Mechanisms of oral tolerance and allergic sensitization to peanut

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La raison, c'est l'intelligence en exercice
L'imagination, c'est l'intelligence en érection

Victor Hugo

Femke van Wijk

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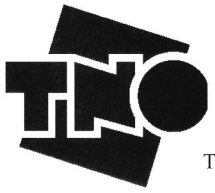

TNO Quality of Life

Mechanisms of oral tolerance and allergic sensitization to peanut

Mechanismen van orale tolerantie en allergische sensibilisatie tegen pinda

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Chapter 1

General introduction

Immediately after birth the mucosa of the gastrointestinal tract, which represents the greatest body surface area exposed to the outside environment, is confronted with a large variety of foreign antigens. The immune system of the intestine now has to meet the task of discriminating between pathogens and harmless antigens, such as food proteins and commensal bacteria, and to respond accordingly. This important job is fulfilled by cells of the gut-associated lymphoid tissue, the largest immunologic organ in the body.

Despite the large extent of food antigen exposure, only a small percentage of individuals experience adverse immunologic reactions to food. This is due to the fact that the normal immune response to dietary proteins is associated with the induction of oral tolerance, which refers to a state of active inhibition of immune responses to an antigen by means of prior exposure to that antigen via the oral route. Abrogation of oral tolerance or failure to induce oral tolerance may result in the development of food hypersensitivity.

1. Food allergy

1.1 Food allergy, from sensitization to clinical manifestations

Adverse reactions to food include any abnormal reaction resulting from the ingestion of a food or food additive and can be divided into toxic and non-toxic reactions (reviewed by Sampson [1, 2]). Toxic reactions are due to factors inherent in a food and will occur in any exposed individual when given in an appropriate dose. Non-toxic food reactions affect only susceptible individuals and are either non-immune-mediated (food intolerance) or immune-mediated (food allergy/hypersensitivity). Food intolerances are adverse physiologic responses caused by some unique characteristics of the host, such as metabolic disorders (e.g. lactase deficiency). Food allergy can be further divided into immunoglobulin E (IgE)- and non-IgE-mediated reactions. IgE-mediated food allergy (also referred to as Type I food allergy) accounts for the majority of food allergic reactions and is characterized by the presence of antigen-specific serum IgE antibodies that can reside on mast cells and bind circulating antigen.

The induction of Type I food allergic reactions is summarized in Figure 1. After ingestion, food proteins cross the epithelial barrier of the gut and come into contact with the immune system of the gut. Protein fragments are processed by antigen presenting cells (APCs) and displayed on their surface in association with major histcompatibility complex (MHC) class II molecules that can be recognized by a specific T cell receptor. In the presence of interleukin (IL)-4, allergic sensitization is subsequently initiated by the differentiation of naïve antigen-specific Thelper (Th) cells into effector Th2 cells. Typically, Th2 cells produce a cocktail of cytokines including IL-4, IL-5, IL-10, and IL-13 that, among other actions, encourage B cells to develop into IgE-producing plasma cells. Secreted antigen-specific IgE antibodies are distributed systemically and bind to the high affinity

receptor FcεRI on mast cells and basophils. Upon re-exposure to the offending food, cross-linking of the IgE antibodies provokes degranulation of mast cells and release of mediators (such as histamine, cytokines and proteases) leading to a variety of cutaneous (urticaria, angioedema, eczema), gastrointestinal (nausea, vomiting, abdominal pain, diarrhoea), respiratory (cough, wheeze), and systemic symptoms (collapse due to hypotension). These anaphylactic reactions occur within minutes to hours after ingestion of the food [3] and are potentially life-threatening. At present, food anaphylaxis is the leading single cause of anaphylactic reactions treated in emergency departments in Westernized countries [4].

1.2 Prevalence and diagnosis of food allergy

Food allergy has emerged as a major health problem in the western world due to the severity of the reactions and dramatic increase over the past decades. The prevalence of food allergy is greatest in the first years of life, with up to 8% of children younger than 4 years of age experiencing food allergic reactions [5]. However, most childhood food allergies generally resolve spontaneously with age and epidemiological studies suggest that about 2% of the adult population suffers from the disease [6]. Interestingly the perceived prevalence of food allergy is substantially higher than the actual prevalence. Up to 30% of the general population believes they have a food allergy [7].

In the diagnosis of food allergy, the medical history attempts to establish which food is involved. Skin prick tests and *in vitro* assays are performed to identify IgE sensitivity for specific foods. Although the skin test response and allergen-specific IgE serum concentrations seem to be predictive for clinical reactivity [8-10], the double-blind placebo-controlled food challenge remains the golden standard to confirm symptomatic food allergy [8].

1.3 Peanut allergy

Although dozens of foods have been implicated in food allergy, eight food products account for 90% of food allergic reactions: cow's milk, eggs, peanuts, tree nuts (walnuts, hazelnuts, almonds, cashews, brazil nuts, pecans and pistachios), soy, wheat, crustaceans, and (shell)fish [4, 11]. The food allergies most commonly associated with life-threatening anaphylaxis (those to peanuts, tree nuts, fish and shellfish) are the ones least likely to be outgrown.

Peanut allergy deserves special attention for several reasons. Peanut dominates the list of food allergens implicated in fatal and near-fatal allergic reactions and as many as one-third of peanut-sensitive patients have severe reactions [12]. Unlike most other food allergies, peanut allergy tends to be persistent which means a lifelong problem for about 80% of the peanut allergic population [13].

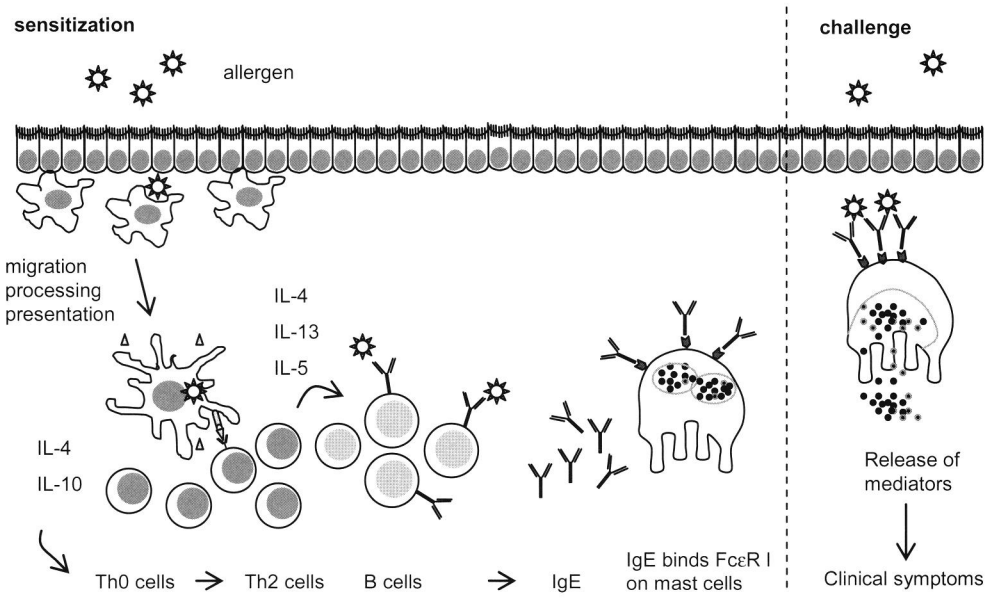

Figure 1. IgE-mediated hypersensitivity. An allergen that crosses the mucosal barrier is taken up and processed by APCs and subsequently presented to Th cells. Appropriate stimuli delivered by activated APCs and cytokines in the local environment initiate the development of Th2 cells, which produce cytokines such as IL-4 and IL-13 and stimulate the production of IgE antibodies by B cells. These allergen-specific IgE antibodies have the ability to bind to the high-affinity receptor FcεRI on mast cell surfaces (sensitization). Upon subsequent exposure to allergen (challenge) the membrane-bound IgE molecules are crosslinked by interaction with allergen, which stimulates the release of preformed and newly synthesized mast cell mediators. These mediators initiate a cascade of events, which are responsible for the clinical symptoms.

Avoidance of peanut exposure is hampered by the common use of peanut as an adulterant in different food products, such as cookies and chocolate, and these hidden peanut allergens are often responsible for accidental ingestion [14]. Furthermore, trace amounts of peanut, as low as 100 µg, have been reported to provoke allergic symptoms in patients [15, 16]. Unfortunately, several recent studies have demonstrated that the prevalence of peanut allergy is increasing [11, 17, 18].

1.4 Why are we not all allergic? Risk factors for the development of food allergy

Several factors that affect the development of IgE-mediated food allergic disease have been identified. The most important determinant seems to be genetic predisposition. An atopic phenotype (predisposition to mount IgE responses *per se*) in one or both parents substantially increases the chances of atopy in the offspring [19]. However, the majority of infants, even those genetically predisposed to atopy, are still spared from allergic diseases. Furthermore, it appears not to be genetically programmed against which particular proteins

an allergic response will be developed [20]. Therefore, it is generally assumed that against a genetic background of increased susceptibility, environmental factors also affect the outcome of allergic disease. These factors include, among others, the age at which food antigen is introduced, formula versus breastfeeding, dietary composition, composition of the gut microflora, and gastrointestinal infection status [21]. Since it has become clear that immunologic responses to food proteins can arise already *in utero* [22], prenatal factors, such as maternal allergen ingestion during pregnancy, may also play a role [22].

1.4.1 The hygiene hypothesis

It is apparent that all forms of allergic disease are increasing in prevalence and a number of hypotheses have been postulated to explain this increase in prevalence. One of these theories, the so-called 'hygiene hypothesis', suggests that due to a modern lifestyle the immune system of people in Western societies is no longer challenged by several infectious diseases of viral, bacterial, fungal and parasitic origin. These infections encountered early in life would "train" the maturing immune system thereby protecting against the development of allergic responses to innocuous environmental substances such as food [23]. In many epidemiological studies the protective effect of number of siblings, day care attendance, pet ownership, and farm residence has been confirmed. However, the hypothesis is mainly based on this circumstantial evidence and there is little experimental support. It now becomes apparent that the original hygiene hypothesis may be too simplistic to describe and explain the complex interactions between micro-organisms and the (developing) immune system.

An alternative interpretation of the hygiene hypothesis is that perturbations in gut microflora composition early in life caused by lifestyle changes result in disruption of mechanisms that are involved in maintaining mucosal barrier function, immune regulation, and development of tolerance. Data supporting this "altered microbiota" interpretation include the correlation found between altered fecal microbiota and allergic disease [21, 24]. Furthermore, in experimental animal models it has been shown that oral tolerance cannot be induced in germ-free mice [25] and that antibiotic-induced changes in the microflora lead to increased susceptibility to food allergy induction [26]. The underlying mechanisms associated with the hygiene hypothesis will be discussed in paragraph 8.3 of this chapter.

1.5 Therapy

Once the diagnosis of food allergy has been established, the only remedy is strict avoidance of the offending food as no definitive therapies are yet available. Present treatments only address the symptoms of an allergic reaction and try to prevent the occurrence of an anaphylactic shock.

Current therapeutic strategies focus on prophylactic medication in high risk individuals as well as immune-modulation of established food allergic responses to alleviate

chronic allergic responses and ideally, to acquire desensitization. A recent clinical trial showed that monthly injections of humanized recombinant anti-IgE increased the threshold for allergic responses in peanut-allergic patients [27]. However, this treatment cannot cure food allergy and life-long injections are required. To cure food allergy the gut mucosal immune system has to be "re-educated" in order to (re)-establish oral tolerance. Unfortunately, traditional immunotherapy used for pollen and bee-sting allergy, is unacceptable in food allergy because of the high incidence of side effects [28]. In the past years several novel therapeutic approaches have been tested including vaccination with plasmid DNA [29], peptide and genetically modified allergen immunotherapy [30], immunomodulatory bacterial or viral agents [31, 32], cytokines and cytokine modifiers [33], and complementary medicine such as Chinese herbs [34] some with promising results. Further elucidation of the mechanisms underlying allergic sensitisation to food proteins is crucial for future therapy development.

2. Food allergens

Foods are composed of proteins, lipids, carbohydrates, and micronutrients such as minerals and vitamins. Major food allergens, in general, are water-soluble glycoproteins ranging from 10 to 70 kDa that are abundant in the food and possess multiple IgE binding epitopes [35]. Interestingly, only a limited number of the numerous proteins in a given allergenic food act as allergens and it remains to be elucidated why even in atopic individuals predisposed to mounting allergic responses only a small proportion of food proteins are associated with allergic disease.

2.1 General properties of food allergens

Since allergen-specific IgE plays such a critical role in the etiology of food allergic disease, the presence of allergen-specific IgE-binding epitopes, either linear or conformational, is of crucial importance. Until now, however, there has been no indication for common structural characteristics of IgE (B cell) epitopes [36].

On the other hand, analysis of a variety of allergenic foods has resulted in the identification of certain biochemical properties shared by many but not all food allergens [37]. It became evident that most food allergens are very stable proteins that are resistant to the effects of food processing and digestion. This seems to make sense since food allergens have to be absorbed in a relatively intact form in order to be able to trigger an immune response [38]. The observation that many food allergens contain intramolecular disulfide bonds that are shown to be important to their allergenicity [39] has led to the assumption that protein structure may be an important factor in the ability of an allergen to resist denaturation. In addition, some physiochemical properties shared by many food allergens

such as binding various types of ligands, glycolisation, interaction with lipid structures, and aggregation may additionally enhance stability and promote allergenicity [40]. Importantly, thermal processing as well as other processing events can dramatically alter the structure and characteristics of food proteins, causing either an increase or decrease in allergenicity [41]. Most recently, it has been demonstrated that certain biochemical functions, such as enzymatic activities, may predispose a protein to become an allergen [36].

It is clear that several features will collectively determine whether a protein will have the characteristics required to stimulate an immune response of the vigor and quality necessary for the induction of allergic sensitization.

2.2 Classification of plant food allergens

Animal and plant food allergens belong to very few of the several thousand known protein families. Plant food allergens can be classified into superfamilies and families based on their structural and functional characteristics [42]. They are predominantly found in the cupin and prolamin superfamilies and in the protein families of the plant defense system. The cupin superfamily includes the 7S and 11S seed storage proteins, the vicilins and legumins, respectively. The proloamin superfamily consists of proline and glutamine containing storage proteins, cysteine-rich 2S albumin storage proteins, non-specific lipid transfer proteins, and cereal a-amylase and protease inhibitors, all stable to thermal processing and proteolysis. The pathogenesis-related (PR) proteins of the plant defense system represent a collection of unrelated protein families. In addition, there are some unrelated families of structural and metabolic plant proteins that harbor allergenic proteins, such as the profilins.

2.2.1 Peanut allergens

Peanut (*Arachis hypogaea*) belongs to the family *Leguminosae* and consists of albumins and storage proteins which, comprise about 87% of the total protein content [43]. At least ten peanut allergens have been described [44-46], and their official names given by the IUIS Allergen Nomenclature Subcommittee are *Ara h 1-8*, *Ara h* Oleosin, and *Ara h* Agglutinin (see Table I). The major peanut allergens *Ara h 1*, *Ara h 2*, and *Ara h 3*, which are recognized by IgE of the majority of peanut-allergic patients, have been purified, characterized and their IgE-binding epitopes have been identified [47-52]. *Ara h 1* is a 63.5 kDa glycoprotein belonging to the vicilin family with at least 23 IgE epitopes. *Ara h 2* (17 kDa) has been identified as a member of the 2S albumin storage proteins containing at least 10 IgE epitopes and has been found to act as a weak trypsin inhibitor [53]. *Ara h 3* is an 11S legumin-like storage protein of 56 kDa containing 4 linear IgE epitopes. The major allergens *Ara h 1* and *Ara h 2* have been shown to be extremely resistant to digestion [54, 55]. *Ara h 4*, previously described as a distinct peanut allergen is now considered to be the same allergen as *Ara h 3* [52].

The molecular weights of the peanut allergens *Ara h 2*, *Ara h 5*, *Ara h 6*, *Ara h 7*, *Ara h 8*, and *Ara h* Oleosin are in the same range of 14 to 18 kDa. Both *Ara h 6* and *Ara h 7*, like *Ara h 2*, belong to the conglutin protein family, which is related to the 2S albumin family and have the characteristically conserved cysteine residues of this group of proteins [44]. Interestingly, *Ara h 5* (a profilin) and *Ara h 8* (from the PR-10 family) show cross-reactivity with birch pollen allergens *Bet v 2* and *Bet v 1* respectively [45, 56].

Table I. A classification of plant food allergens and peanut allergens (adapted from Breiteneder *et al.*[42]).

Protein family	Peanut allergens
Cupin superfamily (storage proteins)	
Vicilins (7S globulins)	*Ara h 1*
Legumins (11S globulins)	
Glycinins	*Ara h 3/4*
Prolamin superfamily	
2S albumins (storage proteins)	
Conglutins	*Ara h 2, Ara h 6, Ara h 7*
Non-specific lipid transfer proteins	
α-amylase/ protease inhibitors	
Prolamins (storage proteins)	
Allergens from the plant defence system	
Pathogenis-related proteins	*Ara h 8*
Proteases	
Protease inhibitors	
Other allergenic structural and metabolic proteins	
Structural proteins	
Profilins	*Ara h 5*
Oleosins	*Ara h* Oleosin
Lectins	*Ara h* Agglutinin
Enzymes	

2.3 The food matrix

In addition to the intrinsic properties of food allergens, the composition of allergenic foods may also play a role in their allergenic nature (reviewed by Lehrer [57]). The presence of interactions between different compounds (proteins, lipids, carbohydrates and non-nutrients) may either influence the digestion process or affect the extremely important immune milieu [58].

In general, allergenic foods contain high protein, high lipid, and low carbohydrate concentrations. The high protein content makes sense since this enhances the likelihood to be exposed to substantial amounts of allergenic protein. High levels of fat may be involved in protecting allergenic molecules during the digestive process and may act as immune-stimulating agents (oil emulsions and other lipid structures are widely used as adjuvants). Furthermore, colocalized proteins, such as certain phytochemicals or lectins, have been shown to act as immunostimulatory and Th2-skewing proteins respectively [58, 59]. Nevertheless, very little is known about the effect of the food matrix on the allergenic potential of proteins and further research is warranted.

3. Animal models of food allergy

In allergic patients, research on food allergy is limited due to the inability of accessing and investigating cell function and associated processes that take place in the gut associated lymphoid tissue or other involved lymphoid organs. Furthermore, for ethical reasons, the process of prospective sensitization cannot be studied in humans. Therefore, animal models may provide a useful tool for investigating the complex mechanisms that are associated with the development of food allergy and for identifying new therapeutic strategies. Additionally, with the introduction of novel foods (including genetically modified foods) there has been a growing interest in the development of experimental animal models to assess the potential allergenicity of proteins.

3.1 Research models

In the past decade, several animal models have been developed for research on food allergy. Studies initiated in small animal models, including guinea pig [60], rat [61], and mouse [62] showed that rodents can be orally sensitized to food proteins with or without the use of adjuvant. Furthermore the allergic responses upon re-exposure to the offending food reflected the human situation [62, 63] and experiments on therapeutic strategies in the mouse model have provided promising results [33, 34]. Recently, larger animals - the dog [64] and swine [65]- that more closely mimic human physiology, anatomy, and allergic disease have been used for food allergy investigation.

3.1.1 The C3H/He(Ou)J mouse model of food allergy

In 1994 it was shown by Snider et al. [66] that intragastric exposure of young mice to food antigen in the presence of a mucosal adjuvant cholera toxin (CT) leads to antigen-specific IgE production and allergic sensitization. This model has been further adapted by Li and colleagues to study peanut allergic responses and to identify potential therapeutics

for food allergy. They have demonstrated that the murine model mimics the clinical and immunological characteristics of peanut allergy in human patients [62].

The mouse strain that was selected for the model is the C3H/HeJ mouse. Due to a point mutation within the coding region of *tlr4* gene that arose through spontaneous mutation between 1960 and 1968, this mouse strain is LPS-hyporesponsive [67]. Interestingly, it was suggested in 1982 that the C3H/HeJ mouse lacks oral tolerance induction [68]. Although it was shown a year later that oral tolerance to soluble proteins can be induced in this particular mouse strain [69], it has been demonstrated recently that the C3H/HeJ mouse is more susceptible to the induction of food allergy than its C3H/HeOuJ wildtype counterpart [26]. Nonetheless, both strains are currently used in food allergy research with a preference for the C3H/HeJ mouse in therapeutic studies.

