMC cultures. Some of these cytokines are involved in T cell subset induction and activation and thus potentially able to modulate disease induction and activity, while other cytokines originate from antigen presenting cells like monocytes, opening up possibilities to modulate antigen presentation. Therefore these cells become targets for the rational development of immunotherapeutical agents in the future.

The protein extracts of *F. velutipes* 4600 and 4622 are able to show immunomodulatory activity, and, as shown by the SDS-PAGE gel, these protein extracts may contain low molecular mass proteins like FIPs or mitogenic lectins that are generally of higher molecular mass [22, 27]. Interestingly, the molecular masses of the known FIPs were estimated to be approximately 15 kDa by SDS/PAGE, as confirmed by the approx. 15 kDa FIP-*Vvo* [18]. Smaller FIPs have also been described, e.g. FIP-*Fve* is approximately 12.7 kDa [20] and LZ-8 from *G. lucidum* is approximately 12.4 kDa [26]. Our *G. lucidum* protein extract also showed a band of approximately 13 kDa accompanied by a 17 kDa protein. *In vivo*, LZ-8 prevents the production of systemic anaphylaxis in mice when administered repeatedly. Kino *et al.* suggested that a reduction of antibody production is the underlying mechanism [19]. Besides FIPs, other immunomodulatory proteins can be present in the extracts. For example, Liau *et al.* showed a lectin with immunomodulatory capacities in *V. volvacea*, the *VVL*, which is a homodimeric protein of approximately 32 kDa [27].

The extraction with acetic acid results in precipitation of many proteins, but the ones that will be able to resist the acidic conditions are most likely the best candidates to survive the gastric environment in vivo and suitable to be used as dietary components. However, since many different proteins like FIPs [20, 26], lectins [27, 29], glycoproteins [30] and non-glycosylated proteins [31] have been found in fungal protein extracts, our protein extracts need to be further purified to attribute any immunomodulatory capacity to one single protein or a combination of proteins.

To test the immunomodulatory capacity of the protein and polysaccharide extracts, we used the hPBMC culture as readout system. hPBMC cultures without addition of stimuli, but with protein or polysaccharide extracts were analyzed for endogenous mitogenic activity that may be present in the selected extracts. Proteins extracted from a fungus can be contaminated with fungus

derived LPS or LPS obtained through the isolation procedures. This LPS can activate hPBMC through Toll-like receptors, and thereby mask or interfere with the immunomodulating capacity of the protein extracts. Polymyxin B was added to the cultures to block all present endotoxin as is widely shown in literature [7, 32, 33]. The only two protein extracts that were able to induce some activity without the presence of a stimulus, but with added polymyxin B, were extracted from *V. volvacea* and *G. lucidum*. The modulation by a protein extract without the presence of a stimulus could point towards the presence of a FIP or a lectin.

Various stimuli cause preferential stimulation of T cells or monocytes in hPBMC. PMA/Ca-I and ConA are widely used to activate T cells, however, both result in a different effector action. When hPBMC are stimulated with ConA only, IFN-γ production was enhanced. Addition of mushroom proteins altered both the IL-12 and IFN-γ production of ConA stimulated hPBMC. The mushroom extracts in ConA stimulated hPBMC showed a tendency to reduce the IL-4 levels, while PMA/Ca-I stimulated cells plus *V. volvacea* proteins significantly upregulated the amount of IL-4. In addition, all protein extracts were able to significantly downregulate the IFN-γ levels, while only *F. velutipes* 4600 was able to do so in PMA/Ca-I stimulated cells. Taken together, the discrepancy between the ConA and PMA/Ca-I activated hPBMC cytokine production suggest that the interaction of the protein extract with T cells may be important. However, although the culturing period of 62 h is sufficient for monocytes to become fully activated, for T cells it might be relatively short to establish the maximal differences in the T cell activation and cytokine production, since functional T cell subsets need approximately three to five days to be generated *in vitro* [10]. In addition, it is possible that lectins are present in the extracts that compete with ConA on the binding site, reducing the activating capacity of ConA. Another possibility is that the proteins present in the extracts can bind to ConA itself, and thereby preventing ConA from binding to its receptor. Future inhibition studies are necessary to prove either of these hypotheses.

Besides a direct inhibiting effect on the T cells, the protein extracts could also act on the monocytes which subsequently downregulate T cell activation. In contrast to the other assessed cytokines, IL-12 is a cytokine that can only be produced by monocytes [8]. Therefore, the overall increase of IL-12 in both ConA and PMA/Ca-I stimulated cells plus the protein extracts suggests that monocytes were activated. Although ConA and PMA/Ca-I are generally considered as T cell stimuli, also monocytes express receptors that can bind ConA, and, in addition, also monocytes contain PKC that can be activated by PMA/Ca-I. It is therefore possible that the exposure to the protein extracts and the stimuli act synergistically, resulting in a significant upregulation of IL-12 by monocytes in 'T cell specific' stimulation conditions. Addition of LPS, which in humans is considered as a monocyte specific stimulus, even further

potentiated the IL-12 levels caused by the protein extracts, again supporting the suggestion that the extracts modulate the monocytes. Whether this immunomodulation is caused by one component within the extracts, or by a combination of e.g. a FIP and a lectin, needs to be further investigated.

A useful tool to show the presence of fungal lectins is the hemagglutination of RRBC [19, 26]. The hemagglutination assay is based on end-terminal sugar residues on membrane bound glycoproteins of erythrocytes. These end-terminal sugar residues can also be bound by sugar binding proteins like lectins. When these proteins bind to the sugars on the surface of two or more erythrocytes, this causes agglutination [34]. We tested the extracts on RRBC with or without pre-treatment with trypsin. Since trypsin removes proteins that have bound to the sugars on the RRBC, proteins present in the extracts will be able to bind to the sugars and therefore result in agglutination [10]. The clear difference between the specific agglutination capacities of the RRBC that were not treated with trypsin and the RRBC treated with trypsin supports the presence of lectins in the acetic acid extracted proteins from *G. lucidum* and *V. volvacea*. Further inhibition tests are necessary to confirm that the protein extracts of *G. lucidum* and *V. volvacea* indeed contain lectins.

Besides the protein fractions, the influence of the polysaccharide fractions obtained from the different mushroom strains was determined. Some of the tested polysaccharide extracts showed significant alterations of the IL-10, IL-12 and TNF-α levels. However, our findings using different mushroom species cannot show the immunomodulatory activity that was described to the mushroom derived polysaccharides in some other studies, possibly due to different extraction procedures [7].

In summary, proteins from *V. volvacea* and *G. lucidum* showed immuno-modulating activity without the presence of any stimulus, despite the fact that neither of them was able to decrease the amount of IL-4 and IFN-γ as effectively as *F. velutipes* 4600 and *C. comatus* in the presence of ConA. Contradictory, only *V. volvacea* significantly increased the IL-4 level, and only *F. velutipes* 4600 significantly decreased IFN-γ after stimulation with PMA/Ca-I. However, the exposure to the protein extracts and the stimuli could act synergistically on the activation of monocytes, eventually leading to a reduction of T cell stimulation. In addition, both *V. volvacea* and *G. lucidum* showed more hemagglutination activity after treatment of the RRBC with trypsin, suggesting the presence of carbohydrate binding proteins. In conclusion, *V. volvacea* and *G. lucidum* posses immunomodulating activity. However, whether this immunomodulation is caused by lectins, glycosylated FIPs or a combination of both requires further purification and characterisation of the extracts.

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SECTION 3

Socio-economic study

Chapter 7: Allergy and Lifestyles

Chapter 8: The FREYAL study

CHAPTER

7

Allergy and Lifestyles

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Abstract

The number of people with allergic complaints has increased over the last decade. This increase of allergic individuals cannot be explained by genetic alterations alone, and it is therefore suggested that the environment and lifestyle must have an additional impact on this increase. Besides a short introduction on the immunological events leading to allergy, different lifestyle factors are addressed in the context of allergic sensitization. Most related papers focus on either a sociological or immunological point of view, whereas here we combine both into a multidisciplinary framework. We discuss relevant lifestyles, including socioeconomic status, leisure activities, general health status, psychological factors, nutrition, alcohol, exposure to allergens, and hygiene and their effect on immunological characteristics of allergic responses. Next to the possible influence of lifestyles on the development of allergies, also coping with an allergy will likely affect lifestyle. Therefore, the impact of allergies on the psychological and economic situation of allergic individuals and their social environment are important.In conclusion, insights in the immunopathology of allergic diseases have been improved, leading to better treatment of the allergic symptoms. However, the development of preventive lifestyles and advise to allergic persons concerning which lifestyles could help relieve or prevent new allergies, is becoming increasingly important. The impact of allergies does not only influence individuals, but also society as a whole.

Introduction

The number of people suffering from allergic complaints has risen dramatically over the last decades. Whether this reflects an increase in the prevalence of food allergies, or is simply associated with an increased clinical awareness of the condition in its extreme form, remains unknown. The definition of allergy according to the World Allergy Organization is: "Allergy is the inappropriate and harmful response of the body's defense mechanism to substances that are normally harmless. It involves the immune system and particularly an antibody called immunoglobulin E (IgE)" [1]. This definition should be taken into account when establishing the number of allergic sufferers, since there is a clear distinction between perceived allergies and clinically proven allergies by detection of allergen-specific IgE antibodies. For example, in studies of self-reported food allergies, the incidence and prevalence of food allergy in Europe is found to vary between 2 and 35 per cent of the population. However, the same review shows that in studies where diagnostic tests for IgE mediated allergies were applied, the prevalence rate was much lower, ranging between 1 and 4 per cent [2, 3]. Either way, the increase of allergic individuals cannot be explained by genetic alterations alone. About 30% of the allergic sensitization cases can be explained by a genetic predisposition, and therefore it is suggested that the environment and lifestyle must have an additional impact on the increase in the number of allergic individuals [4, 5]. In addition, twin studies indicate that genetic factors and environmental control may each account for about 50% of the phenotype expression of allergic disease [6]. In this review, the immuno-pathology of allergies is addressed in the context of different lifestyle factors. Also the impact of allergies on lifestyles of individuals and the involvement of policy makers to acknowledge the necessity of allergy management regulation are discussed. Whereas related papers focus on either a socioeconomic or immunological point of view, here we combine both into a multidisciplinary approach summarized in the conclusion.

Immunopathology in allergy

The allergic response takes two steps before it can cause clinical symptoms in a predisposed individual. This predisposition is due to multiple genes that interact with each other and the environment [7]. This explains why allergic diseases are often found in multiple members of a family. The first step is called the allergic sensitization phase. In this step, the protein that eventually will cause the allergy will enter the human body. The intact or partially digested proteins will be taken up by a specialized antigen presenting cell. These antigen presenting cells will subsequently present

small fragments of the protein in a way that other immune cells can respond. The first responding cells are called T helper cells. In the case of an allergy causing protein, called an allergen, these T helper cells will interact with another immune cell called a B cell and activate these B cells to produce antibodies against this allergen. By producing a special communication protein, called IL-4, the T helper cell instructs the B cell to produce a specific class of antibodies, the IgE antibodies. This last step is essential, since only this antibody class is eventually capable of causing the allergic complaints [8]. These IgE antibodies will bind to another immune cell in the tissue, called the mast cell, and then the end of the sensitization phase is reached. We now consider a person sensitized to a specific allergen.

The second phase is called the challenge phase. Here, a second exposure to the same allergen will lead to the allergic complaints. This response is caused by the binding of the protein to multiple IgE molecules loaded on the mast cell. This binding will cause the mast cell to degranulate and release much of the compounds stored inside the mast cell. One of the released compounds is called histamine, which causes vasodilatation and triggering of nerve cells, leading to e.g. swollen eyes and itching sensations. These symptoms are the hallmark of IgE-mediated allergic responses [8]. However, although the mechanism of the allergic response is quite clear, why these allergens do cause an allergic response in one person, but not in an other person, remains unknown. One possible factor in this distinction is the lifestyle of the individual.

Lifestyles

The term lifestyle comprises values, interests, way of thinking and actions that influence consumer behavior. In other words, lifestyle includes what consumers do and what they think and feel [9]. However, lifestyles are not only based on individual choices, but are a result of an interaction between personal, social and environmental factors [10]. Next, we provide an overview of the relevant lifestyles, including socioeconomic status, leisure activities, general health status, psychological factors, nutrition, alcohol, and exposure to allergens.

Socioeconomic status

Socioeconomic status (SES) includes aspects like ownership, education level, income and professional level. People with a high SES generally have a better overall health status than those with a low SES. This is probably not only due to less access to health care, but also to poor physical and/or mental health. This poorer health arises through various psychosocial mechanisms such as

discrimination, social exclusion, prolonged and/or heightened stress, loss of sense of control, and low self-esteem [11]. Research investigating the link between allergies and SES shows that hay fever and atopic dermatitis were correlated with a high SES, while asthma is related to a low SES [12]. However, SES by itself does not influence the development of allergies, but SES can delay seeking care [13]. In addition, Kaplan *et al.* concluded that higher childhood socioeconomic position, as well as greater educational attainment, was associated with better cognitive function in adulthood [14]. SES should therefore be considered as a marker for the environmental influences that increase the risk of the development of allergies. For example, families with a low SES relatively often include pregnant smoking women, allow indoor smoking or own domestic animals, whereas high SES families frequently extend breast feeding periods [15].

Breastfeeding

During the vulnerable first months and years of life, breast milk is referred to as an irreplaceable immunological resource because it supports passive and active immunity [16]. Besides the effect of the active components in breast milk on the immune cells, also the intestinal microbiota is influenced by breast milk since it allows the growth of bifidobacteria and lactobacilli. Since the intestinal microbiota modulates the mucosal physiology, barrier function and systemic immunological responses, and an altered intestinal microbiota in formula fed children is shown, implications of this alteration can lead to changes in the immunological responses [16].

Large differences are found when breastfeeding rates are compared between nations or ethnic groups. For example, breastfeeding rates in the United States do not meet public health goals and especially the poor non-Hispanic, Black women have the lowest breast feeding rates [17]. Kelly *et al.* show that breastfeeding rates in the United Kingdom are also relatively low. Around 70 per cent of the children is breastfed, but there is a sharp decline within 3 months after birth. They also show that breastfeeding is less common among younger, less-educated, primiparous, and lower-income mothers [18]. However, black African, black Caribbean, Pakistani, Bangladeshi, and Indian mothers were more likely to initiate breastfeeding compared with white mothers [18].

Intestinal microbiota development in infancy

The adult human intestine harbors about 1014 bacteria in a symbiotic manner; the host provides a nutrient-rich environment and the bacteria can confer important health benefits upon the human host [19]. It is shown that a caesarean section in predisposed children might increase the risk of development of food allergy [20]. This supports the concept that

there is a close interplay between colonization of the intestine and the maturation and differentiation of the immune system. Microbes can stimulate the immune system towards a non-allergic response, thereby preventing food allergic complaints [20]. Since large cultural differences are found, it is thought this influence the differences in the development of allergies. The attitudes of European obstetricians to perform a caesarean section in the absence of clinical indication varies within as well as between countries. Obstetricians from Spain, France and The Netherlands are the least likely and those from the UK and Germany the most likely to accept a request for a caesarean section based exclusively on patient's choice. Since the variability can not be explained by the physicians' demographic and professional characteristics, it is likely that cultural factors and perinatal care elements play a role [21].

In addition, the use of antibiotics alters the intestinal colonization and a positive association between antibiotic use and childhood allergy and asthma. However, a large prospective birth cohort study was not able to show an association between antibiotic exposure or vaccination status and eczema [22, 23].

Leisure activities

Leisure time is considered as free time people spent after working hours, used for entertainment and relaxation [10]. The more technologically advanced a nation is, the more citizens are exposed to better lifestyle facilities that make everyday life easier. Moreover, with better job opportunities, and more disposable income, people tend to spent more money on lifestyle products and expensive outdoor foods with high calories. These lifestyle facilities are also linked to the increasing weight of the global population [24, 25]. Physical energy spent during leisure time seems to be negatively correlated with obesity and therefore negatively linked to the occurrence of allergic responses [26]. Exercise-induced asthma occurs in 90 per cent of the asthmatic individuals, and is probably related to the obesity found in asthmatics [27].

General health status and obesity

In order to objectively determine the influence of allergies on individuals, the general health status of these individuals should be taken into account. Statistics show that the more progressive a country is, the higher are the chances for a large part of its population to be affected by obesity, also known as the Lifestyle Disease [24]. The Body Mass index (BMI), relating the body weight of an individual to the square of body length, has been developed as an indicator of overweight and obesity [28]. A BMI above 25 indicates overweight, while a BMI between 18.5 and 24.9 is considered healthy. Research shows that a BMI above 30 (obesity) increases the risk of developing

allergies [29]. In addition, schoolchildren who have a propensity to develop asthma would be more likely to spend more time watching TV because of fear of exercise-induced respiratory symptoms, leading to significant obesity in these children. Obesity, in turn, increases the risk of developing asthma or asthma-related symptoms [30]. In obese people, not only hormone levels [31], but also T cell functions [32] are impaired, leading to associations between a high BMI and health status.

Adjustment of BMI requires education about nutritional behavior, information about aspects of food requirements and should be addressed to the age, growth needs, habitual way of life and the applicability of a new nutritional behavior on a long-term basis [33]. After obtaining the proper information, behavioral changes are essential to establish new nutritional habits in combination with physical exercise leading to weight loss.

Nutrition

Several dietary factors, including higher consumption of processed foods, omega-6 fatty acids and salt, and a lower consumption of omega-3 fatty acids and fresh foods plus lower rates of breast-feeding, all have a potential impact on allergic reactions [34]. Omega-3 fatty acids, which are stored in cell membranes, have an important role in controlling inflammation, but are easily replaced by omega-6 fatty acids, which facilitate the development of inflammation [34]. Also the increased intake of micronutrients like sodium, potassium and magnesium are correlated to the development of allergic responses [34].

Minerals, vitamins and long-chain polyunsaturated fatty acids are important for the immune cells. Furthermore, westernized diets are relatively deficient in antioxidants. Since oxidative stress is found to be associated with the chronic inflammation of asthma, the deficiencies in antioxidants are an important observation [35].

Besides the altered intake of micronutrients, also the intake of exotic foods in the daily diet is proposed as a new source of allergens, called neo-allergens. Examples of such neo-allergens are parvalbumine in e.g. cod fish, threadfin, Indian anchovy, pomfret, and tengirri [36]. Also chayote, rambutan, arguta, pumpkin seeds and custard apple can be considered as neo-allergens [37]. However, also familiar foods like shellfish, lupine flour and wheat flour can be considered as neo-allergens, when they e.g. undergo a change in the food processing [37].