3.2 Predictive models

Potential allergenicity of newly introduced protein(s) has become an important issue in safety evaluation of genetically engineered crops. At present, no single definitive test is available to predict allergenicity of newly expressed proteins. Therefore, a decision-tree strategy (see Fig. 1 chapter 8) has been introduced by the Food and Agricultural Organization (FAO)/World Health Organization (WHO)[70]. This approach relies on evaluating the source (allergenic or non-allergenic) of the gene, amino acid sequence similarity to known allergens, the immunoreactivity of the novel protein with serum IgE from allergic individuals that are allergic to the source of the transferred DNA, and their stability to digestion with proteases from the gastrointestinal tract. In addition, the importance of animal models to assess *in vivo* sensitization has been stressed [71], but unfortunately there are no validated animal models currently available. An accurate predictive model should be able to distinguish between immunogenicity (IgG, IgM, IgA, and cellular responses) and allergenicity (IgE). Validation of the model should include the determination of potency of known allergens and non-allergens followed by novel protein assessment. In this context, controversy exists regarding the use of pure protein versus a protein extract. Novel protein is usually expressed in a vector such as *Escherichia coli* or yeast, and differences in folding and glycosylation between the proteins expressed in the crop and those expressed in the vector may be present. Therefore, the use of purified protein in allergenic potential testing is preferable. Exposure to an extract would even more closely mimic human exposure by preserving glycosylation and/or binding to natural adjuvants found in the crop [72].

Although several animal models are being developed that seem promising, a lot of validation studies remain to be done and it is unlikely that a single animal model will be sufficient to address all issues concerning prediction of allergenicity to humans.

The human gastrointestinal tract comprises of an enormous mucosal surface area (200-400 m^2), which is exposed daily to vast amounts of food antigens, commensal bacteria and pathogens. The immune system of the intestine is therefore constantly trying to avoid potentially harmful adverse responses to food and the commensal flora, while effectively battling infectious microbes.

The usual response to soluble dietary proteins is the induction of systemic immunologic hyporesponsiveness, known as oral tolerance (reviewed by Weiner [73]). The induction of oral tolerance affects a broad spectrum of immunological functions, locally and systemically, to a varying degree. Several mechanisms by which oral tolerance may be mediated have been described, including T cell deletion, T cell anergy (a state of non-responsiveness), and active suppression [74]. There is convincing evidence that a high dose of antigen induces clonal deletion [75] and anergy [76], whereas multiple low-dose feeds are more likely to induce active suppression by regulatory T cells (Tregs) [77]. Nevertheless, the different means of tolerance induction are not mutually exclusive and they may overlap. In addition, many questions remain to be answered regarding the underlying cellular mechanisms of oral tolerance as well as the site(s) of oral tolerance induction.

It is apparent however, that a complex network of interacting gut immune cells is responsible for maintaining the balance between immune activation and tolerance.

4. The gut mucosal immune system

The intestine represents a primary immune organ with several specialized lymphoid structures and cell types. The first line of defense attempts to prevent intact antigens from encountering the immune system (immune exclusion). In addition, a highly developed adaptive immune system regulates the responses to antigens that have crossed the barrier. The anatomy and organization of the intestinal immune system are thought to favour the development of oral tolerance to soluble dietary proteins (reviewed by Mowat [78]).

4.1 Immune exclusion

The primary barrier of the gut that prevents antigens from encountering the immune system is the single layer of gut epithelium, which seals the surface of the gut with tight junctions. In addition, physiological processes, such as proteolysis and acid secretion, limit access of intact antigens to the epithelium. When the integrity of the barrier is disrupted this may lead to excessive immune responses to luminal antigens, resulting in gastrointestinal disease [79].

In addition to these physical and physiological defense systems, antibody-secreting B cells play a key role in maintaining immunological silence. The most prominent antibody at mucosal surfaces is IgA [80]. At least 80% of all plasma cells are located in the intestinal

lamina propria, producing massive amounts of IgA, more than all other immunoglobulin isotypes combined [81]. IgA that is excreted into gut mucosal secretions occurs predominantly as a dimeric complex. This secretory IgA (sIgA) serves as a first line of defense by inhibiting adhesion of microorganisms and by preventing absorption of intact proteins. Binding of sIgA to antigens does not trigger inflammatory processes, thereby preventing the development of chronic inflammation in the intestinal tract. In the gut wall, locally produced IgA can also interact with antigens that have reached the lamina propria and the resulting immune complexes are either taken up by phagocytosis or trancytosed back to the lumen via binding to the polymeric immunoglobulin receptor (pIgR), once more leading to immune exclusion [82].

Hence, the intestines are well equipped to control the quantity of antigen exposure. One has to bear in mind however that the gut epithelial barrier together with IgA secretion limits, but does not exclude, antigens from entering the tissues, while the immune system also constantly samples gut antigens.

4.2 The gut associated lymphoid tissue (GALT)

The GALT can be divided into immune structures that are involved in the induction of immune responses, and isolated immune cells (mostly lymphocytes) scattered throughout the epithelium (intraepithelial lymphocytes) and lamina propria that carry out effector functions. The organized tissues consist of the Peyer's Patches (PPs), the mesenteric lymph nodes (MLNs) as well as smaller isolated lymphoid follicles, which are distributed throughout the wall of the intestines.

4.2.1 Peyer's Patches

In 1677 Johan Conrad Peyer published a detailed article describing raised areas in the intestinal mucosa of several animals. Today these structures are called Peyer's patches (PPs) and they are found in the submucosa along the length of the small intestine. Although PPs development starts during fetal life, postnatal antigen stimulation (by the gut flora) is required for complete maturity [83]. Mature PPs are considered as secondary lymphoid tissue and consist of groups of large B cell follicles and intervening T cell areas. A single layer of columnar epithelial cells separates the lymphoid areas from the gut lumen. This follicle-associated epithelium (FAE) differs from the epithelium that covers the villius mucosa and the most distinguished feature of the FAE is the presence of microfold (M) cells, which are specialized enterocytes. Furthermore, the FAE contains DCs, B cells, T cells and macrophages. PPs were believed to be essential for the induction of intestinal immune responses. They are clearly the most important site for the generation of IgA-committed B cells, but the absolute dependence on PPs as inductive sites has been challenged by recent findings. It is particularly controversial whether PPs are essential for mucosal immune responses and tolerance to soluble antigens. It has been reported that oral tolerance to

ovalbumin (OVA) cannot be induced in mice lacking PPs [84]. However, others have found normal oral tolerance induction to either a low or high dose OVA in the absence of PPs, using different strains of knockout mice, by treatment with neutralizing antibodies, or by excising PPs from the intestine [85-87]. Furthermore, PPs do not seem to contribute significantly to T cell activation following oral administration of soluble antigen [88].

Isolated lymphoid follicles have the appearance of microscopic PPs and their formation, structure and functions seem to be comparable with those of PPs [89].

4.2.2 Mesenteric lymph nodes

The draining lymph nodes of the intestines, the MLNs, are the largest lymph nodes in the body and they are thought to represent the crossroads between the peripheral and mucosal recirculation pathways. There is clear evidence that MLNs have a crucial role in the induction of mucosal immunity or tolerance. Within a few hours after feeding a protein, antigen recognition takes place in the MLNs [90, 91] and naïve T cell activation and division occur predominantly in the MLN [88]. Furthermore, it is impossible to induce tolerance or IgA responses in the absence of MLNs [85, 92].

4.2.3 Effector lymphocytes in the intestines

IgA-committed B cells leave the PPs and migrate to the lamina propria where they, under the influence of IgA-enhancing cytokines (in particular TGF-β produced by T cells), become IgA-secreting plasma cells. Additionally, $CD4^+$ and $CD8^+$ effector T cells accumulate in the lamina propria and within the epithelial cell layer. Their selective homing is dependent on the expression of both the chemokine receptor CCR9 [93] and the integrin receptor $\alpha4\beta7$ [94], which binds the mucosal addressin cell-adhesion molecule 1 [95]. Almost all T cell populations in the lamina propria and overlying epithelium display characteristics of an effector/memory type but are heterogenous with regard to their phenotype and function. Different subtypes include γδ T cells and natural killer (NK) T cells. Together, these effector cells are the first cells to encounter invading pathogens and they ensure the GALT to respond rapidly and effectively to repeated assault by enteric pathogens.

5. Mucosal antigen uptake and distribution

Most ingested proteins are digested within the gut lumen by secreted enzymes. However, a small quantity of intact antigen does escape this enzymatic breakdown and is available for intestinal absorption.

5.1 Crossing of the epithelial barrier

How do soluble (food) antigens gain access to the GALT? M cells in the FAE have long been thought to act as "gateways to the mucosal immune system" by sampling antigens from the gut lumen and delivering them to APCs in the underlying subepithelial dome (SED) of the PPs. However, this accounts mainly for particulate antigen whereas other, equally important, pathways are involved in the uptake of soluble antigens. Cells of the intestinal epithelium have the capacity to transport a small proportion of antigenic material from the intestinal lumen to the underlying tissues by fluid-phase transcytosis. It has been demonstrated that 90% of the endocytosed protein is degraded in lysosomes during transport, whereas 10% is transported via a direct transcellular pathway and stays intact [96]. In addition, a subpopulation of DCs has been identified that takes up apoptotic epithelial cells and its contents such as endocytosed antigens [97]. Finally, it has been shown recently that DCs located in the gut epithelium can sample intestinal antigens directly from the lumen [98].

5.2 Antigen uptake and distribution

After crossing the barrier, the nature of the antigen-induced immune response will be determined by where and how the antigen is taken up and presented to T cells (reviewed by Mowat [78]). The possible fates of orally administered antigens are summarized in figure 2. Antigens can be presented locally in the lamina propria to T cells by MHCII-expressing enterocytes or professional APCs. However, naïve $CD4^+$ T cells are rare in the lamina propria and they do not migrate out of the gut [99]. Therefore, most antigen-loaded APCs from the PPs or gut mucosa will be transported via the afferent lymph to the MLNs where presentation to naïve T cell occurs. It is also possible that free antigen reaches the MLN via the afferent lymph, especially following administration of high doses of antigen. Primed T cells leave the MLNs via the efferent lymph and after entering the bloodstream through the thoriac duct, migrate either to the mucosa to induce local immune responses, or to the periphery for the induction of systemic immune responses. Following high dosing of proteins, free antigen might also gain direct access to the bloodstream from the gut and, after passing the liver, interact with T cells in peripheral lymphoid tissues such as the spleen [91].

Intragastric administration of protein has been demonstrated to lead to antigen presentation in both the gut and periphery [100].

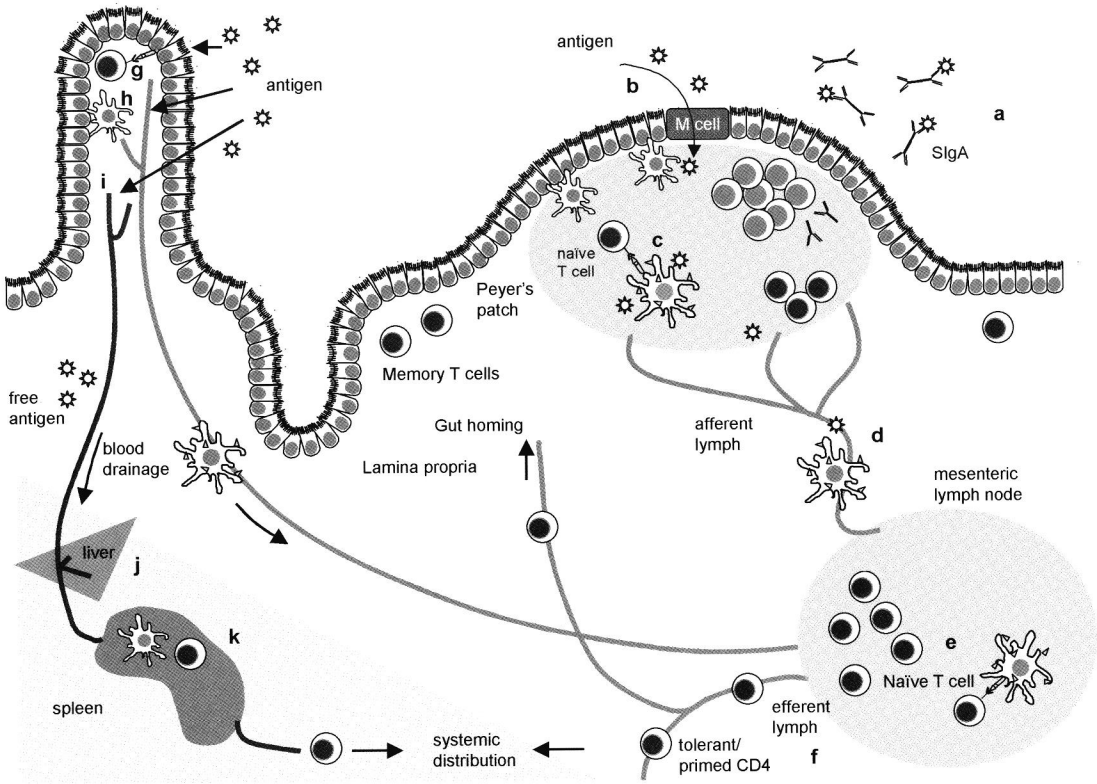

Figure 2. Antigen uptake and presentation in the intestine (adapted from Mowat, 2003). If antigen is not bound by sIgA **(a)** it might enter the SED through M cells in the FAE **(b)** and after been taken up by local DCs presented directly to T cells in the PPs **(c)**. Alternatively, and more likely, antigen-loaded DCs from the PP or free antigen might migrate via the afferent lymph to the MLN **(d)** where subsequent T cell recognition takes place **(e)**. Antigen might also reach the MLN after been taken up by DCs resident in the lamina propria or within the epithelium covering the lamina propria **(h)**. In the lamina propria MHCII-expressing enterocytes might also act as local APCs **(g)**. In all cases, the antigen-responsive CD4+ T cells that acquire expression of $\alpha 4\beta 7$ integrin and CCR9, leave the MLN via the efferent lymph **(f)**, and after entering the bloodstream, exit into the mucosa through vessels in the lamina propria. CD4+ T cells from the MLN might also disseminate from the bloodstream throughout the peripheral immune system. Finally, antigen might also gain direct access to the bloodstream **(i)** and, after passing the liver **(j),** interact with T cells in peripheral lymphoid tissues such as the spleen **(k)**.

6. Antigen presentation

Current evidence suggests that orally administered antigens, similar to antigens administered via other routes, are presented by bone marrow-derived APCs [101]. In the mucosal immune system DCs appear to be the key APCs for the priming of naïve T cells. *In vivo* expansion of DCs using Flt3 ligand enhances oral tolerance induction and antigen-induced expansion of T cells in the GALT [102, 103]. Together with the fact that oral tolerance and priming do not require the presence of other APCs such as B cells [104], these findings implicate that DCs play an essential role in the process of antigen presentation and subsequent activation of naïve T cells.

DCs comprise several subsets that differentially control lymphocyte function and that are present to different degrees in tissues and organs. They can be divided into DCs from myeloid origin (mDCs), including the Langerhans cells and interstitial DCs, and those from a plasmacytoid origin (pDCs), which secrete high amount of IFN-α upon virus encounter. Other mDC or pDC subpopulations are defined by the expression of cell surface markers such as CD11c, CD8α, CD11b, and CD4. In the mouse at least eight different DC populations can be defined [105].

6.1 DC activation

Under normal physiological conditions DCs are "quiescent" or "immature", capable of presenting antigen and inducing tolerance, but being sufficiently responsive to inflammatory stimuli to allow T cell priming and protective immunity when necessary. Maturation of DCs is induced by numerous agents including microbial infection. Activation of DCs by microbes depends on specific recognition of conserved molecules of microbial origin, which are known as pathogen-associated molecular patterns (PAMPs). PAMPs are detected through pattern-recognition receptors (PPRs). These molecules include the members of the Toll-like receptor (TLR) family and the nucleotide-binding site/leucine-rich repeat (NBS/LRR) proteins such as Nod 1 [106]. Ligand binding to these receptors induces upregulation of costimulatory moluecules (CD80 and CD86) and adhesion molecules (such as CD54), increased surface expression of MHCII, and secretion of (pro)inflammatory cytokines (such as IL-12), all contributing to optimal T cell activation. Recently it has been suggested that unlike the upregulation of costimulatory molecules, production of inflammatory cytokines can only occur in the presence of TLR stimuli [107].

Harmless food antigens normally fail to fully activate DCs and therefore induce antigen-specific tolerance [108]. Interestingly, DC migration from the gut wall to the MLN occurs constitutively i.e. in the absence of inflammatory stimuli [109]. This might represent a mechanism by which luminal antigen can be presented in a way that ensures tolerance induction rather than ignorance.

6.2 DC-T cell interaction

It is believed that DCs play a key role in regulating the balance between tolerance and immunity by controlling differentiation of naïve T cells. The outcome of DC-induced T cell differentiation is most likely not due to an intrinsic feature of the DC but rather the result of environmental stimulation, which includes the local cytokine milieu. T cell activation depends on interaction with costimulatory molecules expressed on, and proinflammatory cytokines produced by the DC. The tolerogenic function of DCs appears to involve various mechanisms including deficiency of, or signaling through, costimulatory molecules, expression of death-inducing ligand (in particular Fas ligand), secretion of immunosuppressive cytokines (such as IL-10, TGF-β, and IFN-α), and enzyme induction [110].

6.2.1 Costimulatory pathways

As mentioned, optimal T cell activation requires, besides T cell receptor (TCR)-MHC/peptide complex recognition, a second costimulatory signal that is delivered by cell surface molecules. Antigen presentation in the absence of costimulation results in T cell anergy [111].

The most extensively studied costimulatory pathways are the CD40-CD154 and CD28/CTLA-4-CD80/CD86 interactions, which predominate in the activation of naïve T cells.

CD40-CD154 pathway

CD40 is expressed by DCs and can be upregulated following activation. Its ligand CD154 is expressed on activated T cells. Engagement of CD40 by its ligand has been shown to be critical for DC maturation. Interruption of CD40-CD154 interactions results in inadequate antigen presentation and failure to upregulate adhesion and costimulatory molecules such as CD80 and CD86, thereby affecting T cell priming [112]. Accordingly, DCs lacking CD40 cell surface expression prevent T cell activation, can suppress ongoing immune responses and have been shown to induce IL-10-secreting Tregs [113]. Whether CD40-CD154 interactions also play a role in oral tolerance induction remains controversial. In a study by Hanninen et al, blockade of CD154 did not affect mucosally induced tolerance to OVA [114], whereas Kweon and colleagues showed that oral tolerance cannot not be induced in $CD40^{-/-}$ mice [115].

CD28/CTLA-4-CD80/CD86 pathway

CD28-CD80/CD86 signaling is crucial for the activation of T lymphocytes (reviewed by Croft [116]). CD86 (B7-2) is constitutively expressed on DCs at low levels and rapidly upregulated upon stimulation, whereas CD80 (B7-1) is inducibly expressed later than CD86. CD28 is constitutively expressed on T cells. Engagement of CD28 results in T cell

activation by the induction of IL-2 transcription and also ensures T cell survival. Immature DCs, which express low levels of the costimulatory molecules CD80 and CD86, induce reversible antigen-specific T cell hyporesponsiveness (anergy) or apoptosis [117].

Importantly, CD80 and CD86 can also act as direct co-inhibitors by interacting with cytotoxic T lymphocyte antigen 4 (CTLA-4) on T cells [118]. CTLA-4 expression is rapidly upregulated following T cell activation and the affinity of CD80/CD86 for CTLA-4 is higher than for CD28 [119]. The mechanisms by which CTLA-4 inhibits T cell responses are not clear but might include outcompeting CD28 for B7 binding and/or directly antagonizing CD28- and TCR-mediated signaling within the cell. The critical role of CTLA-4 as a negative regulator of T cell activation is dramatically illustrated in CTLA-4 deficient mice, which die within 3-4 weeks after birth from massive lymphocyte proliferation, infiltration and autoimmune-related tissue destruction [120, 121]. Studies on the involvement of CTLA-4 in oral tolerance show that CTLA-4 is required for the induction of oral tolerance [122-124]. The role of CTLA-4 in the activation of Tregs might also contribute to tolerance induction. Antagonistic CTLA-4-specific antibodies have been shown to abrogate Treg cell function[125, 126].

Interestingly, CD80 and CD86 also seem to influence the immune response through reverse signaling. Recent studies indicate that, following engagement of CTLA-4, CD80 and CD86 can transmit suppressive signals into DCs [127, 128]. Both a soluble form of CTLA-4 (CTLA-4Ig) and a membrane anchored form of CTLA-4 on Tregs can trigger the immunosuppressive pathway of tryptophan catabolism in DCs. Activation of the CTLA-4-B7 signaling pathway results in the production of IFN-γ by DCs, which acts in an autocrine or paracrine fashion to induce indoleamine 2,3 dioxygenase (IDO), an enzyme that depletes tryptophan resulting in reduced T cell proliferation [127]. Hence, bi-directional CTLA-4-B7 interactions may participate in downregulation of T cell responses and induction of T cell tolerance. Together, signaling via CTLA-4 represents an important mechanism to control T cell activation and to maintain tolerance to self and foreign antigens.