Moreno *et al.* show that meal patterns and food choices within each meal varies between adolescents from different European countries. Three meals a day with snacks in between was typical in all countries except

Spain. Snacking between meals occurred in all 10 European countries, especially after school. In all countries, taste is the most important factor for all meal occasions. Other similarities were the influence of parents, especially at breakfast and the evening meal, the importance given to health and convenience. Although the importance of healthy eating is understood, adolescents do not always eat as well as they should. To them, the problem with healthy food is that it is boring, does not taste very nice, does not fill them up when hungry, takes too much effort and is expensive [38].

Stimulant use

The use of stimulants like alcohol and drugs are investigated for their role in many diseases. Both chronic and acute intake of alcohol can cause powerful immune modulation [39-41]. It is shown that alcohol consumption of humans lead to a shift in cytokine production by different T cell subsets [42]. Similar results are shown in mice, who's alcohol intake results in skewing towards allergy promoting T cells [43-45] or direct effects on B cells [46]. The same group also suggests that the antigen uptake in the gastro-intestinal tract is enhanced, therefore resulting in more IgE synthesis [46]. Also the allergen-specific IgE levels in house dust mite allergic individuals are found to be significantly higher in regular alcohol consumers compared to people that do not drink alcohol [47]. Another study shows that although the sensitization prevalence itself is not associated with alcohol consumption, pollen sensitization is observed more frequently in the alcohol consuming group [48]. Overall, these studies show that the underlying mechanisms of alcohol involvement in the development of allergies need further research.

Although to a smaller extent, also the use of other stimulants like marihuana and cocaine are investigated for their influence on allergic responses. Evidence concerning a possible suppressive effect of cannabis on cell-mediated immunity is difficult to interpret. If suppression occurs, it may only be transient, in the sense that recovery can occur [49]. On the other hand, drugs like cocaine and heroin are frequently reported as being associated with asthma deaths [13], but this rise in asthma deaths seems to be a reflection of substance use or alcohol consumption similar to that seen in victims of homicide [50].

Exposure to allergens

The change in hygiene in relation to allergies has been investigated intensively since the hygienic circumstances have been greatly improved in Western Europe [51]. Remarkably, the amount of allergic individuals in developing countries with less hygiene is much lower than in the Western society [19]. In addition, children that grew up on farms seem to develop less allergies [19, 52]. These studies support the so-called hygiene-hypothesis stating that

increased hygiene and the elevated amounts of vaccinations lower the chance of children being infected, leading to less development and activity of the immune system [53]. The less active immune system is therefore probably more prone to respond to otherwise harmless proteins, resulting in allergies [51]. However, many studies are contradicting the hygiene hypothesis. One example is that worm-infections, despite inducing high levels of IgE, seem to protect against developing allergies [34]. Moreover, over the last decennia, not only allergic diseases, but also the autoimmune diseases are rising [19].

Nevertheless, the frequency, dose and duration of the exposure to allergy causing compounds is important, since allergy does not develop when the allergen itself is not encountered. This explains why some allergies, like olive tree allergy, do occur in Spain, while they are hardly seen in the Netherlands. Other examples are the higher commonality of birch pollen allergy in villages, whereas rat and cockroach allergies are more common in large cities [51]. Geographic variables are therefore an important factor when observing the differences between allergics and non-allergics. Furthermore, we distinguish outdoor and indoor exposure to allergens, to be discussed next.

Outdoor allergens
Although house dust mite allergy is the most common allergy in the Netherlands (22 per cent), seasonal allergies are common as well. Onset of hay fever is caused in 15 per cent of the Dutch citizens by birch pollen of the B. pendula birch species [54]. Birch pollen counts occur in the same order of magnitude as grass pollen in West European cities [55]. In line with that observation, timothy grass pollen allergy is found in 15 per cent of the Dutch population [54]. However, considering the small size of pollen allergens (10-12 □m) [56], outdoor allergens will also be largely present indoors.

Indoor allergens
Although many studies on indoor air quality have been carried out in the workplace, few data are available about air quality in dwellings. However, people living in urban areas spend most of their time (85-90%) indoors, where concentrations of many airborne pollutants are higher than outdoors [57]. Indoor allergens can be very powerful sensitizers for a variety of reasons: the allergenic particles are smaller and therefore more inhalable, the allergens are more potent in their enzymatic activity, and exposure reaches very high levels or is perennial [34]. It is shown that children that are exposed early in life are at much higher risk of having asthma than children who become sensitized later [34]. Adult subjects particular

susceptible to indoor air contaminants include people with allergy or asthma, people with chronic respiratory disease, people with a suppressed immune system, and contact lens wearers [57]. Especially house dust mite exposure occurs indoors, making knowledge about household chores inevitable for those at risk. Examples of chores that can influence the exposure are the way of dusting, vacuum cleaning, and doing the laundry. On the other hand, having allergies can influence the ability to perform such household chores, or the necessity to ask for help in performing the chores.

It is generally accepted that smoking harms one's health, since it directly exposes the airways to cigarette smoke. This exposure makes the lung tissue more sensitive to house dust mite allergens [58]. Whether smoking itself increases the ability to develop allergies is unknown, but smoking does result in a faster enhancement of allergies. However, although some groups showed that smoking is associated with increased total IgE levels [58-61], others showed that smoking is associated with reduced allergic sensitization to some allergens [58, 62-64]. In contrast, the house dust mite sensitization was increased [47]. Since eczema is an allergic response in the skin, and house dust mite causes inhalant allergies, the distinction between the effect of smoking on these two different allergic complaints might indicate that the location of exposure might be important.

Impact of allergy

Besides the biomedical perspective, it is also important to interpret and respond to initial physical changes and development of clinical symptoms, their consequences for daily life and the adaptation to cope with all of these aspects. Chronic illnesses, like allergy, are complex and are affected by psychological, social and cultural factors [65]. The decision whether somebody considers himself sick is based on a series of considerations that range from initial symptom recognition, to family, work and other aspects of a person's social life, and finally to help-seeking responses and treatment susceptibility [66]. Symptoms for which people seek medical attention are not always more severe than symptoms that are not checked by medical staff. When these symptoms interfere with the patient's life and when adjustments to clinical symptoms of allergy made by the individual fail, people perceive themselves as sick and seek medical advice. The various adaptations that allergic patients make, provide leads for individual and social actions. Through normalization, allergic patients (including children) seek to retain a normal life by not presenting their allergy as being problematic rather than taking extreme measures of allergen avoidance. It is suggested that through these actions, allergic individuals wish to stay close to their personal identity [67]. However, coping with allergies requires adaptations in a manner in which a person

responds and adapts behaviorally, cognitively, and emotionally [11]. These adaptations comprise for example change of diet, household activities, physical activities, social contacts and economic costs. Besides physical and material adaptations, also emotions are influenced by having allergies.

Psychological impact of allergic responses

Children with asthma feel angry, afraid and frustrated. These children can also experience feelings of deprivation, guilt, loneliness, and anxiety as well as feelings of lack of confidence [68, 69]. Children with such emotional problems show limited performance in physical education, leisure activities, sports and participation in school [68]. Furthermore, stress correlates negatively with children's Quality of Life (QoL) [69]. However, not only children have fears because of their asthma, also their caretakers, both at school and at home, experience concerns about not being able to manage children's symptoms during their supervision [68]. Other social-psychological variables are the attitudes of the parents. Rydström *et al.* state that when the fathers show feelings of liberation and acceptance, this is positively related to the QoL of asthmatic children, while the protective attitude and showing sadness feelings of the mothers results in the opposite effect [69].

QoL studies with adults show that asthma limits activities associated with mobility, self-care, leisure and social life, work and communication. Also the social network is particularly important in diseases like asthma where persons must learn to self-manage their condition at home. Strong social networks can enhance a person's sense of self-efficacy, mastery, self-esteem, and facilitate self-management behaviors [11]. However, when dealing with asthma implies avoiding smoky places like restaurants or pubs, or even more inconvenient, smoking friends, this can have a large impact on one's social network. It is shown that non-supportive networks impede healthy behaviors and influence the QoL [11].

Moreover, family responses to the demands of caring for an asthmatic child can cause detrimental changes to the family as a whole [11]. Siblings often have to adapt to the same changes in lifestyle. Especially when this means that a pet animal has to leave the house, or specific food ingredients can not be eaten anymore, or of a make-over of the home's interior, all members of the household are affected. In addition, the constant need to recognize the signs of inadvertent ingestion can be very stressful [70]. Therefore, it is important that clinicians include family structure, activities, work/home patterns, living arrangements, and the impact of life events in the health examination of an allergic patient [11].

Economic impact of allergic responses

The economic impact of allergies is widespread and affects many sectors of society, with associated costs to allergics, their caretakers and their households, potentially over a lifetime [70]. Miles *et al.* made a framework for the analysis of the economical impact of food allergies. This framework distinguished the following actors or agents: consumers, caretakers, health services, food industry, and employers in general and regulators. All these stakeholders taken together are part of society and of the national economy, and are all interdependent [70]. The costs of allergy can be described under three categories: 1) direct costs, 2) indirect costs and 3) intangible costs [70].

Direct costs of allergic responses

Direct costs are expenses that are made in order to cope with or prevent allergies. Examples of direct costs are medical and non-medical care costs. Direct medical care costs contain allergy testing, medication and hospitalization costs [71]. However, most hospital treatment is expensive, but among food allergy sufferers hospitalization is less common; many people practice self-treatment when ill and are never admitted. In addition, the majority of costs do not belong to the formal health sector [70].

Besides expenses for alternative medicine, direct non-medical costs are an important factor. Examples of direct non-medical costs are travelling expenses to get to the doctor or hospital, but also substituting for chores that can not be performed by the allergic person and requiring childcare [70, 71]. Obtaining information about allergies by internet access, purchasing books, and traveling to training and self-help groups run by patient organizations also belong into this category [70]. There may also be costs associated with unsupervised dietary regimes, which may compromise nutritional status especially in children [70].

Indirect costs of allergic responses

Indirect costs are difficult to establish, since many consequences can be included. Factors included in indirect costs are loss of income due to loss of working days, school days and not being able to perform every day chores within the household [71]. Another definition of areas within the indirect costs is to include lost productivity, restricted activity, adaptations within jobs, and regulation within the food industry [70]. The costs of society of lost production affects both individuals and their employers. Further, indirect social costs may arise through regulation of the food industry, whereas the reliability of food safety can also increase. On the other hand, industry regulation can also increase public and consumer confidence in food products [70], for example, by increasing the clarity of ingredient labels and allergenic

content information. Nevertheless, literature indicates that the indirect costs of asthma associated with time lost from work by the parents, disruption of family life and of the child's school activities are almost twice as high as the direct cost for patients 18 years and younger [68].

Intangible costs of allergic responses
Intangible costs are caused by the loss of benefit arising from pain, suffering and inconvenience, or other effects on QoL. QoL encompasses factors like health, financial security, standard of living, family and friends, and spiritual commitment [70]. An example of intangible costs is the adaptations made within the household to make the QoL of the allergic patient better, like laminate floors instead of carpets, lamella instead of curtains and mattress and/or pillow covers [71, 72]. Also the removal of pet animals from the household is considered as an intangible cost. These adaptations should than improve the health-related QoL, which is the individual's perception of the effects of an illness, and its treatment thereof. It therefore includes aspects of physical, psychological and social well-being and functioning [70]. For example, the fear of an allergic response to food may lead to a diminished QoL. In addition, shopping for non-allergenic foods may be a source of anxiety and stress since alternative foods may be only available in specialist shops and label reading is needed for every product [70]. Other social activities like eating in a restaurant or at home with family or friends may result in accidental ingestion of hidden allergens with unknown consequences. Prevention of this risk by avoiding these situations, affects the social contacts of the household in a negative way.

Policy measures

Novel foods
Besides known allergens, more and more novel foods are entering our society. Novel foods are defined as foods or food ingredients that have no history of safe use in the European Union. This means that foods that are genetically modified, treated with novel processing techniques or introduced as new to the region might contain proteins that classify as allergenic and should therefore be investigated before allowing them to enter e.g. in the European Union [73]. On the other hand, novel foods could be beneficial for allergic consumers since an altered protein could be used as desensitizer, or genetically modified hypo-allergenic foods could be offered [73]. This means that, despite some risks, novel foods have the potential to contribute to food allergy management, when the allergenicity is carefully assessed [73].

Food safety legislation

Food safety regulation and quality improvement can impose significant costs for producers. When a manufacturer can show that the product is of low allergenic potential, the price of the product can be increased when its use is considered to be a socially responsible act. The increasingly strict production conditions may not only impose costs or loss of markets, but also give rise to new markets and diversification. However, the responsibility of the production of low allergenic food is not only dependent on the manufacturer, but also on the suppliers of raw materials [70]. By opting for food-labeling legislation, the World Health Organization is trying to make manufacturers responsible for checking up on the quality control of their raw material suppliers and their own production processes (chain management). Nowadays, this responsibility leads to many 'May contain' declarations on food labels, since this is a relatively cheap way to have a short-term solution to cover the costs involved in meeting the demands of legislation [70]. Allergic consumers experience this as highly undesired practice, since it causes unnecessary restrictions in the diet of food-allergic consumers instead of giving them more security about their food choices [2].

Advisory role

Family, social, and behavioral factors indirectly affect disparities by means of the impact on resources to which persons have access and by the health behaviors they perceive to be important vs. those which they neglect [11]. Therefore, addressing the general population in order to provide them with a better general health should be a governmental issue. In the Netherlands, the recommended half an hour of physical activity a day is not reached by 75 per cent of the adolescents and 50 per cent of the adult population [10]. Promoting physical activity is one of the aims of health education efforts. Therefore, several theories about how to change these lifestyles have been published, recommending to map people's knowledge about the risks of a certain behavior and investigate their and others' attitudes towards this behavior. Based on that, changing unhealthy behaviors can be mediated by problem specific interventions [10]. Ideally, people can improve their health by choosing to change their lifestyle, but such changes are more sustainable if they are supported by changes in the social and physical environment [10].

Reduction of the use of medical services and asthma medications could be reached through environmental interventions and will thereby provide a cost-effective method for improving the QoL of both allergic and potential allergic individuals. However, whether the possible interventions alone will ever be sufficient to replenish the medication, remains doubtful according to Peat. In addition, he claims that it is difficult to estimate the economic effectiveness of preventive strategies, because studies on this matter are still scarce [34]. We therefore propose that governmental advice towards preventive

lifestyle factors would be of interest to both allergic individuals and non-allergic citizens.

Conclusions

As mentioned before, the impact of allergies does not only influence individuals, but the whole society. Although insights in the immuno-pathology of allergic diseases have been improved, the development of preventive lifestyles and advising allergic persons which lifestyles could help to relief or prevent new allergies still needs attention. The determination of which lifestyles actually contribute to allergic sensitization and challenge requires a well-defined combination of allergic status and mapping lifestyles. Therefore, research in which information from blood samples of patients with inhalant allergies and/or food allergies is linked to information from a socioeconomic questionnaire on lifestyles is considered a necessary and timely approach.

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CHAPTER

8

The FREYAL study – Factors Related to Your Allergies and Lifestyle

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Intermezzo

FREYAL, the name of this study, stands for Factors Related to Your Allergies and Lifestyle. The name itself also has a clear link to historical aspects. In ancient history, birch trees had a magical meaning, and were worshipped as sacred trees long before the oak and lime tree. It is the tree of birth, since it is one of the first to get leaves and thereby signaled the farmers that the early wheat could be sowed. The bright white barks were perfect for a magic goal: you could write a wish on it, or something you wanted to ban and subsequently burn it. Another advantage of the birch bark is that it will not easily rotten, since it contains a lot of betuline, which preserves the bark similar to resin preservation. Therefore, the branches of the birch were useful as brooms. In the Scandinavian countries, the birch was the sacred tree of the Goddess Freya. Freya was therefore used as starting point for the study, but an important factor was missing: lifestyle. By adding the "L" in the shape of the birch tree, the name FREYAL was symbolized in the logo of the study.

From February 2007 until February 2008, individuals displaying complaints comparable to allergic symptoms were tested for allergen-specific IgE levels in their blood. Of these individuals, 184 persons filled in an informed consent to perform additional birch pollen and birch pollen cross-reactive food allergen specific IgE level determination. All persons were requested to participate in the second part of the FREYAL study, in which they were asked to fill in a web-based questionnaire concerning their lifestyle.

The FREYAL study intended to follow participants for several years (Longitudinal study). However, only one year of study has been accomplished so far. The original setup of the FREYAL study is described on page 199 till 203. Since the number of participating individuals is less than required, this chapter will be limited to an overview of the longitudinal data base and the results obtained with the current participants. The current data shows that although the proposed 5000 participants is required to obtain conclusive answers, already some lifestyle factors are shown to be different when comparing non-allergics to single and/or multiple allergics, indicating the usefulness of the questionnaire to assess lifestyle differences.

The initial goal of the FREYAL study

The initial goal of the FREYAL study was to assess which lifestyle factors determine the risk to develop a birch pollen related food allergy. The immuno-pathological changes (more details on page 200) at the transition of hay fever towards pollen-related food allergy will be assessed by analyzing the cell surface markers and cytokine expression profiles of peripheral blood mononuclear cells of the respondents. The lifestyle factors will be measured by means of a web-based questionnaire. The respondent population will be carefully selected from participating general practitioners' labs testing the potential allergics on inhalant allergies. People that obtain a negative outcome, therefore without an inhalant allergy, will be asked to participate as non-allergic control group.

This study was designed to combine lifestyle determinants from the questionnaire to the data derived from the corresponding blood cell cultures. This multidisciplinary approach will eventually provide hay fever patients with an advice to adopt a lifestyle that will prevent the onset of cross-reactive food allergies. In our view, this multidisciplinary approach is required to study a multifactorial disease like allergy.

Number of participants for the longitudinal study

Ideally, 5000 individuals should be obtained to get proper statistical power. This number of participants was based on the following assumptions:

1) Participants need to sign an informed consent after reading the study information at home. We accounted for a maximum loss of 20% due to unreturned informed consents.
2) To obtain correlations between lifestyles and allergic outcomes within our study population, we want to analyze the data by means of a split-sample analysis. This means that two-third of the participants will be used to find the initial correlations (Group I), which thereafter will be validated in the remaining one-third of the study population (Group II).
3) Our questionnaire comprises about 30 variables. For multivariate statistical analyses, at least 10 to 15 respondents per variable are required according to the conventional statistical methodological handbooks [1, 2]. This means that the non-allergic group must consist of at least 750 respondents (500 for Group I, 250 for Group 2), which also applies to the hay fever patients plus food allergy.

For patients that suffer from hay fever due to birch trees, literature indicates that 50 – 70% of the birch pollen allergic individuals will develop cross-reactive food allergies [3]. When we initially start with 2500 hay fever patients, and 50% of them will develop cross-reactive food allergies, we will have between 1000 to 1500 participants within the transition group (Figure 1:

group 2 and 5). However, it is unknown how long the transition from hay fever due to birch pollen to cross-reactive food allergy takes. Since this transition group is our primary interest, and we prefer to retain 750 people in this group, 2500 participants with hay fever will be enrolled. This way, all groups will contain comparable numbers of participants, making adequate statistical comparisons possible.

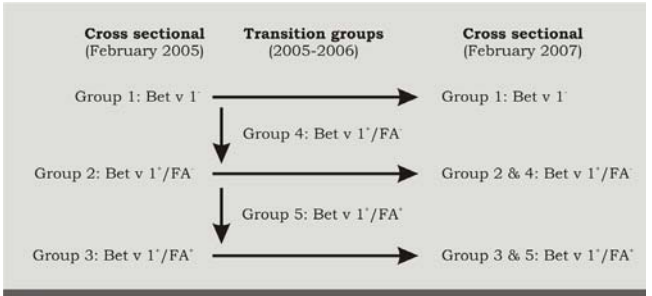

Figure 1. Research groups in the longitudinal FREYAL study. This figure provides an overview of the groups that will be included in the FREYAL study. Group 1 is the control group, containing individuals that thought they experienced allergic complaints, but their IgE levels were below detection limit. Group 2 consists of people that are allergic to birch pollen (Bet v 1⁺), but do not experience any cross-reactive food allergies (FA⁻). The last cross-sectional group, group 3, contains people that have both hay fever due to birch pollen (Bet v 1⁺) and experience complaints towards cross-reactive foods (FA⁺). Over time, people that did not have any allergies at the start, might develop an allergic disease during the study-period. The individuals that fall into this category will be assigned to group 4, whereas people that had no cross-reactive food allergies at the inclusion, but do develop them during the study-period are placed in group 5.

Number of participants for the mechanistic study

The immunopathological parameters should be assessed for at least 200 respondents divided over the five groups described in Figure 1. This number is based on the following assumption: whenever a clear deviation in the regulation or the induction of a single immune response is expected, around 20 individuals per group will be sufficient to perform a proper statistical analysis. However, when an array of variables plays a role in the deviation between allergic and non-allergic individuals, this will probably not lead to statistically reliable results. If this is the case, the obtained correlations will need to be verified in a larger population, which does not fit the capacity of the current lab facilities. This will than be considered as a subject for further research.

Of these 200 people, the peripheral blood mononuclear cells will be used to predict the mechanisms involved in the disease development path, and thereafter, these parameters will be linked to lifestyle factors to unmask factors that alter the risk of developing cross-reactive food allergies.

Assessing allergy-related lifestyle factors

As mentioned in Chapter 7 of this thesis, reduction of the use of medical services and asthma medications could be reached through environmental interventions. However, whether the possible interventions alone will ever be sufficient to replenish the medication, seems to be highly doubtful. We therefore believe that governmental advice towards preventive lifestyle factors would be of interest to both allergic and non-allergic citizens. In order to develop these preventive strategies, the lifestyle factors differing between allergics and non-allergics should be traced. We therefore developed a specific questionnaire for the FREYAL study, based on the literature study described in Chapter 7 of this thesis. The topics addressed in the questionnaire are (number of questions between brackets): demographic variables (12), health and allergies (53), residence and domestic animals (19), nutrition (17), stimulant use (10), hygiene (13), work and education (19), leisure activities (16), knowledge about allergies (4), and socio-psychological variables (3).

Optimizing the FREYAL questionnaire

Assessing whether all questions are interpreted in the right way is necessary to gain reliable and valid answers to the web-based questionnaire. Face-to-face testing of the questions is an accepted way to test whether the questions are interpreted in the intended way by the respondent [4, 5]. An additional advantage of this interview method is the direct contact between the respondent and the interviewer, adding physical appearance or gestures as information to judge whether the questions are answered in the intended way. Indistinctness can immediately be addressed and lead to the reformulation of the question into a clear one. By asking the respondents what kind of questions they missed or disliked, the questionnaire was optimized.

The FREYAL study was designed as a survey. Therefore, the preliminary study was set up in an identical way. The questionnaire contained both closed questions and semi-closed questions. In closed questions, all possible answers are numbered. In semi-closed questions, the opportunity to fill in a personal answer is added, next to pre-coded answer categories.

The preliminary study

The respondent population was divided into three categories: 1) non-allergic individuals, 2) people with hay fever, and 3) people with hay fever and one or more food allergies. By discriminating on the basis of the allergic status, it was intended to assess the mutual relationship between lifestyles and consumption patterns on the development of food allergies. The minimum

age of the respondents was set at 18 years. Hay fever mostly starts at the adolescent age, and the cross-reactive food allergies develop somewhat later. However, to avoid double informed consents and ethical objections, only adults are asked to participate. In addition, in this age category, new lifestyles develop when leaving the parental home, for cohabitation or living on one's own.

The sampling of respondents was performed according to the snowball method. This method starts with a small group of people belonging to a target group, and asking them to name people that also fit the specific target group.

To ascertain that all determinants were correctly sorted out, three test interviews were done. Thereafter, the final questionnaire and interview methods were established for all subsequent interviews. All interviews were performed at the respondents' homes. On average, the interviews of non-allergic individuals took 25 minutes, of hay fever patients 40 minutes and of people with multiple allergies 45 minutes.

This preliminary study resulted in a validated questionnaire that was suitable for the subsequent FREYAL study. The questions were found to be clear and the routing of the questions correct. Some respondents indicated that the questions concerning the socio-psychological variables were difficult to understand. Two experts on this matter were consulted and the questions were subsequently adjusted.

Conclusions of the preliminary study
The main conclusions based on this preliminary study were:
1) Despite their allergic complaints, allergic individuals judged their health to be very good or fairly good, 2) the hay fever group had a significantly higher BMI than the other two groups, 3) no differences in smoking, alcohol consumption, nutritional status and playing sports were shown between the non-allergic individuals, hay fever patients and hay fever patients with cross-reactive food allergies, and 4) the quality and quantity of cleaning was positively correlated to the prevalence of allergies.

The longitudinal FREYAL study data base

The FREYAL study was conducted in collaboration with the general practitioner (GP) laboratory in Velp, The Netherlands, which took care of the blood collection and the allergy testing. Whenever a GP requested an inhalant allergy test at the GP laboratory, the patient was asked whether he or she was willing to participate in the FREYAL study. In case the patient was

interested, he or she was asked to give permission to store the remaining serum after the requested allergy testing, and was provided with an information brochure about the FREYAL study to read at home. Whenever the patient felt like participating in the full FREYAL study, he or she was asked to return the completed informed consent form to Wageningen University. Once this informed consent was received at Wageningen University, the researcher contacted the GP laboratory to complete the relevant allergy tests.

The blood of every participant of the FREYAL study was first screened on the presence of allergen-specific antibodies (IgE) against birch trees. Whenever birch pollen-specific antibodies were detected, additional analyses of apple-, carrot-, and/or celery-specific antibodies were carried out. In addition, every participant was asked to fill in the web-based questionnaire to establish their current lifestyles. This way, socio-economic lifestyle factors could be linked to the presence of allergen-specific antibodies. In doing so, we would be able to point out lifestyles that should be avoided in order to prevent the development of birch-pollen specific cross-reactions to food compounds.

For the FREYAL study, the website www.freyal.wur.nl was launched. Via this website, people could register for the questionnaire. After registration, the volunteers received an email containing a link to their personal questionnaire.

Whenever a participant started the questionnaire, and could not answer all questions at once, the link mentioned above would lead him or her back to the questions to be answered later on. In this way, we hoped to increase compliance to answer all questions. Another advantage of using a web-based questionnaire, is that questions are tailored to individual circumstances and that irrelevant questions are not shown, thereby making each questionnaire personal.

Data reduction

By reducing the number of variables, the statistical power of testing for differences between the individuals based on their allergic status was increased. The reduction method used for each new variable is described below.

Number of allergies

The number of allergies was derived from five dichotomous (Y/N) questions about allergies. Each category (hay fever, food allergy, HDM allergy, other allergies, and no allergies) was given one point if answered with “Yes”, resulting in a scoring range from 0 to 4.

Living environment
Eight dichotomous (Y/N) characteristics of the living environment were combined into three variables: 1) City, 2) Rural area and 3) Close to water.

Infection, surgery and antibiotics
Four questions about whether the respondents ever experienced a serious infection, with or without the application of antibiotics and whether the respondents ever underwent surgery with or without antibiotics were grouped by giving each of the above questions answered with "Yes" one point. Therefore, this variable ranged from 0 to 4.

House dust mite (HDM)-free household maintenance
This variable contained questions about the isolation of the house, aeration of the living room and bed room, the presence of loose rugs on the floor, and the type of window and floor furnishing. For all questions, answer options leading to better cleaning results to minimize the HDM concentration, were added. Therefore, the higher the resulting number, the lower the exposure to house dust mites within the domestic living environment. These items were initially weakly related (α=0.39), but after the removal of aeration, a Cronbach's α of 0.71 was obtained.

Nutritional awareness
The common variance of four propositions about nutritional awareness was assessed by using factor analysis. One factor accounted for 40% of common variance, resulting in a Cronbach's α of 0.65. Hence, answers to these four propositions were summed to form the new variable Nutritional Awareness; the higher the number, the higher the nutritional awareness of the individual.

HDM-free sleeping environment
The questions about using an anti-allergic mattress cover, removing dust from the mattress by vacuum cleaning, regular washing of bed covers, and the temperature at which the bed linen was being washed were summed into the variable HDM-free sleeping environment. The higher the number, the lower the exposure to HDM while sleeping in bed.

Sensible hygienic behavior
Thirteen dichotomous (Y/N) characteristics of the household cleaning habits were combined. Examples are "The bathroom is cleaned at least once a week", "The dishcloth is daily replaced by a clean one", "After cutting meat or fish I always wash my hands with soap", and "The refrigerator is thoroughly cleaned at least once every 6 months". The common variance of sensible hygienic behaviors was assessed by using factor analysis. One factor accounted for 20% of the common variance, resulting in a Cronbach's α of 0.69. Each

variable answered with "yes" added one credit to the score, eventually leading to a maximal score of 13, corresponding to very sensible hygienic behavior, whereas a low score indicated less sensible hygienic behavior.

Allergy knowledge
To obtain a variable corresponding to the knowledge participants gained about their allergy, we combined the questions on whether people were members of an allergy association and whether they searched for information about specific allergy-related topics. Therefore, the higher the value, the more knowledge they had about allergies. These questions were shown to be highly related, as the resulting Cronbach's α was 0.82.

Restraints due to allergy
The four items "I experience my allergy as a disease", "It is difficult to live my life with my allergy", "I have to cancel a lot because of my allergy", and "I think it is hard to cope with my allergy" are all dealing with social restraints because of allergies and appeared to be highly related (Cronbach's α was 0.74). Therefore, these items were equally weighted and combined, leading to a variable that showed a worse social restriction when the number was higher.

Influence of Allergy on social contacts
The four items "People in my living environment reckon with my allergy", "My allergy does not influence the amount of social contacts I have", "My social environment has accepted my allergy", and "I would only start a love affair when he/she accepted my allergy", were all dealing with how allergies influence the social environment. Although weakly related (Cronbach's α of 0.26), these questions were equally weighted and combined, leading to a variable that showed a large influence of allergies on the social environment when the number was higher.

Locus of control
The statements used to assess the locus of control were adapted from Phares [6]. Seven pairs of statements were presented to assess whether an individual had internal or external control beliefs. Of any pair of statements, participants were forced to choose one. Each choice for the internal statement added one point to the internal control scale. Therefore, the higher the sum of the statements, the more a participant believed in an internal control.

Overview of lifestyle variables after data reduction
Table I shows an overview of all lifestyle variables obtained after data reduction. Statistical differences between the non-allergic, single-allergic and multiple-allergic participants are described lateron.

Table I. Overview of average lifestyle scores after data reduction

Lifestyle variables	Answer categories	Mean			
		All respondents (n=75)	No Allergy (n=26)	One Allergy (n=21)	Multiple Allergies (n=28)
General health	Min.: 1; max.: 7 [a]	2.17	1.92	2.33	2.29
Healthy enough for activities	Min.: 1; max.: 5 [b]	1.69	1.40	1.76	1.89
Health compared to others	Min.: 1; max.: 6 [c]	3.08	3.38	3.00	2.86
BMI score	Min.: 1; max.: 3 [d]	2.32	2.42	2.19	2.32
Allergic family members	Full range	1.83	1.81	2.52	1.32
Surgery and infection risk	Min.: 0; max.: 4	0.68	0.77	0.71	0.57
HDM-free living environment	Min.: 0; max.: 20	15.15	14.81	13.81	16.46
Number of pets		1.32	0.85	1.81	1.39
Regularity in eating habits	Min.: 0; max.: 35	22.28	21.96	22.48	22.43
Nutritional awareness	Min.: 0; max.: 13	11.71	12.85	11.24	11.00
Exposure to tobacco smoke	Min.: 0; max.: 4	1.23	1.38	1.29	1.04
Alcohol use	Min.: 1; max.: 8 [e]	2.20	2.23	2.52	1.93
HDM-free sleeping environment	Min.: 0; max.: 4	2.29	2.19	2.14	2.50
Sensible hygienic behavior	Min.: 0; max.: 13	9.84	9.42	9.62	10.39
Knowledge about allergy	Min.: 0; max.: 12	n.a.	n.a.	3.10	3.00
I experience restraints	Min.: 1; max.: 5 [f]	n.a.	n.a.	2.08	2.27
Influences my social contacts	Min.: 1; max.: 5 [g]	n.a.	n.a.	3.57	3.63
Internal locus of control	Min.: 0; Max.: 7	3.31	3.04	3.26	3.32
Cantril scale	Min.: 0; Max.: 10	7.60	7.85	7.41	7.52

n.a.: Not assessed, not relevant for non-allergic individuals.
[a] 1: very good; 2: good; 3: fairly good; 4: Intermediate; 5: Fairly bad; 6: Bad; 7: Very bad.
[b] 1: always; 2: most of the time; 3: Sometimes; 4: Seldom or never; 5: No opinion
[c] 1: Much worse; 2: Little worse; 3: equal; 4: little better; 5: Much better; 6: No opinion
[d] 1: too low; 2: healthy; 3: too high
[e] 1: 0; 2: ≤5; 3: 5-14; 4: 15-24; 5: 25-34; 6: 35-44; 7: 45-54; 8: 55 or more glasses/week
[f] 1: Totally disagree; 2: Largely disagree; 3: I neither agree nor disagree; 4: Largely agree; 5: Totally agree
[g] 1: Totally disagree; 2: Largely disagree; 3: I neither agree nor disagree; 4: Largely agree; 5: Totally agree

Results

Allergen-specific antibody testing in serum

In total 184 persons that went to the GP with symptoms that resembled allergic complaints, were tested in a sequential manner as depicted in Figure 2. When a person responded to the mixture of ALATOP allergens (positive), this meant this persons had IgE isotype antibodies against one or more allergens. Since it was a qualitative test, after a positive result, further allergen-specific tests needed to be performed to obtain quantitative IgE levels. Of the 184 persons positive for the ALATOP, 87 persons had no allergen-specific IgE and were therefore considered as non-allergic. The other 94 individuals were thereafter tested for the house dust mite, cat, dog, herb, tree pollen, grass pollen or fungal panel as depicted in Figure 2. Table II shows the number of positive individuals for these and sequential birch pollen and apple, celery and carrot IgE. A substantial number of individuals was allergic to more than one allergen.

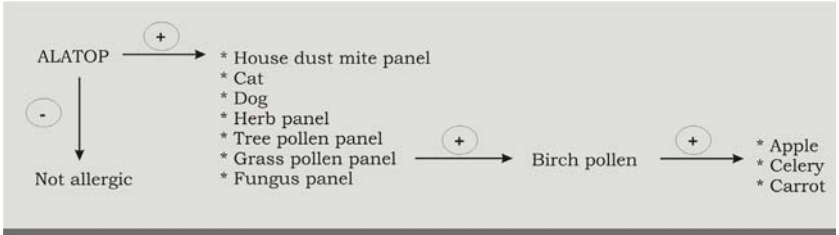

Figure 2. Allergen-specific IgE testing schedule

Table II. Number of allergic respondents by allergy type

House dust mite	65
Grass pollen	39
Cat	36
Herbs	20
Dog	18
Fungi	3
Tree pollen	54
Birch pollen	54
Apple	37
Celery	11
Carrot	9

A substantial number of individuals was allergic to more than one allergen.

It is noteworthy that all individuals responding to the tree pollen panel also responded to the birch pollen, indicating that the birch tree was a major sensitizer in our study population. In addition, 35 of these 51 individuals (70%) already developed a cross-reactive food allergy to apple, whereas only 20% and 17% responded to celery and carrot, respectively.

The amount of allergen-specific IgE present in the serum is generally classified in six classes: class 0) < 0.35 kU/L, class 1) 0.35 – 0.69, class 2) 0.7 – 3.4, class 3) 3.5 – 17.4, class 4) 17.5 – 50, class 5) 50- 100, and class 6) > 100 kU/L. The higher the class, the more allergen-specific IgE antibodies are present in the serum. Since the maximal detection limit of allergen-specific IgE has a cut-off at 100 kU/L, observations with class 6 are often depicted as "100 kU/L". As shown in Figure 3, most individuals that responded to apple, carrot or celery, also displayed a >100 kU/L on the birch pollen specific IgE level.

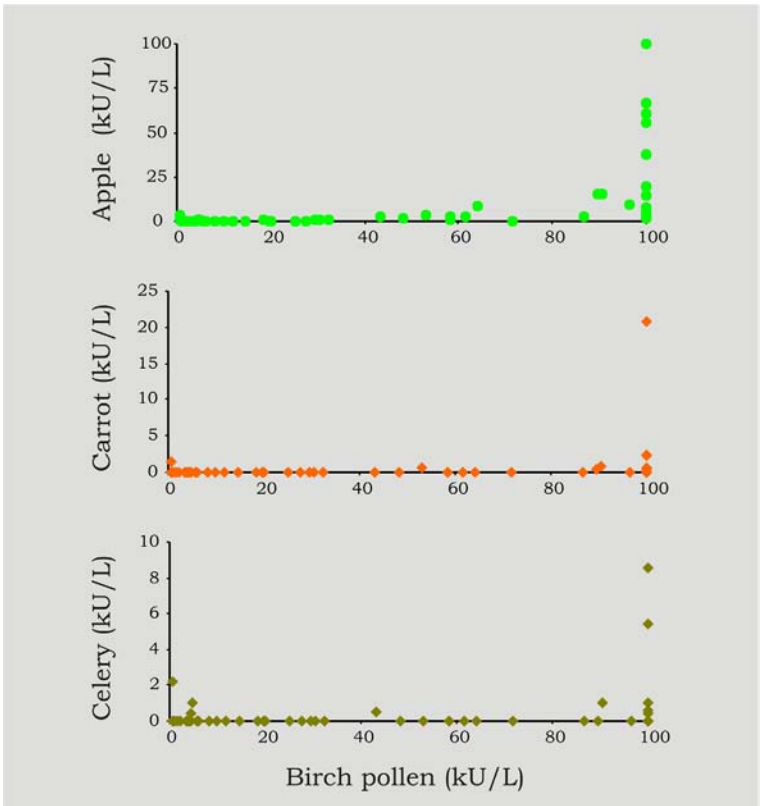

Figure 3. Relation between levels of birch pollen specific IgE and cross-reactive food allergens in apple, carrot and celery.