In the past years, the B7-CD28 family has been extended with newly identified costimulatory (ICOS- B7-H2) and, in particular, inhibitory pathways such as B7-DC/PD1-B7-H1, and BTLA-B7-H4 [129] and it is not unlikely that more pathways will be discovered in the future. Although our understanding of these pathways is still rudimentary, it is apparent that a precise balance between all the different pathways determines the outcome of T cell responses.

6.2.2 Cytokines

Signaling via secretion of cytokines represents an additional way of DC-T cell cross-talk. Maturation of DCs leads to the secretion of high levels of IL-12, which induces the generation of mainly Th1 effector cells. In an immature state DCs can produce IL-10 and TGF-β, which have been shown to contribute to tolerance induction [130] [131] and the generation of Tregs.

6.3 Mucosal dendritic cells

Under steady state conditions DCs from the intestine migrate constitutively from the lamina propria and/or PPs to the MLNs and are responsible for tolerance maintenance to luminal antigens. Although intestinal DCs are not inherently tolerogenic, it is believed that due to the unique local immune environment in mucosal tissues, DC subsets in the GALT have distinctive immune-modulating capabilities.

In the MLNs and PPs three subsets of "conventional" $CD11c^{hi}$ myeloid-lineage derived DCs have been described [132] [133] in addition to several "unusual" phenotypes. DCs in the PPs seem particularly capable of producing IL-10 and inducing T cells to secrete high levels of both IL-10 and IL-4, compared with mDCs from non-mucosal sites [134]. The lamina propria also contains several unusual subsets of DCs, including some that are similar to the IL-10-inducing DCs that have been described in the PPs [135]. DCs from the MLNs of antigen-fed mice produce IL-10 or TGF-β and preferentially stimulate antigen-specific $CD4^+$ T cells to produce IL-10 and/or TGF-β [131]. It is unknown however whether these DCs are all derived from DCs migrating from the PPs and/or lamina propria.

Recently, the plasmacytoid-derived DCs, characterized by the phenotype $B220^{hi}$, $GR\text{-}1^{hi}$, $CD11c^{intermediate}$, $CD80^{lo}$, and $CD86^{lo}$, have gained interest due to their presumed "highly tolerogenic" capacity. De Heer and colleagues have demonstrated that in the lung mucosa mDCs are important for inducing T cell division and priming, whereas pDCs suppress the generation of effector T cells [136]. pDCs reside in the PPs, MLNs and lamina propria and they have been shown to secrete IFN-α and IL-10 and to express high levels of IDO, thereby driving the differentiation of Tregs and immune suppression [130, 137].

An additional feature shared by MLN and PP DCs is the ability to induce the selective generation of gut tropic T cells by increasing α4β7 integrin expression on T cells [138].

It remains controversial why intestinal DCs have unusual characteristics, but it seems likely that precursor DCs are locally modified by cytokines such as IL-10 and TGF-β, which are both abundantly present in the GALT [78]. Finally, it is important to realize that, when activated by the appropriate stimuli, DCs in the intestine can acquire a mature phenotype accompanied by IL-12 production, increased migration, and the induction of effective immunity.

7. Mucosal regulatory T cells

In the absence of pathogenic stimuli, the mucosal microenvironment together with the unique features of mucosal DCs favour the development of T cells with regulatory properties. Several types of mucosal T cells have been shown to play important roles in general immune suppression, oral tolerance induction and maintenance of gut immune homeostasis. Tregs were first described in the 1970s, when they were considered to be mainly $CD8^+$ and were referred as 'suppressor' T cells [139]. The recent wave of research has rehabilitated the old concept of suppressor cells and has brought important new insights into the field of tolerance. Although $CD8^+$ T cells may act as suppressor cells, solely $CD4^+$ T cells have been shown to be crucial in the development of oral tolerance [140, 141]. Several Treg subsets have been described and a distinction has been made between Tregs that emerge during T cell development in the thymus (naturally occurring Tregs), and Tregs that develop following antigen encounter in the periphery (adaptive or induced Tregs). Further subdivisions of these subsets are based on the expression of surface markers and the production of cytokines [142]. Tregs exert their regulatory function either via direct cell-cell contact or via secretion of suppressive cytokines. Mucosal administration of antigen seems to trigger all types of Tregs and in Table II an overview is presented of the different T cell subsets, which are believed to be involved in the regulation of immune responses to orally administered proteins.

7.1 Adaptive regulatory T cells

Adaptive Tregs, similar to Th1 and Th2 cells, arise from naïve precursors and can be differentiated *in vitro* and *in vivo*. They have been shown to be specific for food antigens, bacterial flora antigens, pathogens, and some self-antigens. *In vivo*, their generation appears to be controlled by the activation status of the DC and cytokines in the environment. *In vitro*, adaptive Tregs can be generated by repeated antigen stimulation in the presence of IL-10 plus IFN-α (Tr1 cells)[143], or TGF-β (Th3 cells)[144]. Adaptive Tregs function through the release of suppressive cytokines (IL-10 and TGF-β) and migrate to inflamed tissue and peripheral sites to maintain peripheral tolerance at mucosal surfaces [145].

TGF-β and Th3 cells

One of the most significant advances in immune suppression has been the demonstration that the pluripotent cytokine TGF-β plays a central role [146]. The TGF-β family consists of three members, TGF-β1, TGF-β2, and TGF-β3, which all have similar roles in the regulation of immune cells. TGF-β directly inhibits the proliferation and differentiation of naïve T cells. Furthermore it has been shown to inhibit maturation of DCs, thereby indirectly affecting T cell responses (reviewed by Gorelik and Flavell [147]). TGF-β is also known to play an essential role in maintaining normal intestinal homeostasis [148].

Furthermore, although high and low dose tolerance can be induced in TGF-β null mice [149], a number of studies have demonstrated that several aspects of oral tolerance can be inhibited by anti-TGF-β antibody administration *in vivo* [150, 151].

Table II. An overview of Treg subtypes that may play a role in regulating responses to oral antigens

Treg subtypes	Characteristics	Mechanisms of suppression
Adaptive $CD4^+$ Tregs		
Tr1 cells	IL-10 (TGF-β) production	IL-10 (and TGF-β) secretion
Th3 cells	TGF-β (IL-10, IL-4) production	TGF-β secretion
Naturally occurring $CD4^+$ Tregs		
$CD4^+CD25^+$ T cells	*Fox p3*, CD25, GITR, and CTLA-4 expression TGF-β and IL-10 production	CTLA-4 signaling Membrane-bound TGF-β TGF-β and/or IL-10 secretion
Other Tregs		
$CD8^+$ T cells	CD8αα or CD8αβ	TGF-β, IL-4, or IL-10 secretion
NK T cells	NK1.1 expression Recognize CD1d IL-4 and IFN-γ production	IL-4 and IL-10 secretion Induction of Tregs
γδ T cells	γδ TCR IL-4 and IFN-γ production	Unknown (homeostatic role)

Th3 cells from a unique Treg subset, which primarily secretes TGF-β, provides help for IgA and has suppressive properties for both Th1 and Th2 cells *in vivo* (reviewed by Weiner [144]). Antigen-specific Th3 cells are induced following feeding of low dose antigen and it is thought that the gut microenvironment, rich in TGF-β and Th2 cytokines (especially IL-4), stimulates Th3 immune deviation [144]. Th3 cells express CTLA-4 on their cell surface and secretion of TGF-β is triggered by CTLA-4 signaling. The suppressive effect of Th3 cells is abrogated by injection of anti-TGF-β [152], confirming that Th3 cell function is dependent on TGF-β. Recent data suggest that, by secreting TGF-β, Th3 cells may also contribute the development and/or expansion of other Tregs such as Tr1 cells and $CD4^+CD25^+$ T cells [153, 154].

IL-10 and Tr1 cells

IL-10 is a well-known immunoregulatory cytokine that was originally described as a factor that could inhibit Th1 effector function [155]. IL-10 is also a potent inhibitor of proinflammatory cytokine production, APC activation, and IL-12 production [156, 157] and is associated with the induction of $CD4^+$ T cell anergy [158]. Suppression by IL-10 occurs both through inhibition of antigen presentation and through direct effects on T cells [158]

A distinct type of Tregs, the Tr1 cell, is characterized by secretion of high levels of IL-10 and significant amounts of TGF-β, but no IL-4 or IL-2 upon stimulation. Tr1 cells proliferate poorly following polyclonal or antigen-specific activation [158]. The differentiation of Tr1 cells *in vivo* is controlled by functional subsets of immature DCs, which produce high levels of IL-10 and low levels of IL-12 [159]. DCs in the intestine seem particularly capable of secreting high amounts of IL-10 and they have been shown to selectively induce Tr1 cells [134]. Tr1 cells are able to suppress T cell proliferation and function, and their suppressive effect can be reversed by neutralizing IL-10 and/or TGF-β [160]. In humans, self-reactive Tr1 cells have been isolated from healthy donors [161]. Interestingly, it has been demonstrated that Tr1 cells display an enhanced and selective capacity to migrate to inflamed tissues [145, 162, 163]. This peculiar migration of Tr1 cells is not dependent on the type of tissue, the type of inflammation, or the presence of the specific antigen.

It is clear that IL-10-secreting Tr1 cells play an important role in controlling immune responses in the intestine. IL-10-deficient mice spontaneously develop inflammatory bowel disease and Tr1 clones can prevent the development of this disease [164]. Furthermore, antigen-specific IL-10-producing Tr1 cells have been shown to be induced in PPs and MLNs by feeding proteins [152, 165]. However, data on requirement of IL-10 in oral tolerance induction are controversial. Whereas oral tolerance cannot be achieved in IL-10 knockout mice [87], oral tolerance can be induced normally in mice treated with IL-10-depleting antibodies [166]. Furthermore, anti-IL-10 cannot abrogate established tolerance *in vivo* [166].

7.2 Naturally occurring $CD4^+CD25^+$ regulatory T cells

Phenotypic markers, rather than cytokine profiles, have been used to define a subset of naturally occurring Tregs, the $CD4^+CD25^+$ Tregs. These T cells, first described by Sakaguchi et al [167], originate from the thymus and account for around 5 to 10% of peripheral T cells. They constitutively express high levels of CD25 (IL-2α receptor), CTLA-4, and glucocorticoid-induced tumor necrosis factor receptor (GITR) [168]. However, at present, the most distinctive marker is the recently identified transcription factor *Foxp3*, which is predominantly expressed in $CD4^+CD25^+$ Tregs and seems to have a key function in their development and function [169]. The expression of CD25 by natural Tregs also appears to be crucial for their generation, survival and function rather than reflecting their chronically activated state [170, 171]. In contrast to adaptive Tregs, $CD4^+CD25^+$ Tregs migrate preferentially to lymphoid organs, mostly to the spleen [172], which suggests that $CD4^+CD25^+$

Tregs control the proliferation and the differentiation of naïve T cells [145, 173]. The $CD4^+CD25^+$ T cell repertoire is biased to recognize 'self' and therefore this Treg subtype has been mainly associated with maintaining peripheral self tolerance [167, 174]. It is unknown whether they also recognize foreign antigens. However, thymic selection seems not to represent the solely mode by which $CD4^+CD25^+$ Tregs can be generated because it has been recently shown that low dose antigen application in the absence of costimulation can convert naïve T cells into $CD4^+CD25^+$ Tregs [175].

Mechanisms of suppression by $CD4^+CD25^+$ regulatory T cells

In vitro studies have demonstrated that $CD4^+CD25^+$ Tregs suppress the proliferation of $CD4^+$ and $CD8^+$ T cells by a mechanism that is independent of TGF-β and IL-10 secretion and presumably involves direct cell-cell contact. The cellular interactions that have been implicated in cell contact-dependent suppression are membrane-bound TGF-β [176] and CTLA-4 [125]. CTLA-4 may interact with CD80/86 on activated T cells, which directly prevents activation of the T cell via 'outside-in' signaling, or may interact with CD80/86 on DCs, resulting in activation of IDO and indirect suppression of T cells [177]. $CD4^+CD25^+$ Treg-mediated suppression can be inhibited *in vitro* by activation of APCs through TLR binding (LPS or CpG) resulting in IL-6 production and/or GITR ligand expression. Activated T effector cells expressing IL-6 receptor and/or GITR will bind IL-6 and GITR ligand, which makes them insensitive to the action of suppressor T cells [177].

Data indicate that more than one mechanism of $CD4^+CD25^+$ Treg suppression is operating *in vivo*, possibly depending on the microenvironment and the nature of the immune response. Postulated mechanisms include IL-10 secretion [178], TGF-β secretion (suppression of $CD8^+$ cells)[179], and CTLA-4 interaction [180].

Studies by Powrie and colleagues were the first to demonstrate that CD25-expressing T cells play a suppressive role in mucosal immune reponses [181, 182]. By now, there is also consistent evidence for the generation of $CD4^+CD25^+$ Tregs after oral antigen administration [183-185]. However, the function of $CD4^+CD25^+$ Tregs in control of responses to oral antigens remains to be established.

7.3 Other mucosal T cells with regulatory properties

$CD8^+$ suppressor cells were the first identified population of Tregs thought to be involved in oral tolerance [186]. Distinct functional subsets of $CD8^+$ cells have been described that may contribute to oral tolerance induction [187-189]. However, their functions have not been clearly defined and there is no absolute requirement for $CD8^+$ T cells in the induction or maintenance of oral tolerance [190].

Another T cell subtype in the immunoregulatory network is the NK T cell. It has been reported that oral tolerance induced by feeding antigen can be transferred by NK T cells from the liver [191]. Furthermore, a more recent study suggests that NK T cells are required for the induction of oral tolerance to nickel [192]. In this study, the NK T cells were shown to be indispensable for the induction of Tregs that, once induced, mediated their regulatory function independently of NK T cells. Nevertheless, oral tolerance can be induced in mice lacking NK T cells, indicating that NK T cells are not essential for the induction of oral tolerance [193].

Finally, γδ T cells seem to play a role in some models of oral tolerance. Low dose oral tolerance can be prevented or even abrogated by depleting γδ T cells *in vivo* and oral tolerance can be transferred by γδ T cells isolated from fed mice [194, 195]. Moreover, they are thought to play an important homeostatic role in regulating potentially harmful immune responses in the intestine [196].

7.4 Bystander suppression

An important phenomenon in the context of Treg-mediated immune suppression is the concept of bystander suppression; Tregs induced by fed antigen can suppress immune responses stimulated by a different antigen, as long as the fed antigen is present in the anatomic vicinity [197]. This process can occur by the production of regulatory cytokines, direct contact with the responding T cell, or by 'deactivating' the APC that is attempting to stimulate the third party T cell [74]. The concept of bystander suppression has been shown for $CD4^+CD25^+$ T cells, Th3, Tr1, and $CD8^+$ T cells and probably plays an essential role in the maintenance of a generally suppressive environment in the gut.

One of the interesting questions in the field of immune regulation that remains to be elucidated is the relationship between the various populations of regulatory T cells that have been described. It is not clear whether or how the different subsets influence each others development and/or function and how they collectively orchestrate immune regulation.

8. Food allergy: when mucosal immunity goes wrong

As discussed in the previous paragraphs, the mucosal immune system has adopted numerous mechanisms of regulation, including an intimate interplay between multiple cell types and the creation of a generally suppressed environment, to avoid immune responses to harmless proteins and to induce tolerance instead. Obviously, it is hypothesized that a breakdown in oral tolerance mechanisms or a failure of oral tolerance induction results in food hypersensitivity. In this paragraph, factors that may be involved in undermining oral tolerance, and favour the induction of allergic sensitisation to food proteins, will be discussed.

8.1 Antigen availability

To be able to induce allergic responses, proteins have to cross the intestinal epithelial barrier in substantial amounts and in a relatively intact form. As discussed in paragraph 5.1 there are several mechanisms that control the uptake of intact proteins and disruption of these mechanisms may increase the risk of hypersensitivity responses.

8.1.1 Digestion

One of the biochemical characteristics shared by many food allergens is that they are relatively stable proteins and resistant to denaturation [38]. Several studies have shown that major allergens, such as *Ara h 1* and *Ber e 1,* are resistant to digestion [39, 198, 199]. Accordingly, anti-ulcer drugs, which hinder gastric digestion, promote the development of food allergy in both mice [200] and humans [201, 202].

8.1.2 Intestinal integrity

In general, the intestinal epithelium constitutes an efficient barrier to the penetration of massive amounts of food antigens. However, in young children the mucosal epithelial barrier is not fully developed and there is good evidence that antigen absorption in the neonatal period is higher than in adults. Uncontrolled penetration of antigens in infants represents one of the factors contributing to the development of hypersensitivity responses to food and the high incidence of food allergy in young children [203].

During life, the integrity of the intestinal epithelial barrier may be altered by environmental factors such as inflammation and/or infection. In inflammatory conditions cytokines disrupt tight junctions of epithelial cells, leading to increased permeability of the epithelium and increased paracellular transport of luminal antigens [204]. Both IFN-γ and TNF-α have been shown to directly affect barrier function [205, 206] and TNF-α has accordingly been implicated in cow's milk allergy [207]. In line with these results, infection with *Helicobacter pylori* has been demonstrated to increase the relative proportion of intact protein transported (without disturbing tight junctions) [208]. Consequently, *Helicobacter pylori* infection inhibits oral tolerance induction, which is prevented by a gastro-protective agent that reinforces digestive epithelial barrier integrity [209].

The release of IgA represents another non-specific barrier mechanism. Low levels of IgA have been associated with food allergy in humans [210] and mice [211] and IgA-deficient individuals are predisposed for the development of food allergy [212].

8.2 Activation of Th2 responses

A central finding from experimental models and human studies is that allergic diseases are due to an aberrant immune response mediated through Th2 effector cells and their cytokines including IL-4, IL-5, and IL-13. Numerous studies have found a close relationship between food allergy and Th2-skewing and between tolerance and Th1-

skewing [213-216]. Th2 and Th1 responses have always been considered to be mutually exclusive and reciprocally regulated [217]. However, whether Th1 responses actually counter-regulate Th2 responses *in vivo* remains controversial [218] and it is now believed that the original Th1/Th2 concept in allergy is oversimplified.

Th2 cells are induced by mature DCs and adoptive transfer of DCs isolated from spleen and PPs of food allergic mice induces antigen-specific IgE in naïve recipients, demonstrating that DCs play a pivotal role in allergen-specific IgE responses [219]. It is unknown however which mechanisms underlie Th2 polarization by DCs. Factors that have been proposed to favour the generation of Th2 responses include the absence of IL-12 production (Th2 would be a default pathway), quality and intensity of costimulatory molecule expression, and the presence of IL-4, IL-25, IL-13, IL-6, and histamine [220].

8.3 Regulation

There is strong evidence that the fine balance between effector T cells and Tregs is decisive in the development of allergy. Tregs appear to be less frequent and/or less efficient in atopic patients and Tregs have been shown to prevent allergic disease in experimental models [221, 222]. The questions remain how the balance is disturbed in allergic disease and, just as important, how the delicate balance is normally maintained.

It has been proposed that microorganisms and the normal commensal flora of the gastrointestinal tract play a prominent role in the prevention of allergic sensitisation [223] probably by inducing Tregs [23]. This hypothesis is supported by several epidemiological and experimental studies (reviewed by Prioult *et al.* [224]).

As mentioned in paragraph 6.1, microbial pathogen-associated molecular patterns are recognized by PRRs on APCs. To date the TLRs represent the best-characterized PRRs and a number of TLRs have been implicated in protection from allergic disease [224]. Recent data suggest that TLR4 signaling is involved in prevention of allergic sensitization to peanut. TLR4 mutant mice were shown to be more susceptible to food allergy than wildtype mice. Furthermore, TLR4 wildtype mice exhibited allergic symptoms similar to TLR4 mutant mice after reduction of the bacterial flora by antibiotic treatment. In addition, co-administration of CpG, a TLR9 ligand, prevented allergic sensitization to peanut in the TLR4 mutant mice [26].

Another interesting relationship is that between allergy and helminth (worm) infection. Both are associated with Th2, IgE, and mast cell responses but recent evidence suggests that intestinal helminth infection may protect from allergic disease. Anti-helminthic treatment increases the risk of allergy symptoms in humans [225] and helminth infection has been shown to prevent allergic sensitization to peanut in mice [226]. The mechanism of protection may involve the induction of IL-10 production [227].
Together, these data have led to a high interest in prophylactic and therapeutic approaches based on the stimulation of immunoregulatory networks by microbial agents. Treatment

with probiotics and helminthic parasites are two promising approaches currently being investigated.

8.4 Persistence of the allergic response

A number of food allergies, such as peanut allergy and tree nut allergy are rarely outgrown and in time additional allergies may develop [228]. Several mechanisms have been described, which are involved in maintenance and aggravation of allergic disease. Upon provocation with the offending allergen, intestinal permeability and transepithelial transport increase [229, 230], which may induce further sensitization to the allergen (positive feedback mechanism) or to other co-ingested antigens. The increase in intestinal permeability seems to be mainly effected by mast cell degranulation [231], while CD23-mediated transport is responsible for increased transepithelial allergen uptake. Antigen-specific CD23 (FcεRII) is expressed by intestinal epithelial cells and upregulated following allergic sensitization [230, 232]. CD23 mediates not only the rapid transport of antigens but also their protection from intracellular degradation [233].