Respondents profile

Of the 184 blood donors, 87 individuals classified as non-allergic, 29 individuals were single-allergics, and 68 were multiple-allergic. Of these individuals, 19 non-allergics, 21 single-allergics and 28 multiple-allergics also completed the questionnaire. To enlarge the non-allergic group, seven non-allergic individuals that fitted the demographic characteristics of randomly selected allergic individuals were asked to fill in the questionnaire. In total, 26 non-allergic individuals, 21 single-allergic individuals, and 28 multiple-allergic individuals filled in the questionnaire. Our respondent population therefore contains an under-representation of the non-allergic individuals and an over-representation of the single-allergic individuals. This section gives an overview of the socio-economic characteristics of these 75 respondents. The socio-economic characteristics comprise gender, age, household size, and educational level.

Gender and age

The total respondent group contained 39% males and 61% females. Within the non-allergic group and the multiple-allergic groups this ratio was the same as the overall ratio, whereas the single-allergic group contained approximately the same number of males and females (see Table III).

The overall age ranged from 19 to 78 years. All three groups contained a couple of people older than 35 years, but these individuals were tested in the same way and therefore still included. The overall average age and the average age within the single-allergic group was 33 years, whereas the average in the non-allergic group was somewhat higher, and in the multiple-allergic group somewhat lower (see Table III).

Table III. Socio-economic profile by allergy group

	Total n=75	No allergy n=26	One allergy n=21	Multiple allergies n=28
Gender (%)				
Male	39	38	43	36
Female	61	62	57	64
Age (years)				
Average	33	36	33	29
Lowest	19	19	19	20
Highest	78	78	51	59
Household size (%)				
1 person	24	31	19	21
2 persons	39	42	29	43
3 or more persons	37	27	52	36
Educational level (%)				
Lower education	8	8	5	11
Intermediate education	45	42	52	43
Higher educational	47	50	43	46

Household size
The term household refers to all individuals that live in the same dwelling. Overall, 24% of the participants lived in a one-person household, whereas 39% and 37% were living in two-person or three-and-more person households, respectively, as shown in Table III.

Educational level
The educational level was based on the following categories: 1) Lower education (primary school, LBO and VBO), 2) Intermediate education (MAVO, MULO, MBO, HAVO, VWO and HBS), and 3) Higher education (HBO, university).

Overall, 8% of the respondents were educated at a lower level, 45% had an intermediate education level and 47% had a higher educational level. Comparable ratios were found when subdividing the respondents into groups based on their allergic status (Table III).

Allergy-causing agents

Hay fever patients
The respondents were asked to indicate to which pollen they had a proven IgE-mediated allergy. Table IV shows that the majority of hay fever allergic individuals was allergic to birch trees (59%), followed by hazelnut trees (21%) and alder trees (14%), whereas only few individuals were allergic to grasses.

Table IV. Types of hay fever in hay fever-allergic individuals (n=29)

Trees	Latin names	Allergics (%)	Age (low-high)*
Birch	*Betula Pendula*	59	3-48
Hazelnut	*Corylus avellana*	21	10-32
Alder	*Alnus glutinosa*	14	15-32
Elm	*Ulmus hollandica*	10	10-34
Oak	*Alnus incana*	7	10-34
Ash	*Fraxinus excelsior*	7	10-15
Grasses			
Bluegrass	*Poa pratensis*	10	0-34
Bermuda grass	*Cynodon dactylon*	7	0-34
Ryegrass	*Lolium perenne*	7	0-34
Orchardgrass	*Dactylis glomerata*	7	0-34
Timothy grass	*Phleum pratense*	7	0-34
Velvet grass	*Holcus lanatus*	7	0-34

*Age (low-high): the age range at which participants developed allergic complaints.

The age at which the allergic individuals developed the first symptoms of hay fever indicated that not only teenagers, but also thirty-year olds developed hay fever.

Food allergic patients
The respondents were asked to indicate to which food they had a proven IgE-mediated allergy. Table V shows that the majority of food allergic individuals was allergic to apple (50%), followed by cherry (36%) and pear, peach and peanut (29%). Some participants indicated to also be allergic to kiwi, brazil nut, apricot, carrot, strawberry, plums, cabbage, lactose, spinach, chocolate, wheat, and celery.

The age at which the allergic individuals developed the first symptoms of food allergy indicated that not only teenagers, but also adults of different ages developed food allergy.

Table V. Types of food allergies in food-allergic individuals (n=14)

	Allergics (%)	Age (low-high)*
Apple	50	15-48
Cherry	36	15-55
Pear	29	15-50
Peach	29	15-55
Peanut	29	20-32
Preservatives	14	30-32
Hazelnuts	14	20-26
Food dye	14	6-30
Melon	7	20
Tomato	7	36

*Age (low-high): the age range at which participants developed allergic complaints.

People allergic to animals, house dust mites or fungi
The respondents were asked to indicate to which animal they had a proven IgE-mediated allergy. Table VI shows that the majority of individuals was allergic to HDM (78%), followed by cats (48%), dogs (26%) and rodents (22%).

The age at which the allergic individuals developed the first symptoms of HDM or animal allergy indicated that not only teenagers, but also adults of different ages developed these allergies.

Table VI. Types of animal allergies in animal allergic individuals (n=23)

	Allergics (%)	Age (low-high)*
HDM	78	0-40
Cats	48	0-29
Dogs	26	0-29
Rodents	22	0-29
Horses	13	0-10
Insect venom	4	5
Fungi	4	12
Birds	4	5

*Age (low-high): the age range at which participants developed allergic complaints.

Healthy lifestyle

The lifestyle variable distribution was tested for normality with the Kurtosis analysis and skewness of the variables. Since the variables failed to show a normal distribution, all statistical analyses used to compare the different groups were performed with the non-parametric Kruskall-Wallis test. Taking into account that our study sample contained a small number of participants, $p<.10$ was considered to be significant.

Regularity in eating habits

Participants were asked whether they regularly ate breakfast, lunch and dinner, and whether they consumed fruit and snacks in between the meals. For each day, consuming breakfast, lunch, dinner, and two pieces of fruit, 5 credits were given. This led to a maximal score of (7 days times 5 credits) 35 credits. Therefore, the higher the number, the more regularly participants consumed food. No significant differences could be observed between the three groups (see Figure 4).

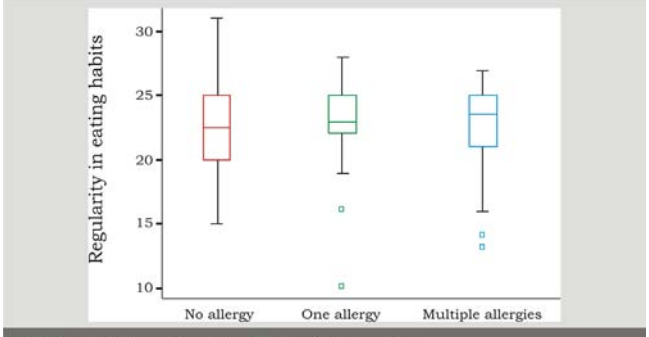

Figure 4. Regularity in eating habits by allergy type

Body Mass Index
The Body Mass Index (BMI) was calculated by dividing the weight in kilograms by the length in squared meters. Thereafter, the BMI was transformed into three categories: 1) too low BMI (<18.5), 2) healthy BMI (18.5 – 25) and 3) too high BMI (>25). Overall, most participants showed a healthy BMI, and all three categories were equally divided over the three groups. There was no significant difference between the groups (see Table VII).

Table VII. Body Mass Index classification by respondent group

BMI	Total	No allergy	One allergy	Multiple allergies
	n=75	n=26	n=21	n=28
< 18.5 (%)	4	0	10	4
18.5 – 25 (%)	60	58	62	61
> 25 (%)	36	42	28	35

Exposure to cigarette smoke
To assess the exposure to smoke, the questions about current or past personal smoking, smoking of members within the household and smoking during childhood were converted into the variable "Exposure to cigarette smoke" by adding all variables unweighted. As shown in figure 5A, most participants scored one point on the exposure scale, meaning they either smoke(d) themselves, or someone in their household was/has been smoking. When the data were divided based on the allergic status, the largest group of non-exposed people belonged to the multiple-allergic group. However, no significant differences between the non-allergic, single-allergic or multiple-allergic individuals were observed on exposure to smoke.

Alcohol intake
The participants were asked how many glasses of alcohol they on average consumed per week. The possible answers were: 1) None, 2) less than 5 glasses, 3) 5 to 14 glasses, 4) 15 to 24 glasses, 5) 25 to 34 glasses, 6) 35 to 44 glasses, 7) 45 to 54 glasses, and 8) 55 or more glasses. To obtain a continuous scale, all categories were replaced by the midpoint of each possible answer category. For example, 5 to 14 glasses was replaced by 10, 35 to 44 glasses by 40, etc.

As shown in figure 5B, 15 individuals hardly ever consumed alcoholic beverages, whereas the largest group consumed less than 5 alcohol containing drinks per week. Comparing the alcohol intake within the three allergy groups, the Kruskall-Wallis test showed a significant difference ($p<.10$). The alcohol intake in the multiple-allergic group was the lowest, followed by the non-allergic group.

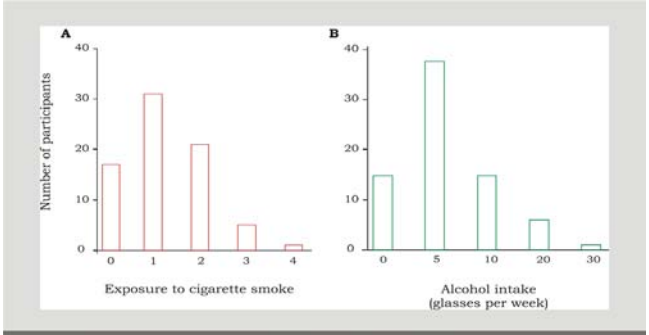

Figure 5. Exposure to tobacco smoke and alcohol intake

Time spent on sports and hobbies

More than 60% of the respondents spent time on sports every week. Approximately 50% of the respondents used its leisure time to participate in one or more hobbies. However, neither time spent on sports, nor time spent on hobbies was significantly different between the groups according to the Kruskall-Wallis test (see Figure 6). This was mainly due to the large standard deviation in the non-allergic group.

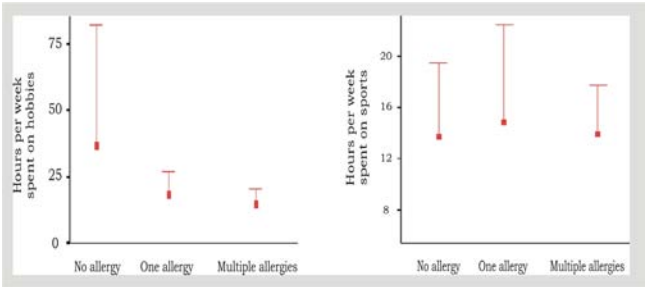

Figure 6. Time spent on sports and hobbies by allergy group
Error bars show the upper level of the 95% CI of the mean.

Well-being

All respondents were asked to imagine a ladder. The top of the ladder represented the 10 which equals the best life one could imagine. The 0 at the bottom represented the worst life one could imagine. Which grade would one then give one's overall life? On average, the participants graded their lives at 7.6. When the groups were divided based on their allergic status, the non-allergics graded their life at 7.8, the single-allergics at 7.4 and the multiple-allergics at 7.5 (Figure 7). These grades were not significantly different.

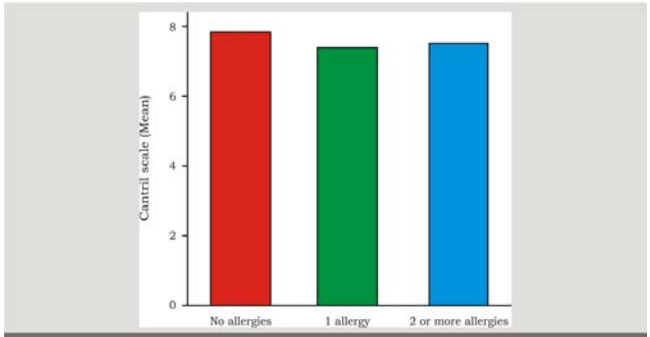

Figure 7. Cantril scale by allergy type

Internal locus of control

Seven pairs of statements were given to assess whether a participant possessed a more internal or external locus of control. The higher the number, the more a person tended to have an internal locus of control. The overall Internal locus of control score was 3.31, which indicated that the participants were not primarily internally or externally oriented, since the score could range from 0 to 7. When comparing the three groups, no significant difference could be observed (Figure 8).

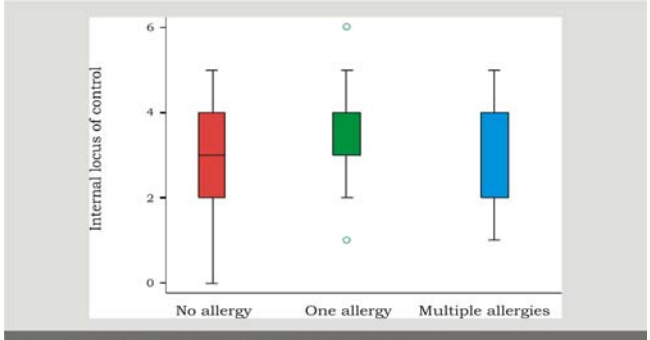

Figure 8. Internal locus of control by allergy type

Allergy within the family

Respondents were asked which family members in the first degree suffered from allergies. All indicated allergic family members were summed to obtain the total number of allergic family members. A significant difference ($p<.10$) between the number of allergic individuals in the first degree of family members was shown when comparing the three groups. The rank order showed that the multiple-allergics had less allergic relatives than the non-allergic individuals, and the single-allergic individuals had the highest number of allergic family members (see Figure 9).

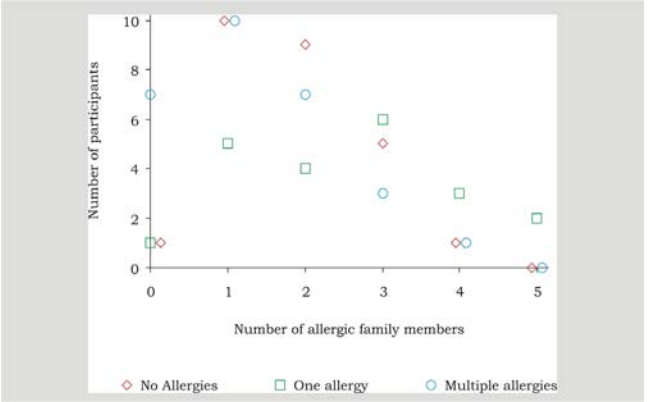

Figure 9. Allergic family members by allergy type

Lifestyle, health and well-being

Lifestyle differences between healthy controls and allergics

Non-allergics versus allergics.
To test for differences between allergic and non-allergic individuals, an independent sample t-test was performed. In this analysis, all allergic individuals, independent of the number of allergies, were grouped and compared to the non-allergic group. The analysis showed that Perceived health ($p<.05$), Opinion about own health ($p<.01$), Health compared to others ($p<.05$), Nutritional awareness ($p<.05$) and the Cantril scale ($p<.10$) were significantly different between the allergic and non-allergic individuals. This means that people without allergies were more positive about their health than allergic individuals, allergics more often had to cancel things because their health hold them back, non-allergics felt healthier compared to others than allergics did, non-allergics seemed to be more aware of nutritional values, and non-allergics graded their life higher than the allergic individuals.

Non-allergics versus single allergics.
To test whether lifestyle variables differed across non-allergic and single-allergic individuals, an independent sample t-test was performed. Perceived health ($p<.10$), Opinion about own health ($p<.05$), Health compared to others ($p<.10$), and the Cantril scale ($p<.10$) were still significantly lower, whereas also the genetic component (number of allergic family members) was found to be significantly higher ($p<.05$) when comparing single-allergic to non-allergic individuals.

Single versus multiple allergics.
To test for differences between single- and multiple-allergic individuals, the independent t-test was also performed on these two groups. The t-test showed a significantly higher number of allergic family members in the single-allergic group ($p<.01$). In addition, the alcohol intake in the single-allergic group was significantly higher than in the multiple-allergic group ($p<.05$). However, the multiple-allergic group showed a significantly lower effort in maintaining the household HDM free ($p<.05$).

Cluster analysis of all participants
To assess which lifestyle factors added to the risk of becoming allergic, we performed a cluster analysis on all participants' data. First, the proximities were calculated to determine the starting point of the data clustering, thereafter the data was clustered stepwise, and finally, the data was separated into two clusters: cluster 1 contained 38% of the participants, cluster 2 contained 62% of the participants. However, although both clusters differed in seven lifestyle factors, the number of allergic individuals between these two clusters did not differ. Table VIII shows the average lifestyle factors of the two clusters, concluding that individuals in cluster 2 showed more health-risk reducing behavior than the individuals in cluster 1.

Conclusions and discussion

Although the number of respondents at the time of analysis was relatively low, we did observe a number of differences. Whether these differences are robust needs to be assessed by completing the response of the initially determined number of participants. After these participant numbers are met, the data set can even be used to categorize the population based on the differences in allergic complaints, e.g. food allergy, hay fever, HDM allergy etc., to establish lifestyle differences between non-allergics and allergics. Besides a cross-sectional approach as applied in this chapter, also an analysis of the longitudinal data base is desirable. In a longitudinal approach, the lifestyles can be correlated with the chance of transition of hay fever to cross-reactive food allergies.

Although the participant recruitment of the preliminary study was different from the FREYAL study 2007, leading to a different participant population, both studies showed that allergic individuals judge their health to be good to fairly good. Since the preliminary study only compared hay fever patients to non-allergics, conclusion 2, 3 and 4 (see page 202) cannot be directly compared to the FREYAL study 2007.

Table VIII. Cluster analysis of all participants

Lifestyle factor	Cluster (average score)	
	1	2
General health	Good	Good
Healthy enough for activities	Most of the time	Most of the time
Health compared to others	Equal	Equal
BMI score	Healthy	Healthy
Allergic family members	2	2
Number of pets	1	1
Alcohol intake	= 5	= 5
Internal locus of control	3	3
Number of allergies	1	1
Cantril scale	7.3	7.8
Health risk reducing behavior		
Surgery & infection risk	0.50	0.80
HDM free living environment	12	17
Regularity in eating habits	20	24
Nutritional awareness	10	13
Exposure to tobacco smoke	1.39	1.13
HDM free sleeping environment	2.04	2.46
Sensible hygienic behavior	9	10

Of the 184 individuals that agreed with the additional allergy-testing on their blood, only 68 people completed the questionnaire on the internet. This low compliance might be due to (a combination of) different factors: 1) individuals assuming they were allergic, but did not get confirmation for this from the laboratory, might lose interest; 2) despite the fact that the information brochure was complete and written in a comprehensive way, it might still have been too long and too detailed for some individuals; 3) the used internet-based questionnaire might have scared off people; 4) the length of the questionnaire might have reduced the number of individuals that complete the questionnaire.

Although the 184 blood donors were a random sample of the general population, the 68 people that filled in the questionnaire were slightly biased. To be more precise, the non-allergic group accounted for 47% in the blood donor group, whereas their relative contribution decreased to 35% in the group that completed the questionnaire. However, the percentage of multiple-allergic individuals remained equal (38%), as a result of an increased percentage of single-allergic individuals (from 16 to 28%). The low participation of the non-allergic individuals might be explained by the fact that unless people are involved or affected by a certain phenomenon, like an allergy, they are less willing to participate in a study concerning this phenomenon.

Approximately 25% of the people with allergy-like complaints were proven to be tree pollen-allergic. All tree pollen allergics were also allergic to birch trees, which is in agreement with the statement that the birch tree is a major sensitizing tree in The Netherlands [7]. In addition, approximately 70% of the birch tree allergic individuals also displayed a cross-reactive apple allergy, which is in agreement with the findings of Bohle et al., who show that more than 70% of the birch pollen allergics become allergic to stone-fruits, nuts or certain vegetables [3]. Furthermore, we showed that mainly individuals that displayed a high birch pollen-specific IgE level, also displayed a cross-reactive response to apple, carrot and/or celery.

In the cluster analysis shown on page 218, the first nine items are not different between the two clusters. In the eventual analysis of the completed FREYAL study database, the equal items should be removed after the first analysis, and the remaining items thereafter tested on the split sample population. Although in this chapter these items might be irrelevant, after further completion of the database, these might again change.

Although a child without atopic parents has a risk of around 10% to develop an atopic disease during life, this risk increases to 20% and 50% when a child has one or two atopic parents, respectively. When both parents have the same atopic disease, the risks even increases to 70%. Also having one allergic parent and one allergic sibling increases the chance to become allergic to 30% [8]. Interestingly, the number of allergic family members in our study was shown to be significantly higher in the single allergics group, whereas the multiple allergics group even contained less allergic family members than the non-allergic group.

A similar phenomenon was observed in the alcohol intake in our study; the alcohol intake was shown to be significantly higher in the single-allergic group, whereas the multiple-allergic group consumed less than the non-allergic group. Alcohol intake in humans has shown a large impact on immune cells [9-11]. To our knowledge, no literature is available in which alcohol intake has been studied in groups with a specified number of allergies.

This discrepancy between the single- and multiple-allergic individuals might be due to the type and severity of the allergies. For example, a person with a 'mild' allergy like hay fever might be better capable to maintain their quality of life than a person with severe asthma, whereas the quality of life of people with food allergies that run the risk of getting an anaphylactic shock might be even less. This means that an individual that has multiple 'mild' allergies might still be better capable to maintain their quality of life than a person with one severe allergy.

The difference between allergic complaints and risks even further complicates research concerning lifestyles. For example, a person suffering from HDM allergy probably cleans the house more thoroughly than a non-allergic or food allergic individual to avoid unnecessary exposure to HDM. This means that a difference in cleaning can be both a cause or a result of the developed HDM allergy. We used a cluster analysis, since this technique does not require the distinction between cause or result.

Taken together, lifestyle differences between non-allergic and allergic individuals can be identified, and would even be better definable when the type of allergy would be categorized into e.g. food allergy, hay fever, and HDM allergy. This means that the data set needs to be completed in order to obtain more conclusive data to provide allergic individuals with advice towards a preventive lifestyle.

Acknowledgements
We would like to thank Dr. Ir. Iteke Weeda for her expert help on the development of the FREYAL questionnaire.

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Appendix 1: Questions used for data reduction

Living environment

- Which of the following characteristics apply to your living environment?
  City, residential area, village, countryside, agricultural area, forest rich area, by the sea, near a fresh water lake, other:...

Number of allergies

- Which of the following allergies do you have?
  Hay fever, food allergy, animal allergy, house dust mite allergy, fungus allergy, other allergies, I do not have any allergies.

Infection, surgery and antibiotics

- Did you ever had a surgery? Y/N
- Did you get an antibiotics treatment after the surgery? Y/N
- Did you ever experience a severe infection? Y/N
- Did you get an antibiotics treatment for this infection? Y/N

House dust mite (HDM)-free household maintenance

- What kind of kitchen do you have?
  Closed kitchen, open kitchen, no kitchen.
- Which of the following parts of your house are (partially) isolated?
  Walls, roof, floors, windows.
- How many days per week do you aerate your house and bed room(s)?
  0-7 days and not applicable.
- Do you have loose carpets on the floors?
  Y/N
- What kind of window decoration do you have?
  Hard decoration: luxaflex, lamella, etc.; Soft decoration: curtains, lace curtains etc.; both hard and soft; none, not applicable.
- What kind of floors do you have?
  Hard floors: parquet, laminate, etc.; Soft floors: carpet; both hard and soft; none, not applicable.

Nutritional awareness

- I prefer fresh products to frozen products. Y/N
- I like to try out recipes from other countries. Y/N
- I eat before I get hungry, which means I am never hungry during mealtimes. Y/N
- It is important to choose food because of their nutritional value than of their taste. Y/N
- I compare the product information on the labels of different foods before I buy them. Y/N
- It is healthy to eat at least two pieces of fruit every day. Y/N

- An adult woman needs at least 100 grams of fats every day. Y/N
- Whenever possible, I buy biological products. Y/N
- I often use instant mixtures for cooking, like for soup, sauces and batter. Y/N
- I limit the number of snacks I eat. Y/N

HDM-free sleeping environment

- Do you have an allergen-free mattress cover? Y/N
- Is your mattress maintained mite-free through vacuum cleaning? Y/N
- How often are your bed sheets being washed?
  Once a week or more, once per two weeks, once per three weeks, once per four weeks, once per five weeks, less than once per five weeks.
- At which temperature are your bed sheets being washed?
  Less than 30˚C, 30˚C, 40˚C, 60˚C, 90˚C.

Sensible hygienic behavior

- The bathroom is cleaned at least once a week. Y/N
- The toilet seat is cleaned at least once a week. Y/N
- A quick cleaning throughout the house is performed daily. Y/N
- There is at least one fixed cleaning day. Y/N
- The house is being vacuum cleaned at least once a week. Y/N
- Dust is usually taken away with a wet cloth. Y/N
- The dishcloth is replaced daily. Y/N
- Each room has its own cleaning cloth. Y/N
- Vegetables and fruits are always washed before they are eaten. Y/N
- After slicing fish or meat, hands are always washed with soap. Y/N
- Dinnerware that was exposed to raw meat or fish are always kept away from dinnerware that serves cooked meat or fish. Y/N
- The refrigerator is thoroughly cleaned once every six months. Y/N
- Food that is expired are always discarded. Y/N

Allergy knowledge

Are you a member of an association of patients? Y/N
Did you gather information about allergy in or on e.g. books, internet or leaflets? Y/N

Locus of control

Statement 1: (choose the one that fits you best)

- Many people can be described as victims of circumstances.
- What happens to people is mainly in their own hands.

Statement 2: (choose the one that fits you best)

- Much of what happens to me is a matter of luck.
- My fate is in my own hands.

Statement 3: (choose the one that fits you best)
- One of the most important reasons why there are wars, is that people have too little interest in politics.
- There will always be wars, no matter how hard people try to avoid them.

Statement 4: (choose the one that fits you best)
- It is silly to think that someone can change the opinion of someone else.
- When I am right, I can persuade others.

Statement 5: (choose the one that fits you best)
- Whenever I have plans, I am almost sure that I can make them come true.
- It is not always wise to plan too far ahead since many things are dependent on luck and coincidence.

Statement 6: (choose the one that fits you best)
- Becoming allergic is due to the behavior of the concerning individual.
- It is impossible to avoid becoming allergic.

Statement 7: (choose the one that fits you best)
- The average citizen can influence governmental decisions.
- The world is lead by a few powerful people and, generally, citizens have little power.

CHAPTER

9

General discussion

General context: The multidisciplinary approach to allergy

The occurrence of various allergies is increasingly becoming problematic for our society. Already 30% of the Western population suffers from allergy, and the prevalence of allergic diseases has increased dramatically over the last 25 years. These complex, multifactorial diseases originate from interactions between the genetic background of the individual (called atopy) modified by exposure to selective factors [1]. In agreement with this statement, this thesis shows that incorporation of additional factors on the link between genetic variation and allergic disease are necessary. The implied disease modifying factors comprise influences from the environment including lifestyle associated factors, influences from the underlying immunopathology, and developmental processes that collectively provide windows of opportunity resulting in individually different phenotypes as observed in allergic patients. In addition to these factors, the importance of a precise characterization of the clinical phenotype, rather than the disease itself, is essential to understand this interaction (see Figure 1). Ultimately, the results should lead to detailed and trustworthy advice towards consumers, patients and authorities.

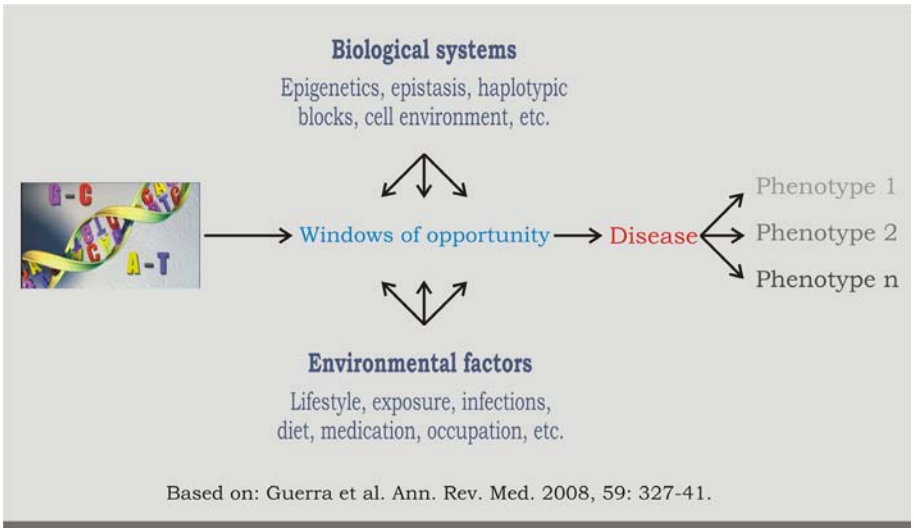

Figure 1. Incorporation of additional factors on the link between genetic variation and allergic disease.

Suitability of mouse models for allergic diseases in humans

There are great ethical considerations which limit the capacity for human studies of type I hypersensitivity reactions. These allergic reactions are increasingly prevalent, persistent and can result in life-threatening anaphylactic responses [2]. This has emphasized the need for other models in this research. Mouse models have been explored for the mechanistic characterization of both the sensitization and challenge phases in the allergic response. Investigations into the cellular and molecular mechanisms in mice have been facilitated by their well-characterized immune system, the availability of species-specific reagents, a fully sequenced genome, and the availability of various recombinant and genetically modified strains. Mice also exhibit a number of similarities to humans in their response to respiratory allergens, including the development of allergen-specific IgE antibodies, eosinophilic lung inflammation, mucus hyper-secretion and airway hyper-responsiveness (reviewed in [3]). However, the immune system of humans and mice are not identical, as there are important differences in the IgG isotypes, their binding affinities for FcγRI, FcγRIII and FcεRI, and the expression of these receptors on subsets of dendritic cells and macrophages [4]. Besides differences in the immune system, mice also possess many limitations. Their vasculature is the actual target of the anaphylactic response, they possess a poorly developed bronchial musculature, and their small size can make it difficult to detect pulmonary reactions associated with the elicitation of respiratory allergy or to achieve the appropriate number of cells required for analyses. In addition to research involved in characterizing the sensitization stage, a number of sensitization-challenge protocols have recently been established using mice (reviewed in [5]). However, also similarities between mice and humans exist since IgE and IgG antibodies of both species bind to FcεRI and FcεRIII with similar affinity and share expression of these receptors on mast cells, macrophages and basophils. Also both species release inflammatory mediators, like histamine, from activated mast cells and macrophages that lead to similar changes in vascular permeability in both species [6]. It is generally accepted that there remains sufficient similarity between humans and mice to indicate that mouse models are useful tools in the understanding the development of allergic disease.

Generally, sensitization and challenge assays typically monitor various respiratory parameters, including Penh, in response to an intranasal challenge. These endpoints can be monitored specifically, in response to the eliciting allergen, or non-specifically, to an agent such as methacholine. The non-specific challenge with methacholine provides an assessment of airway hyperresponsiveness. Lung function measurements are typically complemented with histopathological analysis of the lung and nasal tissue combined with examination of immunochemical (cytokine analysis) and cellular parameters of the bronchoalveolar lavage (BAL) fluid. Since exploration

of these parameters in humans require large ethical considerations, mouse models remain a useful tool to investigate both the sensitization and challenge phases in the allergic response, as described in Chapters 2 and 3. Both chapters describe allergic mouse models, but there are two major differences between the used models (see Chapter 1, Section 6.1). In Chapter 2, we describe a mouse model for allergic asthma, in which we sensitize all mice to OVA-alum, and challenge the control group with aerosolized PBS, whereas the asthmatic symptoms are induced by the OVA-aerosol. The main asthmatic symptoms elucidated in this model for allergic asthma are airway hypersensitivity to methacholine, influx of airway eosinophils, increase production of Th2 cytokines like IL-5 by *in vitro* culture lung tissue cells, and OVA-specific IgE antibodies in serum [7]. In Chapter 3, we used an allergen-induced airway inflammation protocol, in which again all mice were sensitized with OVA-alum, but the mice were subsequently challenged with an intranasal administration of PBS or OVA. Characteristic symptoms in these animals comprise influx of eosinophils in the lungs, IL-5 production by bone marrow derived cells, elevated serum IL-5 levels and the production of OVA-specific IgE levels [8]. Although in Chapter 2 the airway hyperresponsiveness was a key-parameter for the correlations with the other allergic asthma characteristics, the emphasis in Chapter 3 was not on the allergic response in the lungs per se, but on the immunopathology of the involved immune cells and their interactions. This made the allergic inflammation model more suitable for the work performed in Chapter 3 than the allergic asthma model in Chapter 2. The second difference between both chapters, is caused by the genetic background of the used mice. In Chapter 2, we used the congenic mouse strains that are based on the BALB/c and STS mouse strains, whereas in Chapter 3, we have used knock-out and transgenic animals on a C57BL/6 background. In literature, BALB/c and C57BL/6 mice are widely known to express different immune responses under normal and pathological conditions. For example, BALB/c mice display increased susceptibility to tumor genesis, including mammary [8, 9] and colon tumors [10], in contrast to C57BL/6 mice. However, the C57BL/6 mice are more susceptible than BALB/c mice to the induction of experimental, organ-specific autoimmune diseases, such as experimental autoimmune myasthenia gravis [4] and experimental autoimmune uveitis [9]. Furthermore, when infected by an intracellular parasite such as Leishmania major, C57BL/6 mice show a protective Th1 immune responses and resistance to the infection, whereas BALB/c mice show a non-protective Th2 response and are susceptible to the infection [9]. In addition, the STS/A mice display a more resistant phenotype to the induction of lymphomas [10], whereas we showed that the STS/A mice showed a very severe broncho-constrictive reaction after methacholine exposure [11]. In addition, the C57BL/6 mice are generally considered as mice that mainly respond in a Th1 directed manner. However, C57BL/6 mice were shown to be more susceptible to antigen-induced pulmonary eosinophilia than BALB/c mice, irrespective of systemic T helper 1/T helper 2 responses

[12]. The large differences between the expression of disease characteristics stress the importance to be aware of the genetic background of the used mouse strains.

Genetics

Atopic diseases are complex traits that tend to cluster in families, in which a general susceptibility towards allergic sensitization is inherited. Interestingly, these inherited allergies are often directed against multiple allergens, rather than to any single, specific allergen. As described in Chapter 1, genome-wide linkage studies have identified multiple regions of the human genome. Nonetheless, the genetic complexity underlying these diseases has hindered the definitive identification of specific atopy susceptibility genes, and hampered the determination of the contribution of specific candidate susceptibility genes to the development of atopy. However, it has been shown that genetic differences within the human leukocyte associated (HLA) MHC gene loci have an influence on the mechanisms of antigen processing and recognition of the generated antigenic peptides [13]. In addition, other studies of HLA types, indicate that a certain genotype (e.g. HLA II DRB1, DQB1, and DPB1) may lead to a higher susceptibility to both inhalant and food allergies [14]. Also in mice, the loss of oral tolerance or promotion of hypersensitivity to food proteins like peanut proteins has been shown to follow genetic background lines. Comparative studies have indicated that not all mouse strains respond similarly to sensitization and/or challenge with a respiratory allergen. Some of the more commonly used strains include the high-IgE-responding BALB/c mice. It has been shown that the susceptibility to type I food hypersensitivity-reactions, in terms of allergen-specific IgE or anaphylactic symptoms, is dependent on the used mouse strain as C3H/HeJ mice were found susceptible and BALB/c mice were found resistant to food induced hypersensitivity [15]. However, although it is accepted that oral tolerance is partially under genetic control, direct links between genotype and susceptibility towards food allergy are still not found. As described in Chapter 1, many genes have been associated with allergic diseases, but none of these mutations were shown to be the sole responsible cause.