Besides increasing intestinal permeability, mast cells additionally polarize the immune response towards a Th2 phenotype by mediators such as histamine, prostaglandin E2 and IL-4 [234, 235], thereby maintaining or enhancing sensitization. Th2 cytokines also play a role in maintenance of allergic responses by promoting IgE synthesis (IL-4 and IL-13) and by chemoattraction of inflammatory cells such as mast cells (IL-4) and eosinophils (IL-5). Finally, by IgE-mediated stabilization and upregulation of FcεRI, mast cells maintain memory for lengthy periods of time even in the absence of detectable serum IgE [236].

9. Scope and outline of this thesis

The studies described in this thesis have been performed to gain more insight in the factors and mechanisms that determine the outcome of immune responses to orally administered food proteins.

More specifically, the aims of this thesis are:

- Development of an oral tolerance and sensitization model to investigate immune responses to the model allergenic food peanut.
- Investigation of the influence of the food matrix on immune responses to individual peanut proteins.
- Elucidation of the involvement of the CD28/CTLA-4-B7 signaling pathway in oral tolerance and sensitization to peanut.
- Elucidation of the regulatory role of $CD4^{+}CD25^{+}$ T cells and pDCs in responses to orally administered peanut proteins.

In **chapter 2** a mouse model of peanut hypersensitivity is described in which peanut and allergen-specific responses are measured following oral exposure to a whole peanut protein extract in the presence of the mucosal adjuvant cholera toxin. Data from this study suggest that both Th2- and Th1-associated responses contribute to the development of peanut hypersensitivity.

In **chapter 3** the intrinsic adjuvanticity of purified peanut allergens versus a whole protein extract (with or without fat) is tested using the popliteal lymph node assay. Although the purified allergens are demonstrated to lack intrinsic adjuvant activity, it is additionally shown that they are perfectly capable to induce sensitization in the oral model, even in the absence of a food matrix.

In the next part of the thesis the emphasis lies on immune regulation. **Chapter 4** elucidates the importance of the costimulatory pathway CD28/CTLA-4-CD80/CD86 in both the induction of sensitization and oral tolerance by using the fusion protein CTLA-4Ig and the blocking monoclonal antibodies (mAbs) anti-CD80 and anti-CD86.

In **chapter 5** the role of the inhibitory molecule CTLA-4 is investigated using a blocking mAb against CTLA-4. Results described in this study indicate that CTLA-4-signaling plays an important role in regulating the intensity of food allergic responses, but is not essential for the induction of hyporesponssiveness to orally administered proteins.

Chapter 6 continues with the topic immune regulation by investigating the effect of $CD4^+CD25^+$ Treg depletion on the induction of oral tolerance, the induction of sensitization, and the intensity of allergic responses. The data in this chapter reveal a critical role of $CD4^+CD25^+$ Tregs in inducing oral tolerance and regulating sensitization.

Interestingly, disturbing T cell regulation appears not to be sufficient to induce allergic sensitization to food proteins (chapter 5 and 6). Therefore in **chapter 7** the attention is shifted to the DC. In this chapter the role of the pDC -which is thought to possess 'tolerogenic properties'- in immune responses to oral antigens is examined by depleting pDCs before oral tolerance induction or during sensitization. Our data suggest that pDCs play an important role in maintaining immune homeostasis, but are not essential in the induction of tolerance versus sensitization to oral proteins.

Finally, the results and significance of our findings are discussed in **chapter 8**.

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Chapter 2

Mixed antibody and T cell responses to peanut and the peanut allergens *Ara h 1*, *Ara h 2*, *Ara h 3*, and *Ara h 6* in an oral sensitization model

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Abstract

Peanut allergy is known for its severity and persistence through life. Several peanut proteins have been identified as allergenic and are indicated as *Ara h 1-7*. Very little is known about the mechanisms that underlie sensitization to peanut proteins.

The purpose of the present study was to reveal the immune responses that are induced against peanut and the peanut allergens *Ara h 1*, *Ara h 2*, *Ara h 3*, and *Ara h 6* during sensitization, including the very early responses. Humoral and T cell responses against peanut and the peanut allergens were examined in an early and later stage of sensitization in an established murine model of peanut anaphylaxis. Therefore C3H/HeJ mice were orally exposed to two different doses of peanut extract plus cholera toxin.

Oral sensitization to peanut was characterized by an antigen-induced mixed cytokine response in the spleen (IL-4, IL-5, IL-10, and IFN-γ), which could already be observed 7 days after the onset of exposure. Additionally, polyisotypic humoral responses (IgE, IgG1, and IgG2a) against peanut were found in the serum. Moreover, we demonstrated that these Th1/Th2 cytokine and antibody responses were also directed specifically against the major peanut allergens *Ara h 1*, *Ara h 2*, *Ara h 3*, and *Ara h 6*.

This study implicates that both Th1 and Th2 phenomena are involved in the development of peanut allergy in the C3H/HeJ murine model. Furthermore, we show that the present oral model is suitable to examine immune responses to food allergens during different stages of sensitization upon treatment with a whole food extract.

Introduction

Peanut allergy is one of the most common and serious immediate hypersensitivity reactions to foods and is responsible for the majority of cases of fatal anaphylaxis upon ingestion of food [1]. It affects about 1% of the American population [2] and the prevalence of sensitization and clinical allergy to peanut is increasing [3]. Peanut allergy is also known for its persistence through life. It is estimated that only about 10-20% of peanut allergic patients lose their sensitivity to peanut [4, 5] compared to 85% for egg and cow's milk allergy [6-8]

Peanut (*Arachis hypogaea*) belongs to the family *Leguminosae* and consists of albumins and storage proteins, which comprise about 87% of the total protein contents [9]. In the past decade, several peanut proteins belonging to different families of seed storage proteins have been identified as allergenic. *Ara h 1, Ara h 2*, and to a lesser extent *Ara h 3* are considered major allergens since they are recognized by serum IgE of the majority of peanut-allergic patients as has been demonstrated by immunoblotting [10-12]. *Ara h 1* and *Ara h 2* are recognized by serum IgE from >90 % of peanut-sensitive patients [10, 11] while *Ara h 3*-specific IgE can only be detected in about 45 % of the patient population [13]. The three allergenic proteins have been fully characterized, sequenced, cloned and the IgE-binding epitopes analyzed, with each allergen containing at least 10 different IgE-binding sites [13-17]. Besides the thoroughly characterized allergens, at least 16 other peanut proteins were found to bind IgE from peanut allergic sera [18], four of them officially termed *Ara h 4*, *Ara h 5*, *Ara h 6*, and *Ara h 7* and they are recognized by a subpopulation of patients [19].

Over the last few years several oral food allergy models have been established in rodents [20, 21] that can provide a useful tool to investigate the mechanisms involved in sensitization and the subsequent clinical reactions to peanut proteins. Li *et al.* [22] have demonstrated that intragastric exposure of C3H/HeJ mice to peanut extract (PE), in combination with the mucosal adjuvant cholera toxin (CT) induces PE-specific IgE serum antibodies and systemic anaphylactic symptoms upon oral challenge. Furthermore, *Ara h 1*- and *Ara h 2*-specific IgE antibodies were detected and splenocytes from animals sensitized to PE exhibited proliferative responses to *Ara h 1* and *Ara h 2*. The IgE antibodies against *Ara h 2* bound the same major allergenic epitopes as antibodies in the sera of peanut allergic patients, indicating that in this model the allergic response to peanut reflects the human situation.

Classically, type I hypersensitivities, which include peanut allergy, are regarded as Th2-induced IgE-mediated phenomena counterbalanced by Th1 phenomena. However, recent studies on airway hypersensitivity suggest that the Th2 hypothesis may be too simplistic [23]. It has also been shown that a polyisotypic peanut-specific humoral response is observed in peanut-allergic individuals, including elevated levels of serum IgG1, IgG4, and IgA [24]. This implicates that the food allergy responses are not exclusively committed to IgE production. However, in food allergy models, the Th1/Th2 paradigm has lead to an

emphasis on examining the antigen-specific IgE responses and associated clinical outcomes, whereas the events occurring during sensitization to food proteins including polyisotypic antibody responses still have to be elucidated.

Due to ethical and practical reasons, research on the very early phase of sensitization to food antigens cannot easily be performed in humans. Therefore, in the present study, we used a slightly modified murine peanut allergy model [22, 25] to further characterize serum antibody and cytokine responses in an early and later stage of sensitization. We demonstrated that in this model intra-gastric peanut exposure induces polyisotypic PE-specific serum antibody responses and a mixed cytokine response in the spleen, both 1 week and 7 weeks after the onset of exposure. For the first time we revealed that also the purified peanut allergens *Ara h 1*, *Ara h 2*, *Ara h 3*, and the recently identified *Ara h 6* exhibit a mixed serum antibody response and a combined Th1/Th2 cytokine response upon oral exposure to PE plus CT.

Methods

Mice

Female, specific pathogen-free C3H/HeJ Clr mice (4 weeks of age) were purchased from Charles River (Sulzfeld, Germany). Mice were maintained under barrier conditions in filter-topped macrolon cages with wood chips bedding, at mean temperature of 23 ± 2°C, 50-55% relative humidity and 12 h light/dark cycle. Drinking water and standard laboratory food pellets were provided *ad libitum*. The experiments were approved by the Animal Experiments Committee of the Veterinary Faculty, Utrecht University (Utrecht, The Netherlands).

Chemicals and reagents

Chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands) unless stated otherwise.

Preparation of peanut extract and purification of allergens

The peanut extract was prepared from crude peanuts (Runner, Georgia) as described by Koppelman *et al.* [26]. SDS-PAGE analysis confirmed the presence of proteins migrating at the same molecular weights as the purified allergens *Ara h 1* (14%), *Ara h 2* (6.5%), *Ara h 3* (50%), and *Ara h 6* (4.5%) (data not shown). Their relative amounts (expressed as percentage of total protein) were quantified by densitometry as earlier described [27]. The allergens *Ara h 1*, *Ara h 2*, and *Ara h 3* were purified as described by Koppelman *et al.* [26] and *Ara h 6* was purified from a side-fraction.

Sensitization protocol

Oral sensitization to PE (n=6) was performed by intra-gastric dosing of 1 mg or 6 mg PE (in 200 µl PBS) on three consecutive days, followed by weekly dosing (days 8, 15, 22, 29, 36, and 43) of the same amount of PE. On days 1, 2, 3, 8, 15, and 22, 10 µg of CT (List Biological Laborites Inc, California) was co-administered. Oral treatment with PBS + CT (n=6) served as a negative control and positive control mice (n=6) were intraperitoneally (i.p.) exposed to 50 µg PE without an adjuvant (in 100 µl NaCl) on days 1, 3, 7, 9, 11, 13, and 15. Mice were sacrificed at day 8, or 7 weeks after the onset of exposure.

Measurement of serum IgG1, IgG2a, IgE, and IgA antibodies

Blood was collected at weekly intervals and levels of PE and *Ara h*-specific antibodies were measured either by ELISA (IgG1 and IgG2a) or sandwich ELISA (IgE and IgA). Plates (Costar, Cambridge, MA) were coated overnight with 10 µg/ml PE or *Ara h* (for IgG1 and IgG2a) or with 1.5 µg/ml purified rat anti-mouse mAb (BD Pharmingen, Hamburg, Germany) for the sandwich ELISA in carbonate buffer (pH 9.6), followed by 1 h blocking

(37°C) with PBS-Tween/3% milk powder (Elk, Campina Melkunie BV, Eindhoven, The Netherlands). Each test serum was titrated starting at 1:8 or 1:16 dilution and incubated for 1 h (37°C). A presera pool was used as reference value (dilution 1:4). For detection of IgG1 and IgG2a antibodies, AP-conjugated antibodies (Southern Biotechnology Associates Inc, Birmingham, USA) were added (1 h, 37°C). Subsequently 1 mg/ml p-nitrophenylphosphate in diethanolamine buffer was used for the color reaction, which was stopped with a 10% EDTA solution. To measure antigen-specific IgE and IgA antibodies, serum was incubated for 2 h and subsequently a PE- or *Ara h*-digoxigenin (DIG) conjugate solution was added (1 h, 37°C). The coupling of PE or the *Ara h*'s to DIG (molar mixture 1:10) was performed according to the manufacturer's instructions (Boehringer Manheim, Germany). The coupled proteins were separated on a sephadex G-25 column and labeling efficiency was determined spectrophotometrically at 280 nm. After incubation (1 h, 37°C) with peroxidase-conjugated anti-DIG fragments a TMB-substrate (0.1 mg/ml) solution was used and the color reaction was stopped with 2 M H_2SO_4. Absorbance was measured using an ELISA reader ELX800 (BIOTEK Instruments-Inc, Winooski). The reciprocal of the furthest test serum dilution giving an extinction higher than the reference value was read as a titer.

Cell culture and cytokine measurement

Spleen cell suspensions (150 µl of $2.5*10^6$ cells/ml in complete RPMI 1640 with Glutamax-1 (Invitrogen, Life Technologies, Paisley, Scotland) supplemented with 10% FBS (ICN Pharmaceuticals, Costa Mesa, CA) and 2% penicillin-streptomycin (Invitrogen) were incubated with 50 µl PE (200 µg/ml), BSA (100 µg/ml), or *Ara h* (100 µg/ml) in 96-well plates for 96 h at 37 °C, 5% CO_2. In the supernatant IFN-γ, IL-4, IL-5, and IL-10 were determined by sandwich ELISA (BD Pharmingen). Plates (highbond 3590; Costar) were coated overnight with 1 µg/ml rat anti-mouse IL-4, IL-5, or IFN-γ, and the following day plates were blocked with PBS/Tween/casein (BDH, Poole, England) for 4 h at room temperature. Samples and cytokine standards were added in several dilutions and incubated overnight at 4°C. Plates were incubated with 0.25 µg/ml rat anti-mouse IL-4, IL-5, or IFN-γ conjugate for 1 h at room temperature followed by streptavidin-HRP (Sanquin, Amsterdam, The Netherlands) incubation for 45 min. Finally, TMB-substrate (0.1 mg/ml) was added and the color reaction was stopped with 2 M H_2SO_4. Absorbance was measured at 450 nm using an ELISA reader ELX800. IL-10 ELISA was performed in accordance with the manufacturer's instructions.

Statistics

Statistical analyses were performed using one-way ANOVA with Bonferroni as *post hoc* test. Statistical analyses on serum antibody titers were performed on log-transformed data.

Results

Polyisotypic (IgG1, IgG2a, IgE, and IgA) antibody responses to PE upon oral PE exposure

During oral (1 mg or 6 mg) or i.p. exposure to PE, all mice developed PE-specific antibody responses. In mice, IgG1 and IgE antibodies are associated with Th2 responses, whereas IgG2a antibodies are linked to Th1 phenomena [28]. From day 14, all orally and i.p. treated animals showed a PE-specific IgG1 and IgG2a response. On day 21 PE-specific IgE was detected in 60 % of the 1 mg dosed animals and in all 6 mg (oral) and i.p. exposed mice. In the following weeks PE-specific antibody levels continued to rise, resulting in 100% responding animals for IgG1, IgG2a, and IgE (Fig. 1). The levels of the Th1- and Th2-associated antibody isotypes were highly correlated at all time points measured. Similar levels of antibody responses were found in both orally and i.p. treated groups.

Oral dosing of PE plus CT induced a significant rise in PE-specific IgA levels (Fig. 2), with 100 % responding animals in the 6 mg dose group and 60 % in the 1 mg dose group. Remarkably, IgA to peanut was only detectable in 50 % of the i.p. treated animals at a relatively low level (titer 3.0 ± 0) compared to those found in the 1 mg (7.4 ± 0.6) and 6 mg (7.0 ± 1.9) orally dosed groups respectively.

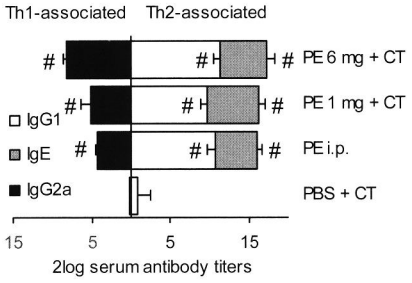
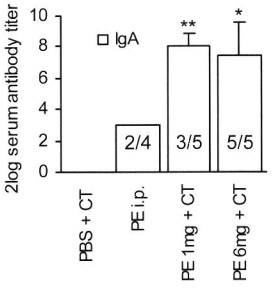


Figure 1. Peanut-specific Th1 (IgG2a) and Th2 (IgE and IgG1) serum antibody titers after 7 weeks of oral treatment with PE + CT (1 mg and 6 mg) or i.p. exposure to PE. Peanut-specific antibody levels were detected using ELISA. The data are presented as mean ^{2} log Ig titer ± SEM of 6 mice per group. # indicate significant differences ($p < 0.01$) from control group (PBS + CT).

Figure 2. Peanut-specific IgA serum antibody titers after 7 weeks of intragastric dosing with PE + CT (1 mg and 6 mg) or i.p. exposure to PE. Peanut-specific antibody levels were detected using ELISA starting at a 1/8 dilution. The data are presented as mean ^{2} log Ig titer ± SEM of IgA positive mice. The numbers of animals developing a specific IgA antibody response are indicated in the bars. * indicate significant differences (*, $p<0.05$; **, $p<0.01$) compared to the control group (PBS + CT).



Mixed serum Th1- and Th2-associated IgG antibody responses to the major peanut allergens Ara h 1, Ara h 3, and to a lesser extent Ara h 2 and Ara h 6

We additionally examined whether antibody responses to the major peanut allergens *Ara h 1*, *Ara h 2*, *Ara h 3*, and *Ara h 6* were also polyisotypic (Fig. 3). Seven weeks of oral treatment with PE plus CT induced comparably high IgG1 and IgG2a responses to the purified peanut allergens *Ara h 1* and *Ara h 3* in both the 1 mg- and 6 mg-dosed animals. *Ara h 2* induced lower IgG1 responses than *Ara h 1* and *Ara h 3*, and it induced a significantly higher *Ara h 2*-specific serum IgG1 titer in the 6 mg compared to the 1 mg dose group (titer 9.2 ± 2.8 and 2.8 ± 1.6 respectively). Furthermore, *Ara h 2*-specific IgG2a antibody titers were not significantly elevated in any of the PE-treated animals. Finally, IgG1 and IgG2a antibody levels against *Ara h 6* were very low in all PE-exposed groups, however the highest levels were found in 6 mg orally exposed group.

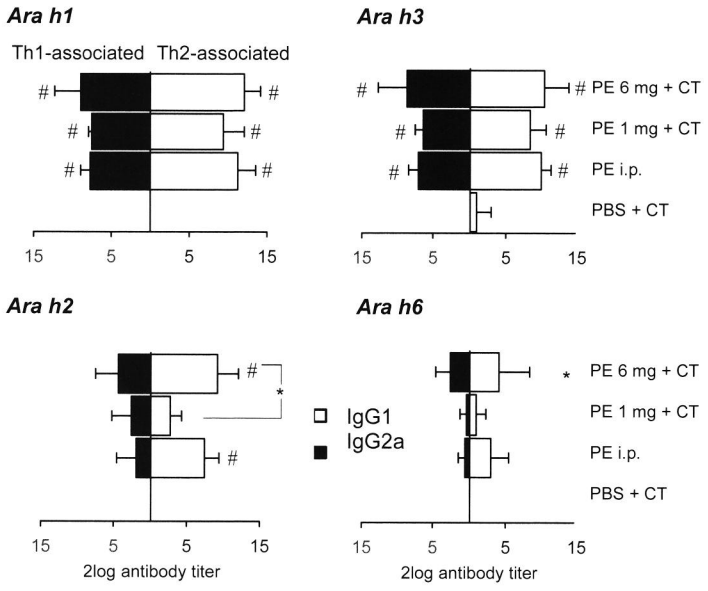


Figure 3. IgG1 (Th2) and IgG2a (Th1) serum antibody responses against the purified peanut allergens *Ara h 1*, *Ara h 2*, *Ara h 3*, and *Ara h 6* after 7 weeks of oral treatment with PE + CT (1 mg and 6 mg) or i.p. exposure to PE. *Ara h*-specific antibody levels were detected using ELISA. Bars represent group means ± SD of 6 mice per group. *, $p<0.05$, #, $p<0.01$ versus orally dosed PBS + CT group.

Oral exposure to PE induces IgE against all four allergens, with levels superior to the IgE levels upon i.p. treatment

In addition to IgG antibodies, *Ara h*-specific IgE antibodies were measured in pooled sera, using a sandwich ELISA (Fig. 4). Comparably strong IgE responses against *Ara h 1*, *Ara h 3*, and *Ara h 6* were observed in both (1 mg and 6 mg) orally dosed groups. Remarkably, i.p. treatment with PE induced lower levels of IgE against these allergens, whereas no difference was observed in PE-specific serum IgE levels between the different treatment groups (Fig. 1). Serum IgE responses to *Ara h 2* were very low in all PE-exposed groups.