In Chapter 2, it has been shown that the recombinant congenic mouse strain system is a useful tool to map and identify asthma-related genes, as the same correlations between asthma-related characteristics are found in humans and mouse models and a broad range of intensity and type of 'asthmatic' responses were observed. Moreover, the different asthma characteristics segregated independently, which will make it possible to map loci implicated in these different characteristics. This was also observed in another disease model (*Leishmania Major*) using the recombinant congenic mouse strains CcS/Dem, in which it was shown that a locus on chromosome 10 showed a

correlation with a locus that controls IgE regulation. Interestingly, this locus of the mouse chromosome 10 was found to be homologous to the human region 12q15 – q24.1 that controls total IgE concentrations in humans [16].

As described in Chapter 1, recombinant congenic mouse strains are generated by intercrosses of F1 mice and subsequent brother–sister interbreeding [17]. In contrast, consomic mouse strains are generated by repeated backcrossing of F1 animals to the parental receiver strain, although only one chromosome is derived from the donor strain. This means that consomic mouse strains only have one differential chromosome segment which is derived from the donor strain. Besides the use of congenic and consomic mouse strains, also other susceptible and resistant strain combinations have been used to perform functional genomic analysis of individual phenotypic extremes within a disease trait. For example, Patel *et al.* showed that a member of the calcium-activated chloride channel (CLCA) gene family was inducible with mucous cell metaplasia, but not with airway hyperreactivity [18]. Moreover, the fact that at least two mechanisms are involved in the airway reactivity to methacholine in our mouse model of asthma, even further complicates the genetic influences in multifactorial allergic diseases.

Molecular aspects of epigenetics

The term 'epigenetics' refers to the study of heritable changes in gene expression that occur without a change in DNA sequence. Heritable defects in gene expression that are not involving DNA sequence changes have been referred to as epimutations. In addition, the term 'epialleles' refers to epigenetic variants of a genetic allele, whereas epigenetic polymorphisms have been described as variations of epigenetic patterns between individuals. Examples of epigenetic polymorphisms are the DNA methylation polymorphisms involved in the variations of DNA methylation across the genome.

Three molecular mechanisms that interact with each other are involved in these epigenetic polymorphisms: 1) the methylation of DNA usually inhibits gene transcription, 2) the association of histon proteins with eukaryotic DNA forms a highly ordered and condensed DNA:protein complex called chromatin, and 3) small RNAs derived from cleavage of double-stranded RNA can silence genes at transcriptional and post-transcriptional levels. In more detail, histones can be modified in different ways, like acetylation, methylation, and phosphorylation of the histone tail domains. This alters the chromatin structure, leading to regulation of gene transcription. In addition, RNA-mediated gene silencing is important in maintaining chromosomal structure, genome defense and gene regulation. Common diseases involve interactions between genes and the environment and epigenetic mechanisms are thought to be an interface between genes and the environment in these diseases. The

environmental factors may impinge on epigenetic mechanisms in gene expression in many ways, including lifestyle associated factors, and thereby explain individual susceptibility to the development of allergic symptoms. Studies in which the epigenetic regulation is investigated, like cohort-driven epigenetic research, have the potential to address key questions concerning the influence of timing of exposure, dose of exposure, diet, and ethnicity on susceptibility to allergy development [19]. Ideally, this type of multi-disciplinary research therefore combines genetics, immunology, sociology and economics.

Extensive research on asthma and allergy have revealed conditions in which various genetic hits that are individually mild are capable of major phenotypic effects when acting in concert within a permissive environmental context [20]. Therefore, understanding how environmental and developmental factors interact with genetic determinants to affect disease susceptibility remain a challenge for geneticists [20].

Immune cells

Modulation of cytokine gene transcription is widely shown to occur by environmental factors, including inhaled or ingested components, adjuvant factors like smoking or diesel particles, and stress and welfare conditions. Differential cytokine production can be the results of either exon mutations or promoter polymorphisms at specific sites. Especially the type- and concentration-dependent binding of transcription factors contributes to the transcription rate of these genes. In addition, multiple genetic defects have been found within different cytokines and their receptors [20]. Since cytokines are produced by all immune cells, not only the interplay between genes, but also the interplay between cells is important in the pathology of allergic diseases. Current literature suggests that the assumed general interplay of specific immune cells should be further refined. Therefore, several immune cell types are further categorized into subsets based on their cytokine production profile, progenitor origin or effector function.

Dendritic cells (DC) are characterized by an unique capacity to stimulate naïve T cells and initiate primary immune responses. Recent studies suggest that DC also play critical roles in the induction of central and peripheral immunological tolerance, regulate the types of T cell immune responses, and function as sentinels in innate immunity against microbes. The diverse functions of DC in immune regulation depend on the heterogeneity of DC subsets and their functional plasticity. The remarkable functional diversity of DC subsets endows the immune system with the flexibility to mount appropriate immune responses, and the dynamics of such a flexible and responsive DC system might be dictated by subset heterogeneity dependent

on maturity and lineage origin, and functional plasticity. However, there are still unsolved mysteries regarding origin, function, and the molecular events governing their characteristic features in allergy (reviewed by [21]). In addition, in murine lung tissue (at least) four types of DC have been described: intra-epithelial, myeloid, interstitial, and plasmacytoid DC, that all perform different functions not only during sensitization but also during established inflammation [22]. Besides the described DC subsets, also T cells (see Chapter 1, Section 1.2) and B cells (see Chapter 1, Section 1.3) subsets are currently being investigated. Since most of these subsets originate from the same precursors, it is likely that factors like cytokines within the direct environment play an important role in directing the differentiation into a particular subset. There has been tremendous progress in understanding the mechanisms for development of Th2-biased responses after exposure to allergens and the characteristics of Th2 cells prominently involved in this process. For example, IL-13 has been shown to be a potent initiator of an inflammatory response, goblet cell hyperplasia, airway hyperresponsiveness, and airway fibrosis in allergic asthma [23]. More recently, two molecules, thymic stromal lymphopoietin (TSLP) [24, 25] and interleukin-25 (IL-25) [26, 27] are shown to be relevant to the development of Th2-driven inflammation and the pathogenesis of asthma. Furthermore, the studies on protective immunity to asthma have been focusing on the role of TGF-β expressed on regulatory T cells and following activation of Notch-HES1 signaling on tolerance induction in the lung [28].

B cells and IgE production

As mentioned in Chapter 1, B-1 B cells are a subset of B lymphocytes ($CD3^-$ $TCR^-CD4^-CD8^-CD19^+B220^+CD5^+$) that are infrequent in lymph nodes and spleen (< 1% of total cells), and reside mainly in pleural and peritoneal cavities where they comprise 10–40% of the total cells. B-1 B cells are claimed to self-replicate in the peritoneal cavity throughout life, and to originate in fetal/neonatal liver from progenitors, possibly distinct from those for 'conventional' B-2 B lymphocytes, which produce T cell dependent immunoglobulin G (IgG) and IgE that mediate acquired immune protection and atopic allergic responses, respectively. In contrast, the main function of B-1 B cells is thought to be the production of natural background IgM antibodies that are present in normal serum and can bind antigen to subsequently activate complement to mediate the first-line 'natural' defence mechanisms during the onset of infection. To indisputably prove the finding in Chapter 3 that B-1 B cells are capable of isotype switching, but not of Ig-secretion in the absence of Th cell contact, elaborate molecular analysis of the used mouse strains should be performed to discriminate the B cell lineage origin of the total and allergen-specific IgE antibodies in serum and compare them to the IgE+ B cells present in the different effector organs. It has been shown that the IgE repertoire in PBMC derived from atopic patients is characterized by

individual rearrangements, without any bias in the variable region of the heavy immunoglobulin chain [29]. However, a distinction between the different B cell subsets was not made. Collins *et al.* state that since the frequency of VH5 and VH6 sequences are higher in atopic individuals than in healthy controls, and the B-1 B cell repertoire might include an over-representation of the VH5 and VH6 gene families, the influence of B-1 B cells in allergic responses requires further research [30]. B-1 B cells have been associated with unusual patterns of V gene segment usage, with low levels of somatic point mutations, and with the production of polyreactive antibodies, and thus B-1 B cells may be responsible for IgE production. Although B-1 B cells are usually correlated to IgM production, their contribution to IgA production is also well recognized. B-1 B cell-derived chronic lymphocytic leukemic cells have been shown to be capable of class switching to IgE under the influence of IL-4 [31, 32]. There is also clear *in vitro* evidence that fetal B-1 B cells are capable of class switching to IgE under the influence of IL-4 and anti-CD40 antibodies [33]; in fact, low numbers of IgE-switched B cells can be identified in the fetus from 20 weeks of gestation. Despite some conflicting evidence, we believe that B-1 cells may be another element in the pathogenesis of some allergic diseases. If future studies continue to support a role for these cells in allergic diseases, particularly in the case of allergic diseases during childhood, this should not be seen as surprising. After all, B-1 B cells constitute >50% of peripheral blood B cells in children under the age of 16. Collectively, the constricted use of the VH gene family members, the requirement of T cells and their cytokines for the immunoglobulin production, the capacity to display the full isotype switching potential, and the more restricted epitope specificities allow the speculation that B-1 B cells could be more responsive to allergenic epitopes, which is consistent with our data.

It is generally accepted that class switch recombination of B cells occurs in lymphoid tissues, after which the allergen-specific IgE producing cells migrate through the circulation into the effector organs. However, differential localization of antigen-specific and by-stander components of IgE response to inhaled antigen in a rat model have been observed [34]. It has been reported that antigen-specific IgE is produced mainly in the draining lymph nodes, but not in the spleen, and the spleen appears to be only involved in by-stander total IgE upregulation [35]. In addition, recent literature suggests that B cells in effector organs like the nasal mucosa can undergo local class switch recombination to IgE when the present allergen stimulates the expression of IL-4 and CD40L by Th2 cells, providing all required signals to direct class switch recombination [36]. In combination with the generally higher allergen-specific IgE fraction in the tissue than in the blood [37], this suggests that allergen-specific IgE might be formed locally, whereas total IgE levels might be derived from splenic B cells. This notion is supported by the results reported in Chapter 3, showing that constitutively expressed IL-5

during development led to a large proliferating $B220^+$ population expressing IL-5Rα, high levels of IgM and low expression of CD138 in the spleen of IL-5 transgenic mice. Currently, CD138 is the single marker for the identification of plasma cells. Plasma cells are the main producers of antibodies and key effector cells of the immune system. Despite their importance, the analysis of plasma cells still suffers from the limited availability of specific markers. Especially since CD138 is widely expressed on various cell types outside the hematopoietic compartment, and furthermore, not expressed on all subsets of plasma cells [38]. Nevertheless, the findings in Chapter 3 suggest that IL-5 transgenesis results in a shift from the conventionally formed B-2 B cells towards B cells that originate from the B-1 B cell lineage. As shown in Chapter 3, B cells underwent massive migration towards the spleen under the influence of the constitutively expressed IL-5 levels. This migration was probably caused by the migration of osteocytes, stressing that alterations in the level of one specific cytokine might influence several cell types leading to a disturbed immune system.

T cells and cytokine production

Although mouse models enable research on the interaction of the immune cells and their interaction with other systemic, e.g. endocrinology, systems [39], the obtained data needs confirmation of the resemblance with the human situation. However, a reliable study of specific immune cells *in vitro* requires a proper resemblance of the *in vivo* situation, e.g. based on the nutrition, cell-cell contact and their responses towards each other and their environment.

Polyclonal stimulations

Literature provides a large array of possible concentrations, stimulation duration and read-out parameters to activate PBMC in a polyclonal manner. However, although these polyclonal stimulations are widely used, no consensus about the proper stimulation conditions seems to be established. A similar array of contradictory findings have been described on the effect of cryopreservation of human PBMC. Therefore, in Chapter 4, a thorough evaluation of the *in vitro* activation capacity of human PBMC by the use of the polyclonal stimuli PMA/Ca-I, ConA and αCD3/αCD28 has been described. Cryopreservation reduced the PMA/Ca-I stimulated PBMC production of cytokines, whereas cryopreservation did not affect the cytokine production of ConA or αCD3/αCD28 stimulated PBMC. Although cryopreservation left the maximal proliferation capacity of ConA or αCD3/αCD28 stimulated PBMC unaltered, it did delay the proliferation. Therefore, we concluded that PMA/Ca-I is suitable as a positive control in experiments where high cytokine production is expected and only fresh PBMC are used. Proliferation and effects on the T cell subsets in long-term PBMC cultures should use ConA or αCD3/αCD28 as positive control. However, as the name already indicated,

these polyclonal stimuli activate T cells independent from their antigenic specificity, likely making them less useful as controls in allergen-specific T cell experiments. Several studies using soluble MHC Class II molecules with a covalently linked peptide to the β-chain of the MHC molecule, to ensure its placement in the peptide-binding groove (Class II tetramers), are performed. Major disadvantages for this technique are the often low frequencies of the epitope-specific T cells, thereby requiring a sufficiently low detection limit, and the need for a separate molecular construct for every class II-peptide tetramer, and as a consequence, new constructs should be designed for every individual [40].

Allergen-specific stimulations

Although there are some major drawbacks, investigation of peptides within individual MHC-II molecules still gains interest, due to the difference in recognition of specific peptides by both the MHC-II molecule and the TCR in different individuals. Recently, it has been described that MHC-II tetramers containing the immunodominant Bet v $1_{141\text{-}155}$ epitope showed a high affinity to three common HLA-DRB1 haplotypes in both non-allergic and allergic individuals. In addition, the MHC-II Bet v $1_{141\text{-}155}^{+}$ $CD4^+$ T cells from allergic individuals displayed surface markers typical for effector T cells ($CD45RO^+$/$CD62^-$/$CCR7^-$), whereas the corresponding non-allergic T cells appeared to be central memory T cells ($CD62L^+$/$CCR7^+$) [41]. The cytokines produced by the MHC-II Bet v $1_{141\text{-}155}$ stimulated T cells of the allergic individuals comprise IL-5, IL-10, but not IFN-γ, whereas the cells of the non-allergic individuals mainly produced IFN-γ and IL-10. These results show that although some drawbacks have to be overcome, gaining more information about the MCH-II peptide binding and subsequent TCR activation continues to be of interest. In addition, Wang *et al.* described that although the predictions of MHC-I peptide bindings are better, the development of MHC-II binding predictions are improving [42]. The findings of Flinterman *et al.* support the concept that all allergic individuals recognized more diverse IgE epitopes which suggests a more polyclonal response [43]. In contrast, they showed that the IgG4 epitope recognition was less abundant and did not relate to the clinical sensitivity of the peanut. Furthermore, although large difference in the recognition patterns were observed between individuals, the individual binding patterns remained largely stable over 20 months, suggesting that over time the same B cells remain responsible for the production of the allergen-specific immunoglobulins [43].

Allergens and the allergic reaction

Protein characterization

Oral tolerance has been shown to be dependent on the nature of the food allergen. For example, water-soluble proteins with an abundance of conformational and non-conformational IgE-binding epitopes largely constitute common allergens related to type I food hypersensitivities [44]. The exact nature of food proteins that enables these to become allergens is not clear, but a number of characteristics have been shown to predispose food proteins towards allergenicity [45]. A food allergic immune response tends to occur against digested proteins which are in a relatively well-preserved state compared to their native conformation. This well-preserved state of food allergen protein stability is established by e.g. high level of intra-molecular disulfide-bond formation, aggregation or extensive binding to ligands, carbohydrates or lipid moieties [46]. It was also found that the role of specific digestive processes, especially gastric proteolysis, on food proteins can confer allergenicity. However, this allergenicity was recently shown to be dependent on the allergen-food matrix interactions as well. The food matrix, which is considered as the non-allergenic component of allergen-containing foods, affects the allergen composition and could therefore affect the allergen degradation during digestion and/or its consequent presentation to the gut immune system.

Isoforms

Besides the potential differences between the MHC-II epitopes in non-allergic versus allergic individuals and the protein characterization as mentioned in Section 2 of this chapter, differences between allergenic epitopes might also be caused by the presence of multiple isoforms of the allergenic proteins. For example, the major allergen in birch pollen, Bet v 1, is actually encoded by 7 genes resulting in a mixture of Bet v 1 isoforms that display a varying IgE-reactivity [47]. This knowledge might be useful in the screening of different Betula species for potential breeding approaches for birch trees with a reduced allergenicity. By planting low-allergenic birch trees, the number of newly sensitized individuals in the temperate climate zone of the Northern hemisphere might decrease in the future. As described in Chapter 8 and in agreement with literature [47], approximately 25% of the people with allergy-like complaints were proven to be birch pollen allergic. In addition, approximately 70% of the birch tree allergic individuals in this study also displayed a cross-reactive apple allergy, as also reported by others [48]. Furthermore, it has been shown that mainly individuals that display a high birch pollen-specific IgE level, also display a cross-reactive response to apple, carrot and/or celery. It is therefore tempting to speculate that the sustained reactivation of IgE producing B cells might eventually lead to small alterations

within the binding sites of IgE antibodies, leading to an increasing chance to bind cross-reacting allergens with an increasing higher affinity. To test this hypothesis, elaborate molecular analysis of the allergen-specific IgE antibodies in serum and IgE^+ B cells in blood should be performed in a longitudinal study, in order to map differences in the binding groove of the allergen-specific IgE antibodies and differences in the cells producing these antibodies.

Allergenicity versus immunogenicity

The term immunogenicity is generally used for the ability of proteins to stimulate the immune system and induce cellular and/or humoral immune responses, including antigen-specific lymphocyte proliferation/differentiation and antigen-specific antibody production. These proteins can be derived from invading infectious organisms, but also from environmental sources, like foods, drugs, house dust mites, and tree or grass pollen. The immunogenicity of these environmental proteins reflect, in addition to the ability to stimulate the immune system and induce a response, some other properties of these proteins, like their absorption or infiltration into the body across the mucosal epithelium and skin (epidermis). In case of allergens, the term allergenicity is often used. The definition of allergenicity is restricted to cases where the immunological responses induced by the protein lead to the provocation of pathological symptoms, such as allergic disorders [49, 50]. Allergic disorders have a large diversity in both immunological mechanisms and pathological symptoms, and are categorized generally into four types. Type I allergy, the focus within this thesis, is also referred to as the immediate type or the IgE-mediated type which has been most extensively investigated. Therefore, its molecular and cellular mechanisms are relatively well understood.