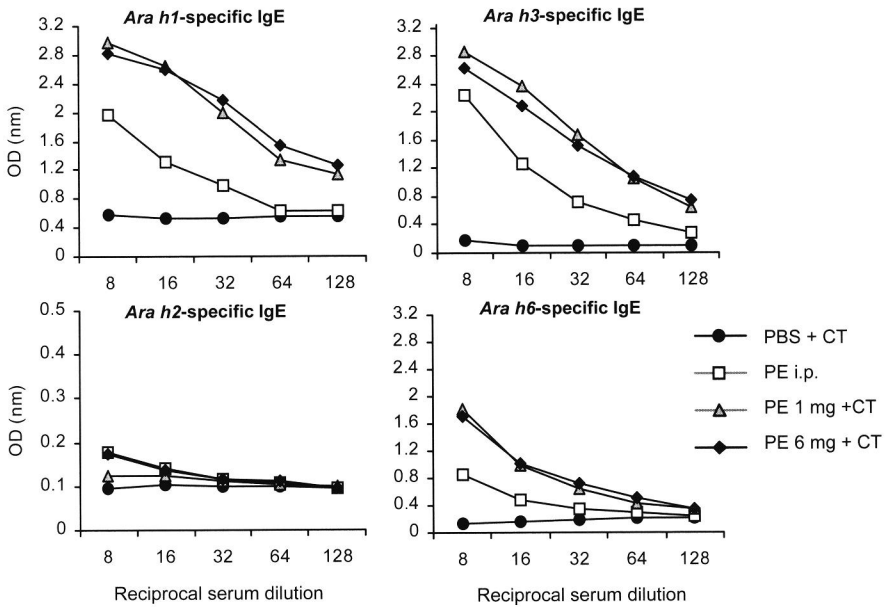


Figure 4. IgE serum antibody responses against the purified peanut allergens *Ara h 1*, *Ara h 2*, *Ara h 3*, and *Ara h 6*, 7 weeks after the initial oral exposure to PE + CT (1 mg and 6 mg) or i.p. treatment with PE. *Ara h*-specific IgE antibody levels were measured in pooled sera (6 mice per group) using a sandwich ELISA.

PE stimulates Th1 and Th2 cytokine production in spleen cultures 7 weeks after the initial exposure to PE

In addition to the humoral response to PE, antigen-induced cytokine production was measured in PE restimulated spleen cell cultures 7 weeks after the onset of exposure. Levels of the Th2-related cytokines IL-4 and IL-5 (Fig. 5A), the Th1-related cytokine IFN-γ and the Tregulatory-associated cytokine IL-10 (Fig. 5B) were determined. In cultures of the PE-treated groups, levels of all four cytokines were elevated compared to the non-PE-exposed group. Oral exposure to 6 mg PE + CT induced high amounts of IFN-γ (2819 ± 603 pg/ml), which were significantly increased compared to the 1 mg dose group and the i.p. treated group. Other cytokine levels (IL-4, IL-5, and IL-10) were also lower in the cultures of the 1 mg dose group in comparison to the 6 mg dosed group. Intraperitoneal injection of PE resulted in a clear IL-5 production, which was higher than in the orally dosed groups. Conclusively, oral PE exposure resulted in a combined Th1/Th2/Treg cytokine production in the spleen with occurrence of superior levels upon 6 mg treatment. Intraperitoneal exposure also induced a mixed cytokine production, but levels of IL-5 and IFN-γ differed from those in orally dosed animals.

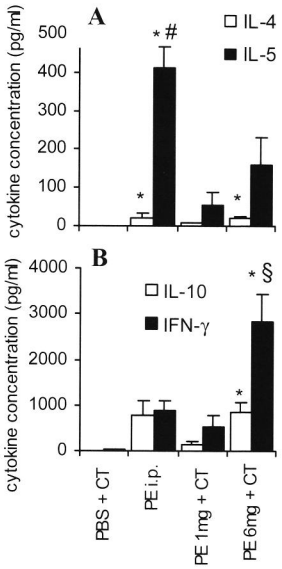


Figure 5. Cytokine levels in splenocyte culture supernatants. Spleen cells from different groups were removed 7 weeks after the initial exposure to PE. Cells were cultured for 96 h in the presence or absence of PE (200 µg/ml). Culture supernatants were collected and cytokine levels of IL-4, IL-5 (A), IL-10, and IFN-γ (B) were determined by ELISA. Non-antigen restimulated cultures showed no cytokine production. Data (of PE-restimulated cultures) are presented as means ± SEM of 6 mice per group. *, $p < 0.05$ versus control group (PBS + CT). #, $p<0.05$ versus orally exposed 1 mg and 6 mg dose groups. §, $p < 0.05$ versus orally dosed 1 mg group.

A combined cytokine response to PE and Ara h 1 in the induction phase of sensitization to peanut proteins in spleen cultures of orally exposed mice

We also examined the production of cytokines 7 days after the initial oral exposure with 6 mg PE + CT for 3 consecutive days. Spleen cells were restimulated with PE or with *Ara h 1*, the allergen that elicited one of the highest antibody responses upon PE exposure. Both PE and *Ara h 1* restimulation of spleen cells induced the production of IL-4, IL-5, and high levels of IL-10 and IFN-γ (Fig. 6). All cytokine levels were significantly higher ($p<0.05$) compared to those in spleen cultures of the control group (PBS + CT), in which no cytokine production could be detected. All *Ara h 1*-induced cytokine levels exceeded the levels detected upon restimulation with total peanut extract, however not significantly. Although restimulation with *Ara h 2, Ara h 3*, and *Ara h 6* also resulted in cytokine production with high levels of IFN-γ (data not shown) *Ara h 1* appeared to be the most potent antigen for restimulation.

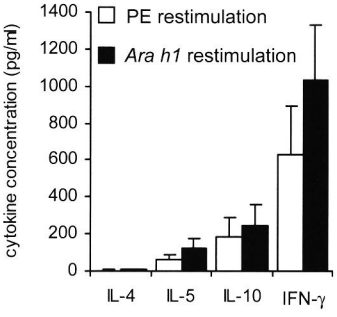


Figure 6. Cytokine levels in splenocyte culture supernatants. Spleen cells from the orally dosed 6 mg group were removed 1 week after the initial exposure to PE. Cells were cultured for 96 h in the presence of PE (200 μg/ml) or *Ara h 1* (100 μg/ml). Culture supernatants were collected and cytokine levels of IL-4, IL-5, IL-10, and IFN-γ were determined by ELISA. Antigen restimulated cultures of the control group (PBS + CT) showed no cytokine production (data not shown). Data are presented as means ± SEM of 6 mice per group.

Discussion

Data from the present study showed that oral sensitization to PE is characterized by a mixed cytokine response and polyisotypic humoral responses. Furthermore, it was demonstrated for the first time that these mixed cytokine and antibody responses are also directed specifically against the major peanut allergens *Ara h 1*, *Ara h 2*, *Ara h 3*, and *Ara h 6*. Additionally, both IL-4 and IFN-γ production were found at the very early phase of the response upon restimulation with allergen, indicating that in this model both Th1 and Th2 phenomena are involved in the development of peanut allergy. After an oral challenge with PE all sensitized mice showed mast cell degranulation (data not shown), which confirmed the PE-allergic status of IgE positive animals.

In type 1 hypersensitivities, such as asthma and food allergies, a shift in the Th1/Th2 balance in favor of Th2 is considered to be a key factor for developing (food) allergic deisease [29-31]. However, recent studies on asthma suggest that the Th2 hypothesis is too simplistic [32] and also in food allergy research the concept of an antigen-specific mixed Th1- and Th2-type cytokine response has gained ground [33-36].

In the present study, intra-gastric administration of PE plus CT for 7 weeks resulted in a dose-dependent mixed cytokine response (IL-4, IL-5, IL-10, and IFN-γ) together with an ambivalent Th2-associated (IgE and IgG1) and Th1-associated (IgG2a) antibody response against PE. Moreover, the major allergens *Ara h 1* and *Ara h 3* and to a lesser extent *Ara h 2* and *Ara h 6* exhibited a similar induction of serum antibody production. These data are consistent with the polyisotypic antibody responses directed to several peanut antigens, including the major allergens *Ara h 1* and *Ara h 2,* found in peanut allergic patients [24]. The presence of different antibody isotypes in our model also suggests that polyisotypic antibody responses are not protective for the development of food allergy.

The diverse nature of the cytokine response after 7 weeks of exposure was also observed early in the response, after 1 week of oral treatment with PE plus CT. Hence, despite the presence of Th2 cytokines, the levels of IL-10 and IFN-γ were remarkably high both in the early and late phase of sensitization. This is in accordance with Morafo *et al.* [37] who showed recently that although the C3H/HeJ mouse seems to be a Th2 responder, peanut-sensitized mice produce reasonable amounts of IL-10 and IFN-γ. Although peanut-allergic children display a high Th2-polarization to peanut antigens compared to Th1-polarization of non-allergic and tolerant children [38], the discrepancy may be explained by the difference in species and the difference between an established response and a developing response. Very few data are available on the effect of Th1-related cytokines on the onset of sensitization. It is known that IFN-γ in the gut induces upregulation of MHC class II on epithelial cells in the small intestine [39] and induces changes in epithelial permeability [40], which might facilitate sensitization responses. Concordantly, Zhang and Michael [39] showed that oral tolerance to BSA is abrogated by IFN-γ treatment.

Interleukin-10 has been described as a general inhibitor of T cell activation, cytokine synthesis [41] and APC maturation [42]. Additionally, the level of IL-10 produced has been directly correlated to the prevention of allergen-induced airway hyperreactivity [43] and to the outgrow of food allergy [35]. On the other hand, food-allergic children display higher levels of IL-10 production in the gut mucosa and the blood than non-allergic controls [44, 45]. In all, the role of IL-10 at the onset of sensitization remains unclear and might be anti-inflammatory in some instances but pro-inflammatory in other circumstances [46]. The early production of IL-10 upon restimulation with PE or *Ara h 1* in our model implies that IL-10 is involved in the induction rather than the suppression of food hypersensitivity.

When interpreting the character of the immune responses in this food allergy model, the role of the adjuvant CT has to be taken into account. Results on the polarizing effect of CT on the immune response of co-administered antigens are contradictive [47]. In general, CT is considered a mucosal Th2-inducer [48, 49]. However, in accordance with several other studies [50, 51] we found no evidence of a selective effect of CT on either Th1- or Th2-associated immune responses since we observed a clear mixed Th1/Th2-induced response to PE. However, CT may be responsible for the high levels of PE-specific IgA that we observed upon oral exposure to PE plus CT but not upon i.p. treatment. Although these results imply that administration via the mucosal route is indispensable for considerable systemic IgA production, co-administration of CT is known to induce IgA switching [52]. Although a defect in IgA production has been associated with a predisposition to allergy [53, 54], allergen-specific IgA antibodies were found to be increased in the serum [24] and saliva [55] of food allergic patients. In our model serum IgA may be an indicative of broken mucosal protective barriers.

Our results demonstrate that the utilized model is suitable to investigate specific humoral and T cell responses against individual allergens present in a whole food extract. Li *et al.* [22] have already shown that *Ara h 1*- and *Ara h 2*-specific IgE antibodies can be detected after intra-gastric dosing with PE. In our study the allergens *Ara h 1* and *Ara h 3* elicited the most vigorous humoral response. The minor allergen *Ara h 6* appeared less potent, but also the major allergen *Ara h 2* only induced moderate antibody responses. However, the recognition pattern of peanut allergens in peanut-allergic patients is also diverse [56]. Besides differences in allergenic potency, a possible explanation for the distinctive magnitude of responses induced by the allergens might be the difference in relative amounts of allergen (*Ara h 3>Ara h 1>Ara h 2>Ara h 6*) which were present in the peanut extract used for *in vivo* sensitization. The use of purified allergens for oral sensitization may overcome this problem, but it will consequently result in ignorance of the foodmatrix.

Strikingly, i.p. treatment induced lower *Ara h*-specific IgE antibody levels compared to the levels observed in both orally treated groups, whereas PE-specific IgE levels were comparable in the three treatment groups. These observations suggest that in this model i.p.

sensitization is less adequate in distinguishing allergenic proteins than sensitization via the oral route.

Taken together, our data indicate that both Th1 and Th2 phenomena are involved in the induction of peanut allergy. Future research will focus on defining the early events that are responsible for sensitization to peanut, by determining the role of costimulatory molecules, cytokines, and adjuvants.

Acknowledgements

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Chapter 3

The effect of the food matrix on *in vivo* immune responses to purified peanut allergens

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Abstract

There is little knowledge on the factors that determine the allergenicity of food proteins. One of the aspects that remains to be elucidated is the effect of the food matrix on immune responses to food proteins.

To study the intrinsic immunogenicity of allergens and the influence of the food matrix, purified peanut allergens (*Ara h 1*, *Ara h 2*, *Ara h 3*, or *Ara h 6*) and a whole peanut extract (PE) were tested in the popliteal lymph node assay (PLNA) and in an oral model of peanut hypersensitivity. In the PLNA, peanut proteins were injected into the hind footpad of BALB/c mice and in the oral exposure experiments C3H/HeOuJ mice were gavaged weekly with PE or allergens in the presence of cholera toxin (CT).

Upon footpad injection, none of the allergens induced significant immune activation. In contrast, PE induced an increase in cell number, cytokine production, and activation of antigen presenting cells. Furthermore, the presence of a food matrix enhanced the immune response to the individual allergens. Oral exposure to the purified allergens in the presence of CT induced specific IgE responses irrespective of the presence of a food matrix.

These results suggest that purified peanut allergens possess little intrinsic immune stimulating capacity in contrast to a whole PE. Moreover, the data indicate that the food matrix can influence responses to individual proteins and, therefore, has to be taken into account when developing models for allergenic potential assessment.

Introduction

Food allergy has emerged as a major health problem in westernized countries and its prevalence is increasing [1]. Type I food allergy is characterized by the production of protein-specific serum IgE antibodies that can reside on mast cells. Upon re-exposure to the offending food, cross-linking of the IgE antibodies provokes degranulation of mast cells and release of mediators, leading to a variety of symptoms.

In the past decade, considerable research has been conducted to characterize the food proteins that are responsible for allergic reactions. Knowledge on the general properties of proteins in relationship to their allergenicity is needed to assess the allergic potential of novel (including genetically modified) foods and foods produced by new processing techniques [2-4].

Foods are composed of proteins, carbohydrates, lipids and micronutrients. Major food allergens, in general, are glycoproteins ranging from 10 to 70 kDa that are abundant in the food and possess multiple IgE binding epitopes [5]. To be able to induce allergic sensitization, allergens have to be absorbed through the gut epithelial barrier in an immunologically intact form [6]. Accordingly, food allergens are generally thought to be resistant to food processing methods and digestion [7]. However, several food allergens have been identified that are sensitive to these processes and conversely, many non-allergenic proteins are equally resistant to degradation as allergenic proteins [8]. At present, the sum of properties that renders a food protein allergenic is unknown.

One of the aspects that remains to be elucidated is the influence of the food matrix on the immune responses to food proteins. It has been hypothesized that the food body, consisting of fats, carbohydrates, and other proteins may affect allergenic potential of proteins [9]. For example, high allergenic foods such as tree nuts and peanuts have medium protein concentrations but high levels of fat [10]. These fats may protect proteins during the digestion process or influence the activation of immune cells, which may result in enhanced allergenicity of a protein. These considerations prompted us to investigate the contribution of the food matrix to immune responses against peanut allergens.

Peanut allergy accounts for the majority of severe food allergic reactions; it tends to be persistent and trace amounts of peanut can induce allergic responses [11]. Several peanut proteins belonging to different families of seed storage proteins have been identified by immunoblotting as major or minor allergens and seven of them have been characterized and officially termed *Ara h 1-7* [12]. We have recently shown that allergen-specific antibody and cytokine responses can be determined following oral or i.p. exposure to a whole peanut extract (PE) [13].

To study the intrinsic immunogenicity and adjuvanticity of purified peanut allergens versus a whole peanut protein extract and to reveal the effect of the food matrix, two different peanut protein extracts (with or without fat), several purified major peanut

allergens (*Ara h 1, Ara h 2*, and *Ara h 3*) and one minor allergen (*Ara h 6*) were used in the current experiments.

To determine intrinsic immune stimulating capacity of the purified allergens compared to a whole peanut extract, the popliteal lymph node assay (PLNA), an established model to test immune stimulation was used. After injection of 1 mg PE or purified allergen, PLN cell numbers, cytokine production and expression of costimulatory molecules (CD80, CD86, and CD54), which is a measure for antigen presenting cell (APC) activation, were determined. Data derived from this study show an important role of the PE food matrix (irrespective of the presence of fat) in activating immune cells, thereby driving immune responses to peanut proteins.

Additionally, the effect of the food matrix on IgE responses against peanut allergens was examined, since the elicitation of specific IgE responses is one of the absolute requirements of food allergens to induce immediate hypersensitivity responses. This was studied in an established mouse model of peanut hypersensitivity, using the relevant oral route of exposure in combination with a mucosal adjuvant, cholera toxin (CT). All purified peanut allergens induced IgE-specific serum antibody responses with only little additional effect of the food matrix. In conclusion, purified peanut allergens possess little intrinsic adjuvanticity in contrast to a whole PE. Furthermore, the results suggest that the food matrix can influence responses to individual proteins.

Materials and methods

Mice

Female, specific pathogen-free BALB/c mice (6 wk of age) and C3H/HeOuJ Ico mice (4 wk of age), bred on a SM R/M-Z CRL VRF1 diet (ssniff Spezialdiaten GmbH, Germany) were purchased from Charles River (Lyon, France). Mice were maintained under barrier conditions in filter-topped macrolon cages with wood chip bedding, at mean temperature of 23 ± 2°C, 50-55% relative humidity and a 12 h light/dark cycle. Drinking water and standard laboratory food pellets (rat and mouse breeder and grower, Special Diet Services, Essex, England) were provided *ad libitum*. No peanut proteins were present in the diets. The experiments were approved by the animal experiments committee of the faculty of veterinary medicine, Utrecht University.

Chemicals and reagents

Chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands) unless stated otherwise. For cytokine measurements, IL-2, IL-4, and IFN-γ capture and detecting antibodies, and IL-10 ELISA kit were obtained from BD Pharmingen (Hamburg, Germany) and streptavidin-HRP from Sanquin (Amsterdam, The Netherlands). BD Pharmingen was the supplier of rat anti-mouse IgE and the Digoxigenin (DIG)-coupling kit was obtained from Boehringer Mannheim (Mannheim, Germany), and anti-DIG-peroxidase Fab fragments from Roche Diagnostics (Mannheim, Germany). Alkaline phosphatase (AP)-conjugated goat anti-mouse, human adsorbed IgG1 and IgG2a were purchased from Southern Biotechnology Associates (Birmingham, AL). Campina Melkuni (Eindhoven, The Netherlands) was the supplier of milk powder used in blocking buffer. Medium used for the *in vitro* restimulation was complete RPMI 1640 with Glutamax-I (Life Technologies, Paisley, Scotland) supplemented with 10% fetal calf serum (ICN Pharmaceuticals, Costa Mesa, CA) and 2% penicillin-streptomycin.

Preparation of peanut extract and purification of allergens

Peanuts from the Runner cultivar (Cargill, Dawson, GA) were kindly provided by Imko Nut Products (Doetinchem, The Netherlands). The PE was prepared as described by Koppelman *et al.* [14]. Briefly, peanuts were ground and the protein was extracted by mixing 25 g ground peanut with 200 ml 20 mM Tris buffer (pH 7.2). After 2 h stirring at room temperature the aqueous fraction was collected by centrifugation (3000 *g* for 30 min) and subsequently centrifuged at 10000 *g* for 30 min to remove residual traces of fat and insoluble particles. The extract consisted of 4.3% protein, 0.2% fat, 0.5% carbohydrates, 0.2% ash, and 94.8% water, as determined by Kjehdahl analysis and acid hydrolysis. For the preparation of fatty PE (PE fat), fat was not removed following centrifugation resulting in a PE/oil emulsion consisting of 3.6% protein, 3.5% fat, 0.4% carbohydrates, 0.2% ash, and 92.3% water.

Protein concentration (PE 30 mg/ml and PE fat 25 mg/ml) was determined with a BCA protein assay kit (Pierce, Rockford, IL). SDS-PAGE analysis of PE and PE fat confirmed the presence of proteins migrating at the same molecular weights as the purified allergens *Ara h 1* (14%), *Ara h 2* (6.5%), *Ara h 3* (50%), and *Ara h 6* (4.5%) and both extracts showed a similar protein pattern (data not shown). Their relative amounts (expressed as percentage of total protein) were quantified by densitometry as previously described [15]. Purification of the allergens *Ara h 1*, *Ara h 2,* and *Ara h 3* was performed according to previously described methods [14, 16] and *Ara h 6* was similarly purified from a side-fraction. Endogenous endotoxin content was measured by Limulus Ambeocyte Lysate assay (Bio Whittaker, Walkersville, MD) and was <0.02 µg LPS/mg protein (=<200 EU/mg protein) for all purified allergen and PE solutions.

PLNA protocol

For the PLNA a standard protocol was used as previously described [17]. Pilot studies were performed to determine the optimal concentration of PE that also served as a positive control. Different doses of purified allergens (0.1 mg, 0.5 mg, and 1 mg) were tested and no differences were found between the different dosing groups for all parameters measured. The results of the 1 mg dose group are presented in the results section. Accordingly, BALB/c mice (n = 4) were subcutaneously injected into the hind footpad with 1 mg of protein (PE, PE fat, *Ara h 1*, *Ara h 2*, or *Ara h 6*) dissolved in 50 µl sterile NaCl and control animals were injected with 50 µl vehicle. *Ara h 3* could not be dissolved at this concentration and was therefore not included in the PLNA. Seven days after protein injection, mice were killed by cervical dislocation, and the draining popliteal lymph nodes (PLN) excised. PLN single-cell suspensions were prepared under aseptic conditions in complete RPMI 1640 and adjusted to $2.5*10^6$ cells/ml.

Oral treatment protocol

C3H/HeOuJ mice (n = 6) were orally exposed to the purified allergens *Ara h 1*, *Ara h 2*, *Ara h 3*, *Ara h 6* (0.1 and 1 mg), PE (1 and 6 mg), or PE fat (6 mg) in the presence of the mucosal adjuvant CT. Oral exposure was performed by intra-gastric dosing of protein on three consecutive days, followed by weekly dosing (4 wk). CT (10 µg) was co-administered on days 1, 2, 3, 8, 15, and 21. At day 30, mice received a double dose of protein. Mice were killed by cervical dislocation 31 days after the onset of exposure.

Cell culture and cytokine measurement

PLN single cell suspensions (150 µl of $2.5*10^6$ cells/ml in complete RPMI 1640) were incubated in the presence of PE (200 µg/ml), *Ara h 1* (200 µg/ml), *Ara h 2* (200 µg/ml), *Ara h 6* (200 µg/ml), Con A (5 µg/ml) or medium alone in 96-well plates (Costar, Cambridge,

MA) for 96 h (Con A 24 h) at 37 °C, 5% CO_2. After centrifugation for 10 min at 150 *g*, supernatant was collected and stored at -20°C until analysis.

In the culture supernatants, IL-2, IFN-γ, IL-4, and IL-10 levels were determined by sandwich ELISA. Plates (highbond 3590) were coated overnight with 1 µg/ml rat anti-mouse IL-2, IL-4, or IFN-γ and the following day plates were blocked with PBS-Tween/3% milk powder for 4 h at room temperature. Samples and cytokine standards were added in several dilutions and incubated overnight at 4°C. Plates were incubated with 0.25 µg/ml rat anti-mouse IL-2, IL-4, or IFN-γ conjugate for 1 h at room temperature followed by streptavidin-horse radish peroxidase incubation for 45 min. Finally, tetramethylbezidine(TMB)-substrate (0.1 mg/ml) was added and the color reaction was stopped with 2 M H_2SO_4. Absorbance was measured at 450 nm. The IL-10 ELISA was performed in accordance with the manufacturer's instructions.

Flow cytometry

For flow cytometric analysis, $2*10^5$ PLN cells were incubated with 50 µl of predetermined dilutions of fluorescin isothiocyanate (FITC)-, CyChrome (CY)-, and biotin-conjugated monoclonal antibodies in 96-well plates (30 min darkness at 4°C). Cells were characterized based on the following monoclonal antibodies: CD54 FITC (3E2), CD80 FITC (16-10A1), CD86 FITC (GL1), isotype controls for CD80 (hamster IgG2 FITC), and CD86 (Rat IgG2a FITC) and MHCII biotin (NIMR-4), all obtained from BD Pharmingen. After incubation with biotin-conjugated monoclonal antibodies cells were washed and incubated with streptavidin-CY (BD Pharmingen) in the same way. Finally, cells were washed, stored in formalin (0.1%) and analyzed within 18 h on a FACScan with standard FACSflow using CellQuest software (BD Biosciences, Franklin Lakes, NJ). Cells expressing CD80, CD86, or CD54 were checked for MHCII expression to assure antigen presenting capability.

Measurement of serum IgE antibodies

Blood of orally exposed mice was collected at weekly intervals and levels of PE- and *Ara h*-specific IgE were measured by sandwich ELISA. Plates (highbond 3590; Costar, Cambridge, MA) were coated overnight with 1.5 µg/ml purified rat anti-mouse IgE in carbonate buffer (pH 9.6), followed by 1 h blocking (37°C) with PBS-Tween/3% milk powder. Each test serum was titrated starting at 1:8 dilution and incubated for 2 h (37°C). A pre-sera pool was used as reference value (dilution 1:4). Subsequently a PE- or *Ara h*-DIG conjugate solution was added (1 h at 37°C). The coupling of DIG to PE or the *Ara h*'s at an optimal labeling efficiency was performed according to the manufacturer's instructions. Briefly, the coupled proteins were separated on a sephadex G-25 column and the concentration of coupled protein was determined spectrophotometrically at 280 nm. After incubation (1 h at 37°C) with peroxidase-conjugated anti-DIG fragments, a TMB-substrate (0.1 mg/ml) solution was used and the color reaction was stopped with 2 M H_2SO_4.

Absorbance was measured at 450 nm using an ELISA reader ELX800 (BIOTEK Instruments, Winooski, VT). The reciprocal of the furthest test serum dilution resulting in an extinction higher than the reference value was read as a titer. Background values were <0.100 (OD).

Measurement of serum IgG1 and IgG2a antibodies

Blood was collected at weekly intervals and levels of PE-specific IgG1 and IgG2a antibodies were measured by ELISA. Plates (highbond 3590; Costar, Cambridge, MA) were coated overnight with 20 µg/ml PE in carbonate buffer (pH 9.6), followed by 1 h blocking (37°C) with PBS-Tween/3% milk powder. Each test serum was titrated starting at 1:8 dilution and incubated for 1 h (37°C). A pre-sera pool was used as reference value (dilution 1:4). After incubation AP-conjugated antibodies were added (1 h at 37°C). Subsequently 1 mg/ml p-nitrophenylphosphate in diethanolamine buffer was used for the color reaction, which was stopped with a 10% EDTA solution and absorbance was measured at 405 nm using an ELISA reader ELX800 (BIOTEK Instruments, Winooski, VT). Background values were <0.100 (OD).

Statistics

Multiple comparisons of group means were performed using one-way analyses of variance with Bonferroni as *post hoc* test. For PLN cell numbers, cytokine levels, and serum antibody levels statistical analysis was performed following logarithmic transformation (to achieve normal distribution), whereas the levels of cell surface molecules were untransformed. A value of $p<0.05$ was considered statistically significant.

Results

PE but not purified allergen induces PLN cell proliferation

To examine the intrinsic immune stimulating capacity and adjuvanticity of purified peanut allergens versus whole PE, the PLNA was used. Mice were injected with 1 mg of protein in the hind food pad and at day 7 total cell numbers of the draining PLN were determined (Fig. 1). After injection with PE fat or PE, total lymph node cell numbers increased significantly (4-fold), and no difference was found between the PE fat- and PE-treated groups. In contrast, no cell proliferation was observed following injection with the purified peanut allergens *Ara h 1*, *Ara h 2*, or *Ara h 6*.

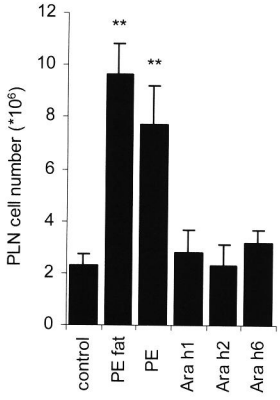

Figure 1. Total cell numbers in the PLN. Mice were injected in the hind footpad with vehicle alone (control) or 1 mg of protein (PE fat, PE, *Ara h 1*, *Ara h 2*, or *Ara h 6*) dissolved in NaCl. At day 7, the draining lymph nodes were isolated and cells were counted. Levels are expressed as group means ± SD of 4 mice per group. **, Significantly different ($p<0.01$) from the control group.

Purified allergen treatment fails to induce cytokine production in Con A-restimulated PLN cell cultures in contrast to PE treatment

To reflect the general activation of T cells, PLN cells were cultured for 24 h in the presence of Con A and the concentrations of the Th2-associated cytokines IL-4 and IL-10 and of the Th1-associated cytokine IFN-γ were measured in the supernatant (Fig. 2). PLN cells derived from PE- and PE fat-treated mice secreted similar high amounts of IL-4, IL-10, and IFN-γ compared to cells derived from control mice. No Th2 or Th1 bias was observed in either of the PE treatment groups.

With respect to the purified allergens, no significant IL-10 or IFN-γ cytokine production was found in PLN cell cultures of *Ara h 1-*, *Ara h 2-*, and *Ara h 6*-treated groups and only *Ara h 6* injection resulted in significant IL-4 production upon restimulation with Con A.

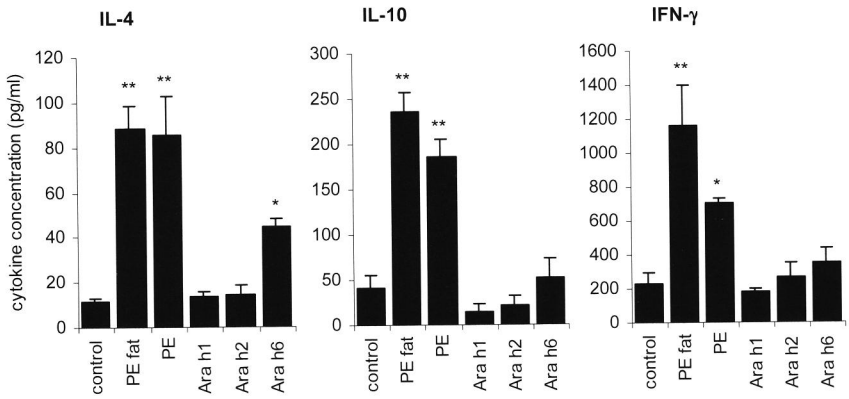

Figure 2. Cytokine production after in vitro restimulation of PLN cells with Con A. Mice were injected in the hind footpad with vehicle alone (control) or 1 mg of protein (PE fat, PE, *Ara h 1*, *Ara h 2*, or *Ara h 6*) dissolved in NaCl. At day 7, the draining lymph nodes were isolated and cultured for 24 h in the presence of Con A (5 μg/ml). Culture supernatants were collected and cytokine levels of IL-4, IL-10, and IFN-γ were determined by ELISA. Data are presented as mean cytokine levels ± SEM of 4 mice per group. *, Significantly different (*$p<0.05$, **$p<0.01$) from the control group.

Cytokine responses induced by restimulation with purified allergen are enhanced by the presence of a food matrix at the time of injection

To examine antigen-specific T cell responses, PLN cells were obtained 7 days after the initial injection with 1 mg of PE, PE fat, or purified allergen and cultured for 96 h in the presence of PE, *Ara h 1*, *Ara h 2*, *Ara h 6*, or medium alone. Th1-associated (IL-2 and IFN-γ) and Th2-associated (IL-4 and IL-10) cytokine production was measured in the supernatant (Table I).

PLN cell cultures derived from PE- and PE fat-treated mice restimulated with PE induced high levels of IL-2, IL-4, IL-10, and IFN-γ. No significant differences in cytokine concentration were found between both groups and a mixed Th1/Th2 cytokine response was observed.

Regarding the purified allergens, only *Ara h 6* treatment resulted in a low but significantly increased cytokine production (IL-2, IL-4, IL-10, but not IFN-γ), whereas *Ara h 1* and *Ara h 2* injection failed to induce considerable cytokine production in PLN cell cultures when restimulated with the corresponding purified allergen.

Interestingly, cells derived from PE- and PE fat-treated groups secreted higher levels of cytokines than cells derived from *Ara h 1*- and *Ara h 6*-treated animals when restimulated with *Ara h 1* or *Ara h* 6. Restimulation with *Ara h 2* only induced low IL-2 production and levels of IL-2 were significantly higher in cell cultures of PE- and PE-fat treated mice than in those of *Ara h 2*-treated mice.

PLN cells cultured in the presence of medium alone showed no production of any of the cytokines examined (data not shown). These results indicate that T cell responses to purified allergens can be influenced by the food matrix.

Table I. Ex vivo cytokine production by PLN cells upon restimulation (96 h) with antigen.

treatment group	restimulation	IL-2 (pg/ml)[a]	IL-4 (pg/ml)[a]	IFN-γ (pg/ml)[a]	IL-10 (pg/ml)[a]
control	PE	4.33 ± 0.63	0 ± 0	0 ± 0	0 ± 0
PE fat		220.9 ± 44.8 **	391.2 ± 27.8 **	650.6 ± 318.1 **	4785 ± 1696 **
PE		225.9 ± 64.2 **	311.1 ± 62.4 **	511.1 ± 183.3 **	4315 ± 1881 **
control	*Ara h 1*	6.9 ± 5.6	0.7 ± 0.67	1.5 ± 1.5	0
PE fat		27.5 ± 4.2 *	43.4 ± 11.8 **	77.1 ± 22.1 *	428.1 ± 142.9 **
PE		21.7 ± 3.4 *	36.9 ± 8.2 **	38.0 ± 14.1	245.7 ± 101.8 **
Ara h 1		8.9 ± 0.7	2.7 ± 0.97 ¶ §	2.1 ± 0.7 ¶ §	27.9 ± 6.7 ¶ §
control	*Ara h 2*	6.4 ± 1.3	0 ± 0	0 ± 0	0 ± 0
PE fat		12.1 ± 1.3 **	1.47 ± 1.4	0 ± 0	0 ± 0
PE		10.8 ± 0.2 *	0.37 ± 0.4	0 ± 0	0 ± 0
Ara h 2		3.8 ± 0.6 ¶ §	0 ± 0	0 ± 0	0 ± 0
control	*Ara h 6*	3.8 ± 1.1	0 ± 0	0 ± 0	0 ± 0
PE fat		41.4 ± 10.9 **	59.9 ± 14.4 **	79.8 ± 46.2 **	508.2 ± 96.1 **
PE		40.2 ± 11.2 **	87.8 ± 44.1 **	28.1 ± 15.7 ** ¶	745.8 ± 281.9 **
Ara h 6		15.1 ± 1.3 *	34.2 ± 2.5 **	0 ± 0 ¶ §	221.3 ± 23.2 **

[a], levels are expressed as group means ± SEM of 4 mice per group

, significantly different (, $p<0.05$; **, $p<0.01$) from control

¶, significantly different ($p<0.05$) from PE fat

§, significantly different ($p<0.05$) from PE

PE but not purified allergen induces expression of costimulatory molecules on APC

Activation of APC is considered as one of the requirements to initiate active immune responses. Therefore, the expressions of the activation markers CD80, CD86, and CD54 were determined on PLN APC, 7 days after protein injection (Fig. 3). Both PE fat and PE injection significantly augmented the percentages of CD80, CD86, and CD54 expressing cells in the PLN, whereas no increase was found following injection with *Ara h 1*, *Ara h 2*, or *Ara h 6*.

Together, these data indicate that purified peanut allergens, in contrast to the whole extract, lack intrinsic immunestimulating capacity and thereby fail to induce significant immune responses in the PLNA.

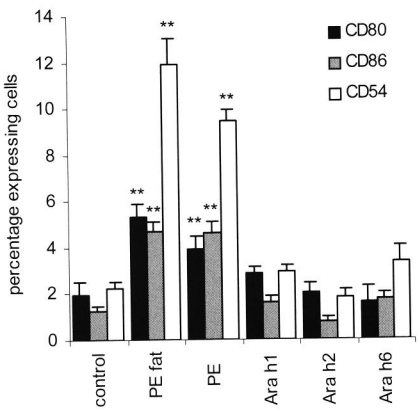

Figure 3. Expression of costimulatory molecules on APC. Mice were injected in the hind footpad with vehicle alone (control) or 1 mg of protein (PE fat, PE, *Ara h 1*, *Ara h 2*, or *Ara h 6*) dissolved in NaCl. At day 7, the draining lymph nodes were isolated and the expressions of CD80, CD86, and CD54 on MHCII-positive cells were determined by flow cytometric analysis. Data are presented as percentages ± SD of 4 mice per group. **, Significantly different ($p<0.01$) from the control group.

Purified allergens induce specific IgE responses upon oral exposure, irrespective of the food matrix

To further explore the role of the food matrix in immune responses to peanut proteins, an established oral model of peanut hypersensitivity was used. In this model, PE- and allergen-specific IgE responses can be determined, using the relevant route of exposure to food proteins [13]. Mice were dosed by gavage for 4 wk with PE (1 and 6 mg), *Ara h 1*, *Ara h 2*, *Ara h 3*, or *Ara h 6* (0.1 and 1 mg) in the presence of the mucosal adjuvant CT.

All purified allergens were able to induce specific IgE responses but only for *Ara h 1* treatment a significant difference was found between the 0.1 and 1 mg dosing group (Fig. 4). Exposure to PE (1 mg or 6 mg) also resulted in *Ara h*-specific IgE responses with levels comparable to those observed after purified allergen exposure. In the control group (oral exposure to PBS + CT) no *Ara h*-specific IgE responses were found.

No difference in antibody responses upon oral exposure to PE or PE fat

To examine the effect of fat content on orally induced antibody responses to PE, mice were orally exposed to PE fat or PE in the presence or absence of the mucosal adjuvant CT. After 4 wk of oral exposure both Th1-associated (IgG2a) and Th2-associated (IgG1 and IgE) PE-specific antibodies were determined in the serum (Fig. 5). In the absence of CT, only low levels of IgG1 and IgG2a and no IgE were found with no differences between the PE fat- and PE-treated group. In the presence of CT, similar high levels of IgG2a, IgG1, and IgE were found in both PE treatment groups. In line with the results found in the PLN, the presence of fat does not affect orally induced immune responses to PE.

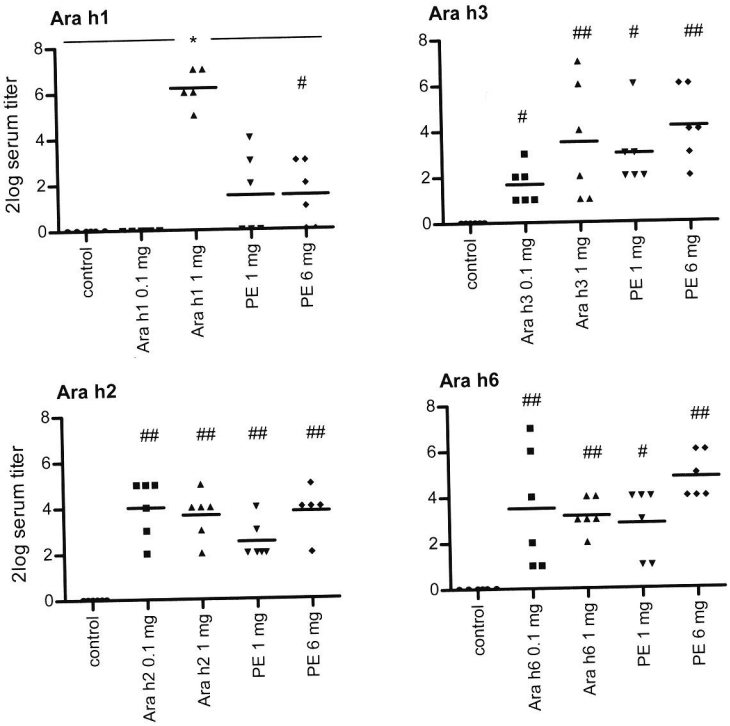

Figure 4. *Ara h*-specific IgE serum levels after 4 weeks of oral treatment. *Ara h*-specific IgE serum levels were determined after 4 wk of oral exposure to the purified allergens *Ara h 1*, *Ara h 2*, *Ara h 3*, and *Ara h 6* (0.1 and 1 mg) or PE (1 mg and 6 mg) in the presence of CT. The data are presented as the mean ^{2}log IgE titer of 6 mice per group (symbols indicate individual animals). #, Significantly different from the control group (# $p<0.05$, ## $p<0.01$).*, Significantly different ($p<0.05$) from indicated groups.

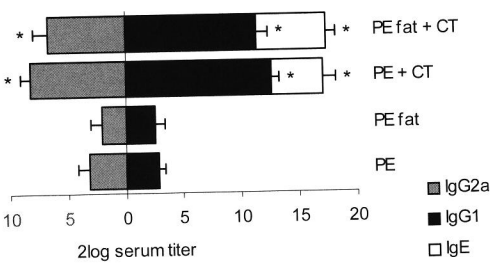

Figure 5. PE-specific serum antibody levels. PE-specific Th1-associated (IgG2a) and Th2-associated (IgG1 and IgE) serum antibody levels were determined after 4 wk of oral exposure to PE or PE fat (both 6 mg) in the presence or absence of CT. The data are presented as the mean ^{2}log antibody titer ± SEM of 6 mice per group. *, Significantly different from corresponding non-CT-treated group ($p<0.05$).