Effect of heat-treatment on the allergenicity

In Chapter 5, the effect of heat-treatment on the allergenicity of Bet v 1 and its homologous PR-10 proteins from carrot and celery was studied. It was hypothesized that the heat-treatment would not affect the T cell epitopes, as these are linear epitopes of approximately 12 to 18 AA, which are not likely to be altered by heat-treatment. Unfortunately, it was not possible to detect any T cell response to the untreated Bet v 1 protein. This is probably due to the low frequency of allergen-specific T cells present in the PBMC fraction obtained from the allergic blood donor. Even in highly sensitized allergic individuals, the frequency of allergen-specific memory T cells has been shown to be not more than 1 in a 100 T cells [51]. When considering the percentage of T cells within the PBMC fraction (60 – 70%) and the $CD4^+$ T cell fraction within the total T cell population (30%), this means that 0.18% of the cells within the PBMC fraction is an allergen-specific T cell, which is probably the reason why this activation could not be detected. Besides the linear T cell

epitopes, the presence of allergenic (conformational) B cell epitopes by the use of an allergen-specific ELISA was assessed. In Chapter 5, we have shown that the inhibition results of recombinant and natural Bet v 1 were different from the results observed in the indirect ELISA. As shown ealier [52], the purification of the natural allergens Bet v 1, Api g 1 and Dau c 1 from birch, celery and carrot, respectively, resulted in a mixture of isoforms. The difference between the inhibition and direct ELISA can therefore be explained by the presence of a more thermostable isoform present in the natural isoform mixture that displayed a higher affinity to IgE. In addition, structural changes of the protein due to heat-treatment affected multiple parts of the protein, resulted in a large difference between the plastic surface bound proteins in the direct ELISA and the free allergens in the inhibition ELISA. This suggests that heat-treated proteins in solution expose epitopes that are hidden again when these proteins are bound to the plate. Besides in our allergenicity tests, purified recombinant allergens are also used in the detection of allergen-specific IgE levels in serum. These tests are considered to be regular diagnostic procedures that use thoroughly characterized allergens. However, it is unknown whether these recombinant proteins are in their naturally folded state. Taken together, the inconclusive data in literature on recombinant proteins as a proper tool to assess whether or not an individual is allergic might result in false negative test-results, whereas the use of allergens isolated from their natural source possibly avoid erroneous chain folding as may occur in heterologous expression systems [52].

Immunomodulation

Immunomodulation is the manipulation of the immune system; it may augment or decrease the magnitude of the immune responsiveness and therefore development of immune-mediated diseases, like allergy. The augmentation of the immune response is known as immunostimulation or immunopotentiation, whereas suppression of the immune responsiveness is called immunosuppression. The necessity of suppression of the function of the immune system is well recognized in several immunopathological disorders, like autoimmunity and can be accomplished by regulatory T cell subsets and the cytokines they produce, including IL-10, TGF-β and IL-17. Conversely, augmentation of the immune response has been a target for increasing the hosts' resistance to the development of diseases, again including allergy. Specific immunomodulation is limited to a single allergen and thus immunopotentiation is used for the development of resistance against exposure to a selected allergen. This is generally known as immunotherapy which is broadly applied to hay-fever patients suffering from grass and tree pollen-related allergies. Non-specific immunomodulation implies for a more generalized change in the immune responsiveness leading to altered host reactivity to many different antigens. Herbal and mushroom

extracts (see Section 5.2) and probiotics (see Section 5.3) are widely applied for the prevention and treatment of allergic diseases.

Immunotherapy

As described in Chapter 1, prevention of allergic diseases can be performed on three different levels: 1) symptom reduction by medication, education, and information; 2) preventing sensitized individuals to become challenged by e.g. allergen avoidance; and 3) prevent atopic individuals of becoming sensitized. Since the mechanism of allergic diseases is largely known, medication to treat allergic symptoms (e.g. anti-histamines, steroids) are available. However, allergen avoidance to prevent mast cell degranulation is far less achievable. Although the avoidance of e.g. raw apple might be quite easy, avoiding the inhalation of birch pollen is almost impossible. However, in these both cases, the severity of the allergic symptoms are relatively harmless. In the case of peanut allergy, inhalation or digestion of a very small amount of peanut might cause life-threatening symptoms [53]. Currently, no immunotherapy is available for food allergy. Therefore, strict avoidance of the allergen is the only way to prevent allergic reactions from occurring, although some experimental therapies show promising results [54]. To date, one of the most promising therapies is the one utilizing anti-IgE antibodies. Several variations of this therapy have been studied in food allergic patients, mostly resulting in an higher acquired tolerance to food allergens (e.g. peanut) before eliciting an allergic response. However, the therapy requires repetition and does not show significant clinical improvement in all patients. Another type of therapy is based upon studies in which high and low dose tolerance are induced by food allergens, in a number of different varieties including conjugation to other molecules, are administered to food allergy patients. These have shown varied results in mouse models and research is continuing on this therapy [55]. A variation on this therapy is the use of recombinant DNA technology to produce forms of a food allergen that possesses a mutation in a known allergenic site (usually a change of one amino acid in the allergenic site). When these altered foods are fed to patients, this should promote tolerance to the near identical natural allergen at the level of antigen recognition and/or presentation [56].

Herbal and mushroom derived immunomodulatory compounds

Besides immunomodulation by the alteration of the allergen-specific recognition, recently, therapeutic effects have been observed in patients using Chinese herb formulations or medicinal mushroom extracts. For example, two Food Allergy Herbal Formula, FAHF1 and FAHF2, have been shown to prevent allergic reactions and to decrease the allergen-specific IgE immune response in mouse models of peanut allergy [57]. However, although these therapeutic compounds have shown promising effects in mouse studies, they

are still undergoing research towards clinical trials. Besides herbal compounds, also fungal compounds can be used as therapeutic formulations. In Chapter 6, we described that the fungal protein extracts of *V. volvacea* and *G. lucidum* contain immunomodulating activity by acting directly on monocytes and thereby modulating T cell activation. Application of these type of food compounds are gaining interest as they can be added to the diet and used orally, without the need to go through clinical trials as ordinary medicines [58]. However, the application of such 'safe' products in individuals with an altered immune state, such as in hospitalized patients, should always be performed with care. Besides differences in effectiveness due to the immune status, also differences in genetic backgrounds might influence the effectiveness and possible risk effects of such 'safe' foods. The fact that unknown immune related disorders might influence the effect of immune directed treatments is also shown with allergen-specific immunotherapy [59]. Although immunotherapy has shown to reduce the allergen-specific IgE levels and increased the levels of allergen-specific IgG and IL-10, minor adverse and a few fatal reactions have been reported [59]. Furthermore, effective immunotherapy has been shown in patients that have symptoms due to one or a few dominant allergens, whereas patients with poorly defined or multiple allergies are advised to deal with symptoms by the use of pharmacotherapy [59].

Immunomodulation by probiotics

Probiotic bacteria have been shown to be a possible alternative for immunotherapy for e.g. allergy as these micro-organisms are considered to promote effects that are beneficial to health. For example, administration of recombinant modified Ara h 1 plus heat-killed *L. monocytogenes* to mice was able to ameliorate clinical symptoms of an established type I peanut hypersensitivity model [60]. This type of therapy, however, is still in the development stage. The largest constituent group of probiotic micro-organisms is the lactic acid bacteria, which have been studied extensively in allergic mouse models and human allergy [61-63]. Additionally, *L. casei* and *L. reuteri* have shown the capacity to prime Th3 and Tr1 regulatory T cell populations which are implicated in tolerance induction. Of all the lactic acid bacteria strains classified, *L. lactis* is generally accepted as an ideal candidate for use in therapy [64]. A major reason for this is that Gram-positive and non-pathogenic L. lactis has been generally regarded as safe. This strain of lactic acid bacteria is widely available in commercial food products and is not perceived as a risk to health in young or elderly individuals [65]. There is evidence that supports the use of *L. lactis* in a potential therapy for food allergy, despite the fact that conventional strains alone can induce Thl immune responses, whereas they have a lesser effect upon down-regulation of Th2 responses [66]. Therefore, the next step would be to augment this property by the use of recombinant *L. lactis* that secretes immunoregulatory cytokines, as investigated by a number

of groups [67, 68]. Taken together, these findings suggest that *L. lactis*, and specifically recombinant strains of this lactic acid bacteria strain, would be an ideal delivery vehicle for restoring levels of bioactive IL-10 or IL-12 in the intestinal immune system in mouse models of type I hypersensitivity [69].

Lifestyle as preventive measure

To further explore the possible prevention measures, also primary prevention has been investigated. Since epigenetics (see Section 2.1 of this chapter) suggested that environmental exposure might play an additional role in the development of atopic individuals, determining lifestyle differences between non-allergic and allergic individuals will eventually deliver preventive lifestyles. The development of preventive lifestyles and advice to allergic persons concerning which lifestyles could help relieve or prevent new allergies, is becoming increasingly important. The impact of allergies does not only influence individuals, but also society as a whole. As described in Chapter 7, literature provides an array of lifestyle factors that have been associated with the prevalence of allergic diseases. However, assessment of a combination of these lifestyles requires the collaboration of various experts from a variety of scientific disciplines. In addition, as shown in chapter 8, lifestyle difference can be identified by the use of a disease-specific designed questionnaire, and would even be better definable when the type of allergy would be distinguishable into e.g. food allergy, hay fever, and HDM allergy. Therefore, the completion of the FREYAL study could provide conclusive data to provide allergic individuals with advices towards a preventive lifestyle.

If preventive intervention is to be at all effective, it would have to be applied early in life, most probably in early infancy. Unfortunately, the understanding of the natural history of the process of atopic sensitization, atopic dermatitis and allergic airway disease is still very limited. The evaluation of risk factors and determinants is a necessary prerequisite for any effective intervention study. Interventions for primary prevention are aimed at a population that is still healthy, but is at risk during diseases. Unfortunately, all predictors investigated so far are insufficiently sensitive and specific. Therefore, possible preventive measures should be recommended only if they are applicable to the whole population, having no risk, and low costs [70]. In this thesis several of these above mentioned aspects have been addressed with the ultimate goal to arrive at new and rationally designed predictive factors. Because individual participants in the FREYAL study did not receive structured preventive or therapeutical advice, any interventional effects were unintended. Differences in atopy relevant lifestyle factors between socio-economic classes were probably a consequence of better knowledge and of experienced atopic individuals of high compared to low socioeconomic status (SES, see also

Chapter 7). This may have favorably influenced the frequency of atopic disorders. During the last decades, two general hypotheses have been proposed in the literature in connection with the observed increase of atopy and asthma. New risk factors connected to nutrition, environmental exposure or lifestyle that were not known several decades ago have become relevant. Also, protective factors related to a more traditional lifestyle in the past have been lost, leading to a greater susceptibility to atopic diseases. Favorable lifestyle habits of individuals with higher socioeconomic status could obviously not influence the genetic risk. Active allergy-preventive lifestyles counteracted most other environmental risks associated with higher socio-economic status. A possible explanation could be that during multiple and critical age-related windows of opportunity times such lifestyle factors may influence (compromise or stimulate) the genetically determined development of tolerance or a permanent allergen-specific intestinal immune response of the Th1 type [70-72].

As discussed before, most attention is currently being given to the medical treatment of allergic patients. However, there is a growing awareness that prevention of allergy deserves more attention. Prevention during the pre-medical phase will be particularly effective through the reduction of costs for health care, and the improvement of the quality of life for patients. This pre-medical phase is of prime interest to future developments in allergy prevention. Essential elements for allergy prevention, as alternatives for the currently applied pharmacological medication, are related to primary prevention but also treatment of established allergic disease by: (1) avoiding contacts with allergens, (2) development of approaches that reduce allergic sensitisation and symptom occurrence (immunomodulation), and (3) changing the lifestyle to avoid contacts with possible adjuvant factors. Counteracting measures require an innovative, flexible and versatile approach due to the complexity of both the problem and potential solutions. Part of this thesis was thus devoted to the study of the indicative and predictive value of relevant markers of immunopathology underlying the development of allergic diseases.

As summarized in Figure 2, this thesis covered two levels of the above described prevention strategies: Chapters 2 and 3 provided insight in the importance of genetic studies and the influence of IL-5 on the development of the adaptive immune system in a mouse model for allergic asthma (primary prevention); Chapters 4 to 6 described the proper way to cryopreserve human PBMC for subsequent allergen-specific and immunomodulatory *in vitro* assays (secondary prevention); and finally, Chapters 7 and 8 summarized lifestyle factors associated with allergic diseases and provided a way to investigate the differences in lifestyles between non-allergic and allergic individuals by means of a customized questionnaire (primary and secondary prevention).

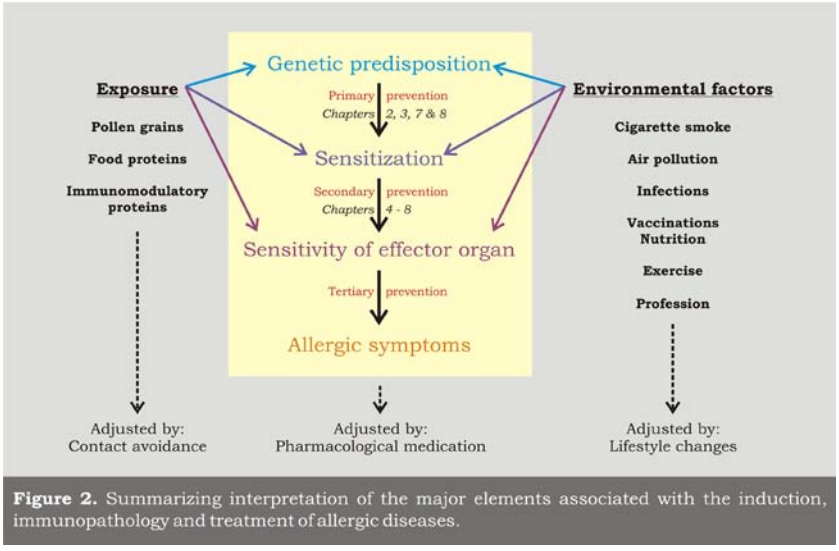

Figure 2. Summarizing interpretation of the major elements associated with the induction, immunopathology and treatment of allergic diseases.

Conclusion

A substantial number of people in Westernized countries are affected by allergic disorders. The mechanism by which the immune system responds to an per se harmless protein in e.g. foods or pollen grains is largely known. However, which triggers cause the predisposition to become sensitized or subsequently challenged remain unclear. Although more and more knowledge is gained on the understanding of antigen presentation, IgE biology, role of regulatory T cells, the hygiene hypothesis, environmental pollutants and immunomodulating compounds in food, no conclusive solutions for the basic immunology in allergic diseases has been established. However, as suggested by the summation above, scientific disciplines need increasingly combined expertise, leading to better understanding of the cross-talk that on multiple levels appear to be important in the development of the multifactorial disease called allergy. Although the prevention of allergic sensitization might turn out to be an utopia, the mitigation of allergic symptoms, the delay of the onset of allergic complaints and/or the delay of the development of cross-reactive allergic responses already constitute significant profits for allergic individuals.

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English summary

English summary

Allergic diseases affect a substantial part of the global population. Although extensive studies have elucidated the allergic mechanism, no conclusive answer has yet been found that will prevent the onset of an allergic disease. Literature suggests that no single factor like a gene mutation, environmental factor or lifestyle component can be hold accountable for the allergic cascade. Therefore, the main goal of this research project was to use a multidisciplinary approach to allergies, by combining information on the genetic components, lifestyles and in vivo and in vitro assessment of the immune cells involved in allergy.

The accomplishment of this multidisciplinary goal required knowledge on the genetic factors involved in the immunopathology of allergic diseases, but also immunological and cell biological knowledge. In addition, we investigated the influence of environmental factors on the allergic response which also required knowledge on sociology and economics to assess involved lifestyle factors. Within the multidisciplinary research areas, allergic responses are studied at multiple levels. For example, the genetic differences can be studied by means of mice with different genetic backgrounds (BALB/c, STS/A, C57BL/6) or in knock-out (CD4 or CD8 knock-out) or transgenic (IL-5 transgenic) mice. These differences in the genetic background on their turn have an effect on the expression of the allergic disease characteristics. Examples of the assessed allergic characteristics comprise antigen-presentation by specialized antigen-presenting cells, the presence of T helper 2 cells, the involvement of Th2 produced cytokines like IL-4 and IL-5, and the isotype switching of B cells towards allergen-specific IgE. Alterations of the allergic characteristics were studied by the use of in vitro cultures of human peripheral blood mononuclear cells (PBMC) that display all above mentioned characteristics when they are properly isolated, cryopreserved and cultured. These alterations were elicited by the use of heat-treatment of allergens or the exposure to fungal derived proteins or polysaccharides. Taken together, these different levels within the allergic individual can on their turn be influenced by environmental and nutritional factors. To study this, lifestyle factors have been assessed by the use of a personalized internet-based questionnaire and these data were thereafter linked to allergen-specific immunoglobulin levels. To further stress the importance of multilevel research within a multidisciplinary study, a more detailed description of the separate chapters within this thesis are combined with their major results, and finalized with future perspectives.

Section 1: Murine allergy studies
Since allergy has a multifactorial onset, we investigated the influence of the genetic background. We used recombinant congenic mouse strains in an ovalbumin model of allergic asthma to establish the impact of the genetic background on the onset of the allergic characteristics. We showed that correlations between e.g. airway hyper-responsiveness and allergen-specific IgE, or IL-5 and eosinophils were not found in every individual strain. This suggested that the asthma traits in this mouse model can be genetically dissociated, potentially leading to the identification of genes involved in (one of) the allergic symptoms. In addition, to get a better insight in the importance of T and B cells, we used WT, CD4 knock-out mice and CD8 knock-out mice in combination with the over-expression of the Th2 cytokine IL-5 in an ovalbumin model of allergy induced airway inflammation. We showed that B-2 cells required CD4+ Th cells to induce allergen-specific IgE secretion, whereas this CD4+ T cell help was not required for the B-1 cells in the spleen to undergo isotype switching. However, under these conditions, these B-1 B cells did not secrete allergen-specific IgE. We suggest that the IgE secreting B-2 cells should be present in the DLN or spleen in order to contribute to the allergen-specific and total IgE levels, respectively.

Section 2: Human in vitro allergy studies
Since observations in mice always needs to be validated in the human situation, an extensive study on the cryopreservation and stimulation of human peripheral blood mononuclear cells in order to read out the immunophenotype, proliferation capacity and cytokines production is described. These parameters give better insight in the function of immune cells, and provided the background to study allergen-specific stimulations and the screening of the immunomodulatory capacity of mushroom extracts. Allergen-specific PBMC cultures in addition to allergen-specific inhibition ELISA have been performed to study the effect of heating on the allergenicity of birch pollen and its cross-reactive allergens like Dau c 1 and Api g 1. We showed that the comparison between purified (isoform mixtures of) native proteins and their recombinant counterparts is complicated by the presence of multiple isoforms. Furthermore, isolated immunomodulatory compounds from several mushroom species that alter the in vitro immune response of human PBMC are described. We showed that our mushroom proteins indirectly altered the T cell-specific cytokine production through their influence on monocyte activation.