Discussion

No definitive characteristic features of food allergens have been identified other than that they need to reach and stimulate immune cells in order to induce sensitization. It has been suggested that both properties may be influenced by the food matrix [9]. In the present study it is shown that purified peanut allergens, unlike a whole PE, possess little intrinsic immune stimulating capacity and that the immune response to these allergens can be adjuvated by the presence of a food matrix. Furthermore, it was demonstrated that the purified allergens are stable enough to get into contact with the immune system after oral exposure, both in the presence or absence of a food matrix.

Although *Ara h 1* and *Ara h 2* are recognized as major peanut allergens they were unable to induce PLN cell proliferation or cytokine production, suggesting little intrinsic immune stimulating capacity. In contrast, PE induced a strong PLNA response (without causing inflammation in the paw) and was also able to adjuvate specific T cell responses against the allergens present in the extract. In line with these results, *Ara h 1* and *Ara h 2* appear to be poor IgG and IgE inducers in mice when injected i.p. (G. Ladics, Dupont, personal communication), whereas i.p. PE injection results in considerable *Ara h 1*- and *Ara h 2*-specific IgG and IgE responses [13].

The actual amount of individual allergens in PE and PE fat was always lower than the 1 mg of allergen injected in a purified form. This may raise the issue that the discrepancy in dose has affected the outcome of the immune response and that the high dose injection (1 mg) has induced non-responsiveness. However, injection of 0.1 and 0.5 mg of purified allergen also failed to induce an immune response in the PLNA (data not shown).

The difference in immune stimulating capacity between the purified allergens and the extract may be explained by selective activation of APC. Generally considered, APC activation and the accompanied upregulation of costimulatory molecules are required for the induction of an active immune response. For contact allergens, which possess high intrinsic adjuvanticity due to their reactive groups [18], it has been shown that they selectively upregulate costimulatory molecules (e.g. CD54 and CD86) on dendritic cells *in vitro* [19, 20]. Furthermore, drugs associated with immune-mediated hypersensitivity reactions were demonstrated to upregulate the expression of costimulatory molecules on APC in the PLNA [21, 22]. Interestingly, none of the purified peanut allergens tested was able to increase expressions of costimulatory molecules on APC, whereas PE injection resulted in an upregulation of CD80, CD86, and CD54. Hence, in contrast to sensitizing chemicals, soluble peanut allergens do not possess intrinsic adjuvanticity and, therefore, they need an accompanying adjuvant (in the case of PE provided by the food matrix) to be able to activate APC and to induce subsequent immune stimulation.

Peanut consists of proteins, carbohydrates and fatty acids and all the different components and their interactions may be responsible for the adjuvant effect. For instance,

it has been described that peanut oils can have an adjuvant effect in a vaccine model [23]. Remarkably, in the PLNA the presence of fat did not seem to play a major role in the induction of the immune response (PLN cell numbers, cytokine production, and APC activation). Besides nutrients, also non-nutritive compounds such as lectins [24] and food contaminants such as LPS [25] and aflatoxins [26] may affect immune responses to food proteins. For future experiments, it would be interesting to test different PE components separately in order to elucidate their immune modulating properties.

Concerning the relevant route of exposure, the food matrix has been suggested to affect the allergenic properties of orally administered proteins by providing adjuvant stimuli to the specialized gut mucosal immune system or by protecting them from digestion [9, 10], but to our knowledge no *in vivo* data are available. In general, oral administration (by feeding or gavage) of soluble food proteins to mice results in peripheral hyporesponsiveness (reviewed by Mayer *et al.* [27]; Nagler-Anderson *et al* [28]) Administration of proteins with adjuvants induces a productive immune response to this normally tolerogenic form of antigen [28]. We have previously shown that, in the present oral model, co-administration of the mucosal adjuvant CT is indispensable to induce antigen-specific IgE responses. Clearly, the intrinsic adjuvant activity of PE (i.e. without fat) is not sufficient to induce sensitization via the oral route [29]. Consequently, it might be impossible to determine the adjuvant activity of the food matrix itself when CT is co-administered. Yet, in the current study the presence of fat did not affect the magnitude or polarization (type 1 versus type 2) of the antibody responses against PE, irrespective of the presence of CT (Fig. 5). Fatty acids have been described as immune modulators following high dose oral supplementation [30, 31], covalent coupling to a protein or peptide [32, 33], or the use of liposome-entrapped proteins [34], but it remains to be elucidated whether fats present in a normal diet have similar immune modulating properties.

Regarding the aspect of digestion, the present results indicate that peanut allergens are at least stable enough to reach the gut associated lymphoid tissue even without the presence of a protecting food matrix. Although it has been shown *in vitro* that polysaccharide- and protein-protein interactions may influence allergen digestion [35, 36], these interactions seemed not to affect the immune response to allergens in the present *in vivo* model. Our data are in accordance with *in vitro* data showing that *Ara h 1* and *Ara h 2* are highly resistant to enzymatic digestion [37, 38]. Furthermore, treatment of purified *Ara h 1* and PE with pepsin resulted in the production of nearly identical sets of digestion products suggesting an intrinsic stability of *Ara h 1* [39]. The current results demonstrate that the major peanut allergens *Ara h 1*, *Ara h 2*, *Ara h 3*, and the minor allergen *Ara h 6* are stable enough *in vivo* to elicit specific IgE responses.

In the past years there has been a considerable interest in the development of experimental animal models to predict the allergenicity of proteins. Our data suggest that assays designed to measure immune stimulation, such as the PLNA, are clearly not suitable to identify allergenic proteins when injected in a purified form, because they do not appear

to posses high intrinsic immune stimulating capacity. Since PE injection did result in immune stimulation, further studies may reveal the feasibility of subcutaneous injection with whole food extract as a tool to identify the adjuvating capacity of compounds within a food matrix.

Following oral exposure all purified peanut allergens were shown to be IgE-inducers, which confirms that they posses IgE epitopes, and that they are able to reach the immune system of the gut in a relatively intact form, both required for the induction of allergic sensitization. However, for the future use of this model as a screening tool, other allergens and especially non-allergens need to be tested. Recently, Gaudry *et al.* [40] have examined several food allergens and non-allergens in a similar oral model and have shown that mice and man similarly respond to major and non-allergens. Although a broader panel of (non)allergens should be tested and the use of an adjuvant remains a topic of debate, the measurement of IgE antibody responses following oral exposure to proteins seems to be a promising tool to predict allergenicity of these proteins. Furthermore, the oral route represents the most adequate route of exposure to study possible *in vivo* digestion and food matrix effects.

In summary, our results indicate that purified peanut allergens possess little intrinsic immune stimulating capacity and that they probably need colocalization of an adjuvant compound to induce sensitization. Moreover, it was shown that the food matrix may affect immune responses to individual allergens and, as a consequence, a possible influence of the food matrix has to be considered in the development of animal tests to predict allergenic potential.

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Chapter 4

The CD28/CTLA-4-B7 signaling pathway is involved in both allergic sensitization and tolerance induction to orally administered peanut proteins

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Abstract

Dendritic cells are believed to play an essential role in regulating the balance between immunogenic and tolerogenic responses to mucosal antigens by controlling T cell differentiation and activation via costimulatory and coinhibitory signals. The CD28/CTLA-4-CD80/CD86 signaling pathway appears to be one of the most important regulators of T cell responses but its exact role in responses to orally administered proteins remains to be elucidated.

In the present study the involvement of the CD28/CTLA-4-CD80/CD86 costimulatory pathway in the induction of allergic sensitization and oral tolerance to peanut proteins was investigated. In both an established murine model of peanut hypersensitivity and an oral tolerance model to peanut, CD28/CTLA-4-CD80/CD86 interactions were blocked using the fusion protein CTLA-4Ig. To examine the relative contribution of CD80- and CD86-mediated costimulation in these models, anti-CD80 and anti-CD86 blocking antibodies were used.

In the hypersensitivity model, CTLA-4Ig treatment prevented the development of PE-induced cytokine responses, PE-specific IgG1, IgG2a, and IgE production and PE-induced challenge responses. Blocking of CD80 reduced, while anti-CD86 treatment completely inhibited the induction of PE-specific IgE. Normal tolerance induction to PE was found following CTLA-4Ig, anti-CD86, or anti-CD80 plus anti-CD86 treatment, whereas blockade of CD80 impaired the induction of oral tolerance.

We show that CD28/CTLA-4-CD80/CD86 signaling is essential for the development of allergic responses to peanut and that CD86 interaction is most important in inducing PE-specific IgE responses. Additionally, our data suggest that CD80 but not CD86 interaction is crucial for the induction of low dose tolerance to peanut.

Introduction

Food allergy has emerged as a major health problem in westernized countries and peanut allergy is one of the most serious of the hypersensitivity reactions to foods in terms of severity and persistence. At present no therapy is available and elucidation of the underlying mechanisms of allergic sensitization may contribute to the development of novel therapeutic strategies.

Under normal circumstances oral administration of food proteins leads to a state of systemic immunologic hyporesponsiveness, known as oral tolerance [1]. Impaired oral tolerance induction or a failure to maintain oral tolerance may result in the development of hypersensitivity responses to food antigens [2]. Dendritic cell (DC)-T cell interactions, leading to either activation or suppression of T cells, represent an important event in controlling the delicate balance between sensitization and tolerance. Activation of T cells requires, besides TCR–MCHII/peptide complex recognition, additional 'secondary' signals provided by costimulatory molecules expressed on antigen presenting cells (APCs). The interaction between CD28 on T cells, and its two ligands B7-1 (CD80) and B7-2 (CD86) on APCs is considered to be the master costimulatory pathway for optimal T cell responses [3]. CD86 is constitutively expressed on APCs at low levels and rapidly upregulated upon stimulation, whereas CD80 is inducibly expressed later than CD86. In contrast to the stimulatory signals provided by CD28, interaction of CD80 or CD86 with the CD28 homologue CTLA-4 induces signals that down-regulate T cell activation. CTLA-4 is constitutively expressed only on $CD4^+CD25^+$ regulatory T cells and is induced on activated T cells and we have previously shown that CTLA-4 signaling plays an important role in regulating the intensity of allergic disease [4].

Type I food allergy is characterized by Th2-associated responses resulting in increased levels of allergen-specific IgE. CD28-B7 engagement has been shown to be required for Th2 cell development [5, 6]. Studies in murine models also suggest a critical role for CD28/CTLA-4-B7 interactions in the production of antigen-specific IgE responses and the development of allergic asthma [7-9]. However, very little is known about the requirement of CD28-B7 costimulation in the development of hypersensitivity responses to orally administered proteins. Although it seems likely that costimulation will be involved in orally induced immune responses regarding the data on airway allergy, the question remains to what extent the CD28-B7 pathway plays a role, and whether CD80 and CD86 are equally involved.

Furthermore, it is still controversial whether B7-mediated costimulation is also involved in oral tolerance induction. It is well established that in the absence of costimulation, tolerance is induced by clonal T cell anergy [10]. However, when low levels of B7 are expressed, they might actually engage CTLA-4 (which has a much higher affinity for both B7 receptors than CD28), thereby contributing to oral tolerance. It has accordingly

been demonstrated by Liu *et al.* that CD86, but not CD80 interaction is essential for low dose oral tolerance induction to Ovalbumin (OVA) [11] Interestingly, other evidence points to a possible role for CD80 signaling in tolerance induction [12].

In the present study we have investigated the involvement of CD28/CTLA-4-B7 pathway in the development of sensitization and tolerance to orally administered peanut proteins. For this purpose, CTLA-4Ig fusion protein, which prevents interactions of CD28/CTLA-4 on T cells with B7 on APCs, and the antagonizing monoclonal antibodies (mAbs) anti-CD80 and anti-CD86 were used in an oral tolerance model and in an established model of peanut hypersensitivity.

We show here that CTLA-4Ig treatment completely inhibits the development of hypersensitivity responses to peanut and that CD86 interaction is most important in inducing optimal peanut-specific IgE responses. Additionally, our data indicate that CD80 but not CD86 plays an essential role in the induction of low dose oral tolerance to peanut proteins.

Methods

Mice

Female specific pathogen-free C3H/HeOuJ Ico mice (4-5 wk of age) purchased from Charles River (Lyon, France) were maintained under barrier conditions in filter-topped macrolon cages. Drinking water and peanut-free laboratory food pellets were provided *ad libitum*. The experiments were approved by the animal experiments committee of the faculty of veterinary medicine, Utrecht University.

Chemicals, reagents and monoclonal antibodies

Peanuts from the Runner cultivar (Cargill, Dawson, GA) were kindly provided by Imko Nut Products (Doetinchem, The Netherlands) and PE was prepared as previously described [4, 13]. The extract contained 30 mg/ml protein as determined by Bradford analysis (Pierce, Rockford, IL) with BSA as a standard.

Cells producing anti-CD80 (HB-301; 16-10A1) and anti-CD86 (HB-253; GL-1) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and antibodies were purified using thiophilic agarose (Seakem, Denmark). Hamster anti-mouse IgG and rat anti-mouse IgG mAbs (Rockland Immunochemicals, Gilbertsville, PA) were used as appropriate isotype controls for CD80 and CD86 respectively. Murine CTLA-4-IgG fusion protein (CTLA-4Ig) was prepared as previously described [8] and kindly provided by Dr. A van Oosterhout (University Medical Center Groningen, Groningen, The Netherlands). Human IgG (hIg, ICN Biochemicals) was used as a control for CTLA-4Ig.

Chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands), unless stated otherwise.

Treatment protocols

Oral sensitization to PE. Mice (n=5) were orally exposed to PBS plus CT (control) or PE plus CT (sensitization). Oral exposure was performed by intragastric dosing of 6 mg of PE plus 10 µg CT on 3 consecutive days, followed by weekly dosing of PE plus CT (days 8, 15, and 22). During sensitization, mice were injected i.p. with CTLA-4Ig (200 µg), anti-CD80 (100 µg), anti-CD86 (100 µg), anti-CD80 (100 µg) + anti-CD86 (100 µg), or the same dose of an appropriate isotype control Ab (human IgG, hamster IgG, or rat IgG) on days -1, 3, 7, 14, and 21. At day 31, all groups received an oral challenge with 12 mg PE and mice were sacrificed on day 32.

Oral tolerance induction to PE. Mice (n=5) were intragastrically exposed to 1 mg PE (to induce oral tolerance) or PBS on 3 consecutive days (day 1-3). Before oral exposures (day 0), mice received an i.p. injection with CTLA-4Ig (200 µg), anti-CD80 (100 µg), anti-CD86 (100 µg), anti-CD80 (100 µg) + anti-CD86 (100 µg), or the same dose of an appropriate isotype control Ab (human IgG, hamster IgG, or rat IgG). Mice were challenged

i.p with 100 µg PE in alum (25 mg/ml), 21 and 35 days after the last oral exposure and mice were sacrificed at day 49.

Measurement of serum IgG1, IgG2a, and IgE antibody levels

Blood samples were collected at various time points and stored at –20°C until analysis. Levels of *Ara h*- and PE-specific antibodies were determined by ELISA (IgG1 and IgG2a) or sandwich ELISA (IgE) as previously described [4]. Briefly, Plates (highbond 3590; Costar, Cambridge, MA) were coated overnight with 20 µg/ml PE, *Ara h1*, or *Ara h 3* (for IgG1 and IgG2a detection) or with 1.5 µg/ml purified rat anti-mouse IgE (BD Pharmingen) in carbonate buffer (pH 9.6), followed by 1 h blocking with PBS-Tween/3% milk powder. Each test serum was titrated (at least 8 serial dilutions) starting at 1:8 or 1:16 dilution and incubated for 2 h. A pre-sera pool was used as reference value (dilution 1:4). For detection of IgG1 and IgG2a, AP-conjugated antibodies (Southern Biotechnology Associates, Birmingham, AL) were added (1 h at room temperature). Subsequently 1 mg/ml p-nitrophenylphosphate in diethanolamine buffer was used for the color reaction, which was stopped with a 10% EDTA solution and absorbance was measured at 405 nm. To measure antigen-specific IgE antibodies, serum was incubated for 2 h and subsequently an antigen-digoxigenin (DIG) conjugate solution was added (1 h at room temperature). The coupling of DIG to the antigen was performed according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). After incubation (1 h at room temperature) with peroxidase-conjugated anti-DIG fragments (Roche Diagnostics, Mannheim, Germany), a tetramethylbenzidine substrate (0.1 mg/ml) solution was used and the color reaction was stopped with 2 M H_2SO_4. Absorbance was measured at 450 nm. The reciprocal of the furthest test serum dilution resulting in an extinction higher than the reference value was read as a titer.

Cell culture and cytokine measurements

Spleen single cell suspensions ($3.75x10^5$ cells in 200 µl complete RPMI 1640 (Life Technologies, Paisley, Scotland) containing 10% FCS (ICN Pharmaceuticals, Costa Mesa, CA)) were incubated in the presence or absence of 200 µg/ml PE in 96-well plates for 96 h at 37 °C, 5% CO_2. After centrifugation for 10 min at 150 *g*, supernatant was collected and stored at -20°C until analysis.

In the culture supernatants, levels of IFN-γ, IL-4, IL-5, and IL-10 were determined by sandwich ELISA. IFN-γ, IL-4, and IL-5 capture and biotin-conjugated antibodies were obtained from BD Pharmingen and the ELISAs were performed as previously described [4]. The IL-10 (BD Pharmingen) ELISA (Biosource, Camarillo, CA) was performed in accordance with the manufacturer's instructions.

Measurement of serum mouse mast cell protease-1 (mmcp-1)

Blood was collected 45 min after oral challenge with PE and serum levels of mmcp-1 were determined using an ELISA kit (Moredun, Scotland). The ELISA was performed according to the manufacturer's instructions.

Statistics

Data were analyzed using SigmaStat statistical software package (SPSS, Chicago, IL). In the oral sensitization study, multiple comparisons of group means were analyzed using one-way ANOVA with Bonferroni as *post hoc* test. In the oral tolerance study, the differences between PBS and PE-exposed group means were determined by using independent-samples *t* test procedure. For cytokine levels and mmcp-1 serum levels, statistical analysis was performed following logarithmic transformation to achieve normal distribution. A value of $p<0.05$ was considered statistically significant.

Results

CTLA-4Ig treatment during oral sensitization inhibits the development of PE- and allergen-specific antibodies and PE-induced challenge responses

In order to investigate the involvement of the CD28/CTLA-4-CD80/CD86 signaling pathway in hypersensitivity responses to peanut proteins, this costimulatory pathway was blocked with CTLA-4Ig during the oral sensitization protocol. After four weeks of exposure to PE plus CT, mice developed PE-specific IgG1, IgG2a, and IgE antibody responses. CTLA-4Ig treatment was associated with a profound inhibition of both Th1-associated (IgG2a) and Th2-associated (IgG1 and IgE) PE-specific antibody levels compared to non-treated or hIg-treated mice (Fig. 1A). Furthermore, CTLA-4Ig treatment prevented the release of mmcp-1 upon an oral challenge with PE (Fig. 1B).

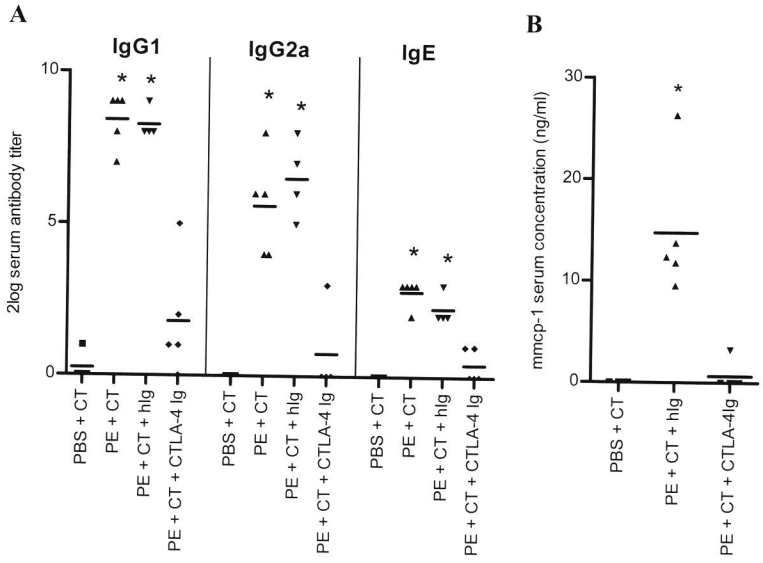

Figure 1. Effect of CTLA-4Ig treatment on PE-specific antibody responses and mast cell degranulation. Mice were orally exposed for 4 wk to PBS + CT (control) or PE + CT (sensitization). During oral exposure indicated groups were treated with CTLA-4Ig or control antibody (hIg). (A) PE-specific Th1-associated (IgG2a) and Th2-associated (IgG1 and IgE) serum antibody levels after 4 wk of exposure. The data are presented as the mean ^{2}log antibody titer of 4-5 mice per group (symbols indicate individual animals). (B) Mmcp-1 serum levels upon an oral challenge with PE. Levels of mmcp-1 were determined in blood samples collected 45 min after the oral challenge. The data are presented as the group mean (symbols indicate individual animals) of 4-5 mice per group. *, Significantly different ($p<0.05$) from the control (PBS + CT) group.