Section 3: Socio-economic study
Besides the identification of parameters that can be used to develop strategies to prevent the onset of the allergic sensitization or the development of cross-reactive food allergens, we investigated which lifestyle characteristics of allergic individuals could be altered to prevent development of (cross-reactive) allergies. To get more insight in the possible influence of lifestyle factors on

the development of allergic responses, we reviewed literature with respect to the mutual influences of allergic responses and lifestyle factors. The term lifestyle comprises values, interests, ways of thinking and actions that influence consumer behavior. In other words, lifestyle includes what consumers do and what they think and feel. However, lifestyles are not only based on individual choices, but are a result of an interaction between personal, social, societal and environmental factors. The relevant lifestyle factors were thereafter used to develop the questionnaire used in the FREYAL study. We showed that lifestyle differences between non-allergic and allergic individuals can be identified, especially when the allergy type would be categorized into e.g. food allergy, hay fever, and HDM allergy.

In conclusion, further exploration of the basic mechanisms and rational design of preventive measures at different levels of integration, will support the formulation of detailed and advices towards consumers, patients and governments to prevent or mitigate allergic diseases.

Nederlandse samenvatting

Nederlandse samenvatting

De titel van dit proefschrift laat zich vrij vertalen als “een onderzoek naar allergie door het gebruik van verschillende wetenschappelijke vakgebieden”. In deze Nederlandse samenvatting wil ik het begrip allergie uitleggen en beschrijven welke verschillende vakgebieden kunnen worden gecombineerd om meer te weten te komen over het ontstaan en het kunnen voorkomen van allergieën.

Allergie en het afweersysteem

De definitie van allergie volgens de gezondheidsraad is “een reactie van het afweersysteem tegen een normaliter onschadelijk antigen dat uiteindelijk leidt tot de productie van IgE”. Om dit voor een ieder een begrijpelijke zin te maken, moeten er een aantal dingen worden uitgelegd. Want wat is een antigen? En wat is IgE? Daarvoor moet ik eerst iets uitleggen over een normaal functionerend afweersysteem. Ons afweersysteem bestaat uit twee onderdelen: de aangeboren afweer en de verworven afweer, die beiden bestaan door de werking van witte bloedcellen. De verworven afweer ontstaat doordat het lichaam in contact komt met een ziekteverwekker of een deel daarvan (door b.v. een vaccinatie) waardoor het lichaam afweerstoffen kan aanmaken. Deze afweerstoffen kunnen dan bij een volgende besmetting snel aan de ziekteverwekker binden, waardoor deze sneller door de witte bloedcellen wordt opgeruimd en je er dus niet meer ziek van wordt. Dit heet immuniteit of resistentie, en is tevens het idee achter vaccineren. Helaas moet het lichaam dus eerst een keer de ziekteverwekker aanpakken zonder de aanwezigheid van afweerstoffen. Dit wordt verzorgt door de aangeboren afweer.

De eerste stap in deze aangeboren afweer is dat de ziekteverwekker wordt herkend als gevaarlijk voor het lichaam. De cellen die alles wat lichaamsvreemd is opmerken, worden antigeen-presenterende cellen (afgekort tot APC) genoemd. Dit zijn witte bloedcellen die een lichaamsvreemde stof opeten en in stukjes knippen. Deze kleine stukjes lichaamsvreemd materiaal noemen we antigenen. Omdat APC zelf niet kunnen inschatten of een antigeen gevaarlijk is, laten zij deze antigenen aan hun buitenkant aan andere witte bloedcellen zien. Een van de cellen die daarop kan reageren is een T cel. Omdat deze specifieke T cel andere witte bloedcellen helpt bij het starten van een afweerreactie, worden deze ook wel helper T cellen genoemd. Zodra deze helper T cel ziet dat het antigeen gevaarlijk is, veranderd de T cel, waardoor hij waarschuwende signaalstoffen (ook wel “cytokinen” genoemd) kan gaan maken. Daarnaast zal hij contact gaan zoeken met een andere witte bloedcel, de B cel. B cellen kunnen na contact met een helper T cel afweerstoffen gaan maken, die later een beschermende werking zullen hebben. Deze werking is tevens afhankelijk van het type afweerstof dat door de B cel gemaakt wordt. Dit type afweerstof wordt mede bepaald door het soort signaalstoffen dat

aanwezig is tijdens het contact tussen de T en de B cel.

De allergische reactie

In het geval van een allergie wordt het antigen een allergeen genoemd. Oftewel, een eiwit dat normaal geen afweerreactie zou moeten oproepen, maar dat in sommige personen wel doet. Hoewel het nog niet bekend is waarom dit in de ene persoon wel allergische klachten geeft, en in een ander persoon niet, is wel bekend hoe een allergie tot stand komt. Het allergene eiwit in bijvoorbeeld berkenpollen komt via de neus het lichaam binnen. Nadat het via de slijmvliezen daadwerkelijk in het lichaam zelf komt, wordt het opgegeten door een APC die onder de slijmvliezen ligt te wachten. Vervolgens gaat de APC op zoek naar een helper T cel die kan beoordelen of dit allergeen gevaarlijk is. In sommige personen wordt dit als ongevaarlijk bestempeld, en er ontstaat dus geen afweerreactie, maar in andere personen wordt dit als gevaarlijk beschouwd. Daardoor zal de T cel veranderen in een T helper 2 cel en kenmerkende signaalstoffen (IL-4 en IL-13) aan gaan maken. Hierdoor komt de B cel tijdens het contact met de T helper 2 cel bloot te staan aan IL-4 en IL-13 en zal daardoor een speciaal soort afweerstoffen gaan maken, die "IgE afweerstoffen" worden genoemd.

Zoals in Figuur 5 op pagina 19 te zien is, is dit een cruciale stap in het ontwikkelen van een allergie, omdat alleen IgE afweerstoffen de vervolgstap kunnen inzetten. Alleen deze IgE afweerstoffen kunnen namelijk binden aan de buitenkant van een andere cel van het afweersysteem, de "mestcel". Wanneer een persoon IgE afweerstoffen heeft tegen een allergeen die gebonden zijn aan een mestcel, noemt men deze persoon "gesensibiliseerd".

De eerstvolgende keer dat een allergeen weer in het lichaam komt (de "challenge" genoemd), en wordt vastgegrepen door de IgE afweerstoffen op de mestcel, zal deze cel ontploffen. Omdat deze cel stoffen bevat die onder anderen zorgen voor jeuk en de aanvoer van vocht, zullen op dat moment allergische klachten zoals een loopneus en traanogen ontstaan. De plek waar het allergeen bindt aan de mestcel bepaald dus wat voor soort allergische reactie ontstaat. Zo is eczeem een allergische reactie in de huid, zit astma in de longen, voedselallergie in de darmen en hooikoorts in de oren, neus en keel.

Allergie Consortium Wageningen

Zoals hierboven beschreven staat is er al best veel bekend over allergische reacties. Waarom wordt er dan nog steeds onderzoek naar gedaan? Het aantal personen met een allergie is de afgelopen decennia drastisch toegenomen. Omdat deze toename te groot is om alleen te worden veroorzaakt door erfelijke

factoren, wordt gedacht dat de veranderde leefomgeving een grote rol moet spelen. En hoewel de allergische gevoeligheid vaak al op jonge leeftijd begint, blijven de allergische klachten vaak levenslang bestaan. Het is daarom ook zeer belangrijk om de manier waarop allergische gevoeligheid ontstaat te onderzoeken. Daarnaast richt het huidige medische onderzoek zich voornamelijk op de herkenning van de verschillende typen allergieën en de uiteindelijke behandeling daarvan. Het Allergie Consortium Wageningen is voornamelijk geïnteresseerd in de periode vóórdat de eerste klachten van (voedsel)allergie opkomen. Het einddoel van het consortium is de allergische reacties zoveel mogelijk te voorkomen.

Hoofdstuk 2 en 3: Muisstudies

In dit proefschrift hebben we gekeken naar verschillende benaderingen van het allergie-probleem. In de eerste twee hoofdstukken heb ik door middel van studies met muizen gekeken naar de invloed van de overerfbaarheid en daarmee de genen die betrokken zijn bij het ontwikkelen van een allergie. We hebben hiervoor muizen gebruikt omdat deze zodanig kunnen worden ingeteeld dat alle muizen van één stam exact hetzelfde erfelijke materiaal hebben. Hierdoor wordt het onderzoek een stuk makkelijker in vergelijking met de mengelmoes die we bij mensen aantreffen. Door verschillende muisstammen waarvan precies bekend is op welke plaatsen het erfelijk materiaal van elkaar verschilt, met elkaar te vergelijken, hebben we gezocht naar delen van het erfelijk materiaal die betrokken zijn bij het ontstaan van een allergische reactie. Dit staat beschreven in hoofdstuk 2. Daarnaast is het mogelijk om in muizen één bepaalde signaalstof verhoogd te laten produceren om zo te zien wat de functie van dat specifieke signaalstofje is. Dit is terug te vinden in hoofdstuk 3.

Hoofdstuk 4 t/m 6: Menselijke witte bloedcellen

Omdat het gebruik van muizen altijd moet worden gestaafd met de situatie in de mens, hebben we in hoofdstuk 4 tot en met 6 gebruik gemaakt van witte bloedcellen die we uit bloed van vrijwilligers hebben gehaald. Omdat het niet altijd mogelijk is om met vers bloed te werken, hebben we eerst uitgezocht hoe de witte bloedcellen het beste ingevroren kunnen worden zodat ze op een later tijdstip gebruikt kunnen worden. Dit staat beschreven in hoofdstuk 4. In hoofdstuk 5 hebben we geprobeerd de structuur van allergene eiwitten in berk, wortel en selderij door verhitting te veranderen. Hiermee zou de herkenning van de helper T cel kunnen veranderen, waardoor er geen IgE afweerstoffen meer aangemaakt zouden worden. Of wellicht zou het allergeen niet meer kunnen binden aan de IgE afweerstoffen op de mestcel, waardoor er geen allergische klachten meer door ontstaan. Daarnaast hebben we gekeken of verschillende stoffen die aanwezig zijn in paddenstoelen gebruikt kunnen worden om de witte bloedcellen van een allergisch persoon te

veranderen. Dit zou bijvoorbeeld kunnen doordat de stoffen uit de paddenstoel ervoor zorgen dat de helper T cellen geen IL-4 en/of IL-13 meer gaat maken waardoor er geen IgE meer ontstaat, of doordat de APC het allergeen niet meer of anders gaat presenteren.

Hoofdstuk 7 en 8: Allergie en leefstijl

In hoofdstuk 7 en 8 hebben we gekeken naar hoe de leefstijl van allergische personen afwijkt van de leefstijl van niet-allergische personen. Wellicht kunnen verschillen in leefstijl de verschillen in gevoeligheid voor het ontwikkelen van allergieën verklaren en zo ook worden gebruikt als mogelijkheid om allergische klachten te verminderen of zelfs te voorkomen. In hoofdstuk 7 wordt een overzicht gegeven van een aantal leefstijlfactoren zoals ras, sociale klasse, geboortemaand, leeftijd van eerste contact met allergenen, geboortevolgorde, familiegrootte, rokende moeder en borstvoeding die in de literatuur genoemd zijn als mogelijke invloeden op de verhoogde aantallen allergieën. In hoofdstuk 8 hebben we deze literatuurstudie gebruikt om een vragenlijst te ontwikkelen waarmee de leefstijl van allergische personen kan worden vergeleken met die van niet-allergische personen. Deze studie noemden wij de FREYAL studie, hetgeen staat voor Factors Related to Your Allergies and Lifestyle. Vrij vertaald betekend dit dat wij op zoek zijn (geweest) naar de relatie tussen de leefstijl van mensen en de eventuele allergieën die zij hebben.

Conclusie

Uit de bovenstaande hoofdstukken hebben wij geconcludeerd dat verder onderzoek naar het mechanisme achter een allergie en het ontwikkelen van voorkomende maatregelen op verschillende manieren moet gaan plaatsvinden om in de toekomst gedetailleerde adviezen aan consumenten, patiënten en de overheid te kunnen geven. Naar ons inzicht ligt de sleutel in de samenwerking van verschillende wetenschapsgebieden, hetgeen terug te vinden is in het beleid en werk van het Allergie Consortium Wageningen.

Dankwoord

Dankwoord

Bizar om te merken dat het schrijven van een dankwoord ook voelt als een afsluiting van een bijzondere periode. Ik ben daarom ook blij met de wetenschap dat het juist dit stuk is waar de meeste mensen meteen naartoe bladeren om te zien wie er allemaal genoemd worden. Omdat ik veel mensen erg veel dank verschuldigd ben, en zeer waarschijnlijk toch iemand ga vergeten, voor een ieder die heeft bijgedragen tot dit proefschrift: Heel veel dank!

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Prescilla

Personalia

Curriculum vitae
List of publications
Education overview

Curriculum vitae

Prescilla Jeurink was born in Nijmegen on 20 November 1979. In 1996, after graduating from the Christian Lyceum in Veenendaal, she started her study in Experimental Zoology and Biotechnology at the Higher Laboratory Education in Utrecht. During this study, she performed a traineeship combining the two study directions in a project investigating the role of Bax and Bcl-2 in malign haematopoiesis at the department Central Haematology of the Radboud hospital under the supervision of Dr. Jules Meijerink and Drs. Aniek de Graaf.

After completing her study, the author started to work on a project on the localization of asthma susceptibility genes in recombinant congenic mouse strains under the supervision of Prof. Dr. Antoon van Oosterhout at the Pharmacology group of the University of Utrecht. During this project, she performed experiments at the WEHI institute in Melbourne, Australia, under the supervision of Dr. S. Foote.

Thereafter, the author started as a PhD student on the project entitled "Mutual influence of consumption patterns on the development of food allergy" funded by the Allergy Consortium Wageningen. The project was carried out at the Cell Biology and Immunology Group, and the group of Economy of Consumers and Households of the Wageningen University, under the supervision of Prof. Dr. Huub Savelkoul, Prof. Dr. Gerrit Antonides, Prof. Dr. Harry Wichers and Dr. Johan van Ophem. During her PhD period, the author spent 6 months in the group of Prof. Dr. Jan Lötvall at the Lung Pharmacology Group of the Göteborg University on a project focusing on the influence of T cell subsets and IL-5 on the distribution of B cells using a mouse model of allergic asthma.

Currently, the author is employed as a scientist in the immunology section of Danone Research - Centre for specialised nutrition, headed by Prof. Dr. Johan Garssen.

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van Oosterhout, A.J.M., Jeurink, P.V., Groot, P.C., Hofman, G.A., Nijkamp, F.P., Demant, P., Genetic Analysis of Antigen-Induced Airway Manifestations of Asthma Using Recombinant Congenic Mouse Strains, Chest 121 (2002) 13s.

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Date:	23 June 2008
Groups:	Cell Biology and Immunology
	Economics of Consumers and Households

Basic courses (minimum 3 credits)	year
WIAS Introduction Course	2004
Ethics for lifescientists	2007
subtotal basic courses:	*3 credits**

Scientific exposure (minimum 8 credits)

International conferences	
Immunological, chemical and clinical problems of Food Allergy, Budapest	2004
Allergy Matters! , Wageningen	2004
American Thoracic Society, Orlando	2004
EAACI congres, Amsterdam	2004
EAACI Summer School, Praag	2005
EAACI Summer School, Antalya	2006
EAACI congres, Göteborg	2007

Seminars and workshops	
NVVI wintermeeting 2003	2003
NVVI wintermeeting 2005	2005
NVVI springmeeting 2007	2007
WIAS science Day	2007

Poster presentations	
Immunological, chemical and clinical problems of Food Allergy	2004
Allergy Matters!, Wageningen	2004
American Thoracic Society, Orlando	2004
EAACI conference, Göteborg	2007

Oral presentations	
NVVI wintermeeting	2003
subtotal scientific exposure:	*17.4 credits**

In-depth studies (minimum of 6 credits)

Disciplinary and interdisciplinary courses	
Advanced Immunology (Utrecht/Rotterdam)	2005
Research Methodology (MGS)	2004

Undergraduate courses (Wageningen University)	
Research methods	2004
Research and Interview	2004
Lifestyles & consumption patterns I & II	2004
Food related allergies	2004
subtotal in-depth studies:	*16 credits**

Skill support courses (minimum of 3 credits)	
Professional skills	
Scientific Writing	2006
Writing a winning grant proposal	2007
Research skills	
FACSCanto II Training Beckton Dickenson	2007
External training of 6 months at Göteborg University, LFG Group	2006
Didactic skills	
Lecturing Pathophysiology	2007
Supervising practicals Cell Biology	2004 - 2006
Supervising practicals Comparative Immunology	2006 / 2007
Supervising 2 BSc and 6 MSc students	2004 - 2007
Tutorship Cel Biology I	2007
Presentatie bij de Nederlandse bond voor Plattelandsvrouwen	2005
Presentatie bij de Christelijk-Maatschappelijke vrouwenbeweging	2006
Management skills	
Organisation of the WIAS Science Day	2007
Membership of WAPS council	2005 - 2006
subtotal skill support studies:	*36.8 credits**

Total number of credit points: 73.2*

Herewith the WIAS Graduate School declares that the PhD candidate has complied with the educational requirements set by the Edcational Committee of WIAS which comprises of a minimum total of 30 credit points.

** A credit point represents a normative study load of 28 hours of study.*

The cover of this thesis was designed by Pieke design.
The cover shows a graphic representation of the skin prick test as within the circles, skin is visible. The skin prick test is used to show whether an individual is allergic or not. For the author, the phenomenon that different allergy types (e.g. hay fever with symptoms in the nose and eyes, and food allergy with symptoms in the gut) can all be detected as a reaction in the skin, remains intruiging. In addition, the multiple layers (white cover and skin) are symbolic for the multiple layers within the thesis. For example, the thesis covers mouse models, immunomodulation and lifestyle, which are all different levels when looking at allergy research. Furthermore, within the immune system multiple levels (like cytokines and immune cells) need to communicate in order to function.

De omslag van dit proeschrift is ontworpen door Pieke ontwerp.
De omslag is een grafische weergave van de huidprik test met de afbeelding van huid in de cirkels. De huidprik test wordt gebruikt om aan te tonen of een individu allergisch is of niet. Voor de auteur is het fenomeen dat alle verschillende typen allergie (b.v. hooikoorts met symptomen in de neus en ogen, en voedselallergie met symptomen in de darmen) als een reactie in de huid kunnen worden gemeten nog steeds indrukwekkend. Daarnaast zijn de meerdere lagen (witte omslag en de huid) symbolisch voor de meerdere lagen binnen dit proefschrift. Een voorbeeld hiervan is het bestuderen van muis modellen, immunomodulatie en leefstijl, welke alle verschillende lagen binnen het allergie onderzoek beslaan. Daarnaast vinden we ook meerdere lagen binnen het immuun systeem (zoals cytokines en immuun cellen) die met elkaar moeten communiceren om goed te kunnen functioneren.