PE consists of a large variety of different proteins but only a number of them have been identified as allergens (termed *Ara h 1-8*) and are recognized by serum IgE of peanut-allergic patients. Therefore, the effect of CTLA-4Ig treatment on IgG1 antibody responses against the purified major peanut allergens *Ara h 1* and *Ara h 3* was evaluated. Exposure to PE plus CT resulted in profound *Ara h*-specific IgG1 responses in non-mAb-treated or hIg-treated mice, whereas these allergen-specific responses were completely inhibited in CTLA-4Ig–treated mice (Fig. 2).

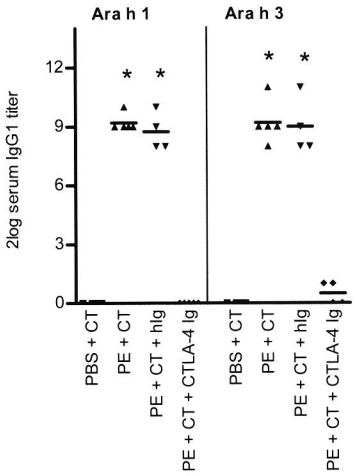

Figure 2. Effect of CTLA-4Ig treatment on allergen-specific IgG1 levels. *Ara h 1* and *Ara h 3*-specific serum IgG1 levels were determined after 4 wk of oral exposure to PBS + CT or PE + CT. Indicated groups were treated with CTLA-4Ig or control antibody (hIg) during the oral exposure protocol. The data are presented as the mean ^{2}log antibody titer of 4-5 mice per group (symbols indicate individual animals). *, Significantly different ($p<0.05$) from the control (PBS + CT) group.

CTLA-4Ig treatment during oral sensitization inhibits PE-induced cytokine production

To analyze the effect of CTLA-4Ig on T cell responses, spleen cells were cultured in the presence or absence of PE and cytokine levels were determined in the culture supernatants. In agreement with previous studies, significant levels of IL-4, IL-10, and IFN-γ were found upon restimulation with PE in culture supernatants of PE-sensitized mice. PE-restimulated spleen cell cultures derived from CTLA-4Ig-treated mice showed significantly reduced levels of IL-4, IL-10, and IFN-γ production compared to levels found in cell cultures of hIg-treated mice (Fig. 3).

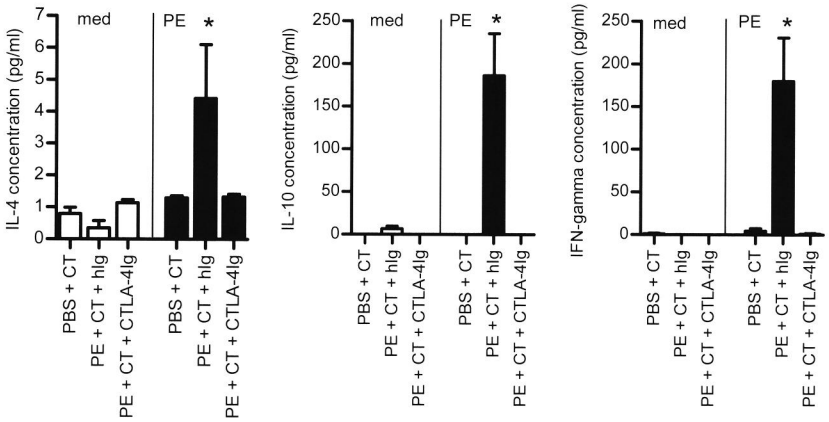

Figure 3. Effect of CTLA-4Ig treatment on cytokine levels in splenocyte culture supernatants. After 4 wk of oral exposure cell suspensions were cultured in the presence (black bars) or absence (white bars) of PE and IL-4, IFN-γ, and IL-10 cytokine levels were measured by ELISA. Data are presented as the group mean ± SEM of 5 mice per group. *, Significantly different ($p<0.05$) from the control (PBS + CT) group.

Either CD80 or CD86 signaling is required for the production of PE-specific IgG antibodies, but CD86 ligation is most important for optimal IgE responses

Because CTLA-4Ig inhibits the interactions of both CD28 and CTLA-4 to both CD80 and CD86 we set out to investigate the specific roles of the individual B7 molecules in the induction of allergic sensitization to peanut proteins. For this purpose mice were treated with blocking mAbs (anti-CD80, anti-CD86, or a combination of both) during the entire antigen sensitization period.

Treatment with either mAb alone had no significant effect on the production of PE-specific IgG1 or IgG2a antibodies in sensitized mice (Fig. 4). However, treatment with the combination of both mAbs during the sensitization period resulted in a large reduction in both IgG1 and IgG2a antibody levels. Interestingly, blockade of CD80 ligation reduced, but did not completely inhibit PE-specific IgE production, whereas blockade of CD86 ligation completely prevented PE-specific IgE responses in 5 out of 6 animals. When both CD80 and CD86 were blocked, no PE-specific serum IgE was induced. These results suggest that either CD80 or CD86 ligation is sufficient to induce IgG1 or IgG2a responses to orally administered PE and that CD86 ligation is most important for the induction of systemic IgE responses.

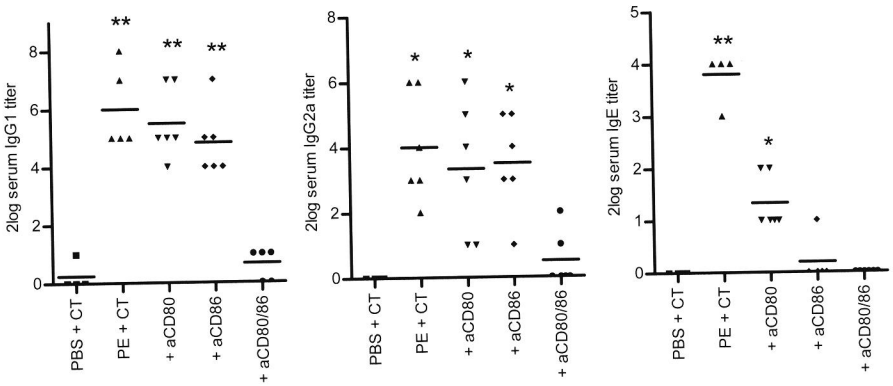

Figure 4. Involvement of CD80 and CD86 in PE-specific antibody responses. PE-specific IgG1, IgG2a, and IgE serum antibody levels were determined after 4 wk of oral exposure to PBS + CT (control) or PE + CT. Mice were treated with anti-CD80, anti-CD86, anti-CD80 + anti-CD86, or with isotype control (PE + CT represents both rat anti-mouse IgG and hamster anti-mouse IgG-treated groups). The data are presented as the mean ^{2}log antibody titer of 4-6 mice per group (symbols indicate individual animals). *, Significantly different (*, $p<0.05$ and **, $p<0.01$) from the control (PBS + CT) group.

CD80 but not CD86 ligation plays a role in oral tolerance induction

Since CD80 and CD86 can also act as coinhibitors by engagement with the high-affinity ligand CTLA-4, they may additionally be involved in the induction of tolerance to orally administered proteins. To investigate this, mice were treated with a single i.p. injection of blocking mAb or control antibody before oral exposure (days 1-3) to PE (oral tolerance induction) or PBS (control). Mice were immunized i.p. with PE plus alum 21 and 35 days later and sacrificed at day 49. Spleen cells were cultured in the presence or absence of PE to measure cytokine production and PE-specific antibody levels were determined in the serum. As shown in Fig. 5, multiple low-dose PE exposures significantly suppressed PE-specific IgG1 and IgE levels as compared to PBS-exposed mice. Anti-CD86, anti-CD80/86 and CTLA-4Ig treatment had no effect on the reduced PE-specific IgG1 and IgE levels found in isotype control-treated mice following oral PE pre-exposure. However, in PE-exposed anti-CD80-treated mice, PE-specific IgG1 and IgE levels were comparable or even significantly higher (IgE) than levels found in PBS-exposed controls, indicating that oral tolerance was abrogated by anti-CD80 treatment. As shown in Fig. 6, oral tolerance induction following low-dose PE exposure is also associated with a decrease in Th2 cytokine (IL-4 and IL-5) production in spleen cell cultures restimulated with PE. In all mAb–treated groups a significant reduction of IL-4 and IL-5 production was found following oral PE pre-exposure, except for the anti-CD80-treated group in which the reduction was not significant, suggesting that CD80 blockade partly inhibits T cell suppression related to oral tolerance induction.

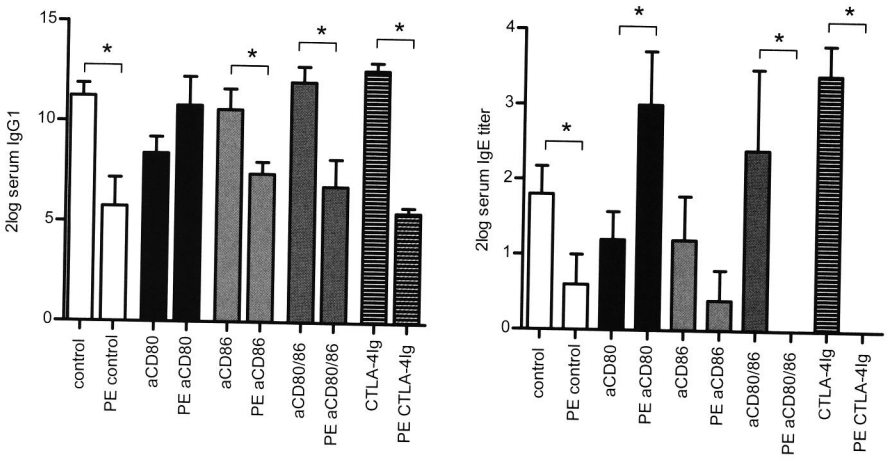

Figure 5. Effect of CD80 and CD86 blockade on antibody production in an oral tolerance model. Mice were orally exposed for 3 consecutive days with PBS or PE. Before oral exposure mice were treated with anti-CD80, anti-CD86, anti-CD80 + anti-CD86, CTLA-4–Ig, or isotype control (the presented control group is representative for the rat IgG, hamster IgG, and humanIg-treated groups). Mice were subsequently immunized i.p. with PE plus alum (2 times with a 2-wk interval) and PE-specific IgG1 and IgE antibodies were measured 2 wk after the last i.p. injection. Data are presented as the group mean ± SEM of 5 mice per group.*, Significantly different ($p<0.05$) from indicated group.

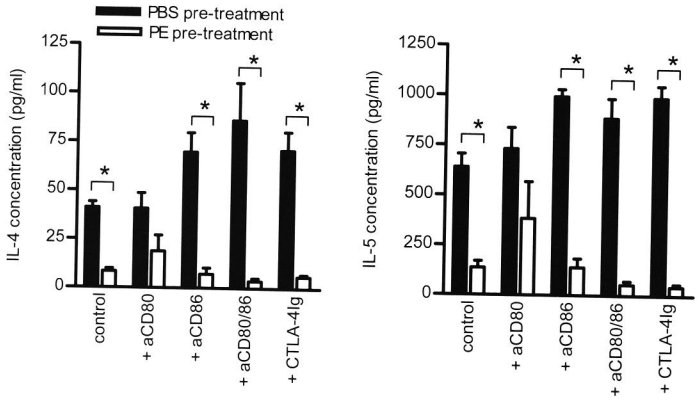

Figure 6. Effect of CD80 and CD86 blockade on cytokine production in an oral tolerance model. Mice were orally exposed for 3 consecutive days with PBS (white bars) or PE (black bars). Before oral exposure mice were treated with anti-CD80, anti-CD86, anti-CD80 + anti-CD86, CTLA-4–Ig, or isotype control (the control group is representative for the rat IgG, hamster IgG, and humanIg-treated groups). Mice were subsequently immunized i.p. with PE plus alum (2 times with a 2-wk interval) and 2 wk after the last i.p. injection, splenic single cell suspensions were cultured in the presence or absence of PE. In the supernatant IL-4 and IL-5 cytokine levels were determined by ELISA. In cultures restimulated in the absence of PE no cytokine production was observed (data not shown). Data are presented as the group mean ± SEM of 5 mice per group.*, Significantly different ($p<0.05$) from indicated group.

Discussion

The CD28/CTLA-4-CD80/86 signaling pathway is the best characterized T cell costimulatory pathway, but it is complex because of the dual specificity of CD80 and CD86 for the stimulatory receptor CD28 as well as the inhibitory receptor CTLA-4. In the current study we investigated the involvement of the CD28/CTLA-4-CD80/86 signaling pathway in oral sensitization and oral tolerance to peanut. Blockade of the CD28/CTLA-4-CD80/86 pathway by treatment with CTLA-4Ig inhibited all antigen-specific T cell, B cell, and challenge responses in an oral model of peanut hypersensitivity, illustrating the essential role of CD28 costimulation in food allergic responses. The present findings are in agreement with previous reports, which have demonstrated a critical role for CD28-CD80/86 signaling in Th2 differentiation and the development of allergic airway responses by treatment with the same CTLA-4Ig fusion protein [7-9]. The strong effect of CTLA-4Ig treatment may not solely be explained by the preventing CD28-CD80/86 interaction; recent studies have demonstrated that CTLA-4Ig ligation of CD80/CD86 on DCs may induce tolerance by stimulating DC tryptophan catabolism via B7 signaling [14, 15].

In the current study, results on the individual roles of CD80 and CD86 in the hypersensitivity model showed that signaling through either CD80 or CD86 was sufficient to generate PE-specific IgG responses, whereas CD86 costimulation was most important for the induction of allergic PE-specific IgE responses. These data are consistent with results obtained in mouse models of allergic airway inflammation, demonstrating that CD86 and not CD80 interaction is essential for the development for systemic IgE responses [16, 17].

Although expression of CD80 and CD86 on APCs and T cells is increased upon encounter with inflammatory stimuli, both CD80 and CD86 are constitutively expressed in small amounts on resting DCs [18, 19]. It has been hypothesized that in non-inflammatory conditions the B7 molecules may play a role in maintaining aspects of immune tolerance [12]. Our data clearly indicate that CD80 but not CD86 is involved in the induction of low dose oral tolerance to peanut proteins. In agreement, Lohr et al have demonstrated that low constitutive expression of B7 on DCs potently limits T cell activation and functions to maintain self-tolerance [20]. This tolerance induction seems to be mediated by interaction with CTLA-4 constitutively expressed on $CD4^+CD25^+$ regulatory T cells (Tregs) [12, 20, 21]. Accordingly, blockade of CTLA-4 in the present oral model resulted in impaired oral tolerance induction (data not shown) en we have recently shown that $CD4^+CD25^+$ Tregs play an essential role in the induction of oral tolerance to PE (chapter 6). Hence, our data suggest that CD80 engagement with CTLA-4 on $CD4^+CD25^+$ Tregs may play an important role in the induction of oral tolerance. The current finding that CD80 is the major ligand involved in oral tolerance induction by interaction with CTLA-4 is (indirectly) supported by several observations. Physiochemical studies revealed that CD80 has a much higher CTLA-4 binding affinity and avidity than CD86 [22] and crystallographic data show that it is unlikely

that CD86 is able to form stable dimers necessary for CTLA-4 binding [23]. Furthermore, CD80 is the major ligand mediating CTLA-4 localization, while CD86 is the main ligand for CD28 concentration at the immunological synapse [24]. Finally, in several mouse models of autoimmunity, administration of anti-CD80 blocking antibodies has been associated with exacerbation of the disease [25, 26]. However, in contrast, a study of low dose oral tolerance showed that CD86 but not CD80 is indispensable for the induction of tolerance to OVA using TGF-β and IFN-γ cytokine production as the readout for oral tolerance [11]. These contradictive results may be due to the use of a completely different oral tolerance and mAb treatment protocol with different readout parameters compared to the current study. Indeed, timing of anti-B7 antibody treatment has been shown to be critical for the effects that are found [26]. For example anti-CD86 therapy prevents the onset and early progression of diabetes in NOD mice, but is not effective during the late phase of disease progression [26]. Nevertheless, CD80 and CD86 appear to have distinct as well as overlapping roles in regulating responses to orally administered antigens and the functional outcome likely depends on the context, including relative expression levels of CD80, CD86, and CTLA-4, the presence of adjuvants, and the dose of antigen.

Blockade of the CD28/CTLA-4-CD80/86 costimulation pathway has been proposed as an effective treatment in allergic disease. In the hypersensitivity model we found that 4 wk of CTLA-4Ig treatment not only prevented the production of antigen-specific IgE antibodies but also strongly reduced total serum IgE levels (data not shown). These data suggest that existing elevated serum IgE levels in atopic patients may be targeted with CTLA-4Ig. However, the complex dual role of this pathway has significant implications for developing clinical treatment strategies. Under certain circumstances, CD80, and to a lesser extent CD86, provide a critical interaction with CTLA-4 to maintain or induce tolerance (as shown for CD80 in the present manuscript) or to downregulate immune responses. Temporal and tissue-specific expressions complicate the prediction of the consequences of B7- blockade. In addition, whereas CTLA-4-B7 interactions (but not CD28-B7 interactions) seem to play an important role in $CD4^+CD25^+$ Treg function, recent data indicate that CD28-B7 interactions (but not CTLA-4-B7 interactions) are essential in the development of $CD4^+CD25^+$ Tregs and for their survival in the periphery [27, 28]. Accordingly it has been shown that $CD28^{-/-}$ and B7-1/B7-$2^{-/-}$ NOD mice develop accelerated diabetes and have markedly reduced numbers of $CD4^+CD25^+$ Tregs [29]. Interestingly, in the current study a significant 2.5-fold decrease in $CD4^+CD25^+$ Tregs was found following 4 wk of CTLA-4Ig treatment (data not shown), confirming that also CTLA-4Ig therapy may result in complicated outcomes, especially when treatment is stopped and $CD4^+CD25^+$ Treg levels may not be restored instantly.

In summary our data extend the knowledge on the involvement of the CD28/CTLA-4-B7 signaling pathway in responses to orally administered proteins and clearly show the different roles of CD80 and CD86. The data indicate that this costimulatory pathway is

indispensable for the induction of oral sensitization and IgE-mediated hypersensitivity to peanut, with CD86 being the most important ligand. In contrast, CD80 but not CD86 interaction is crucial for the induction of low dose tolerance to peanut, probably by engaging CTLA-4.

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Chapter 5

CTLA-4 signaling regulates the intensity of hypersensitivity responses to food antigens, but is not decisive in the induction of sensitization

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Abstract

Although food allergy has emerged as a major health problem, the mechanisms that are decisive in the development of sensitization to dietary antigen remain largely unknown. CTLA-4 signaling negatively regulates immune activation, and may play a crucial role in preventing induction and/or progression of sensitization to food antigen. To elucidate the role of CTLA-4 signaling in responses to food allergens, a murine model of peanut allergy was used. During oral exposure to peanut extract (PE) together with the mucosal adjuvant cholera toxin (CT), which induces peanut allergy, CTLA-4 ligation was prevented using a CTLA-4 monoclonal antibody. Additionally, the effect of inhibition of the CTLA-4 pathway on oral exposure to PE in the absence of CT, which leads to unresponsiveness to peanut antigen, was explored.

During sensitization, anti-CTLA-4 treatment considerably enhanced IgE responses to PE and the peanut allergens *Ara h 1, Ara h 3,* and *Ara h 6,* resulting in elevated mast cell degranulation upon an oral challenge. Remarkably, antagonizing CTLA-4 during exposure to PE in the absence of CT resulted in significant induction of Th2 cytokines and an elevation in total serum IgE levels, but failed to induce allergen-specific IgE responses and mast cell degranulation upon a PE challenge.

These results indicate that CTLA-4 signaling is not the crucial factor in preventing sensitization to food allergens, but plays a pivotal role in regulating the intensity of a food allergic sensitization response. Furthermore, these data indicate that a profoundly Th2-biased cytokine environment is insufficient to induce allergic responses against dietary antigen.

Introduction

Food allergy, which results from adverse immune responses to dietary Antigen, affects about 2% of the American population [1]. Peanut allergy is responsible for the majority of fatal food-induced allergic reactions [2], and the prevalence of peanut allergy seems to be increasing. Recently, progress has been made in the treatment of clinical symptoms in peanut allergic patients [3], but mechanisms responsible for the induction of sensitization to food allergens remain largely unknown.

Mucosal immune responses to orally ingested food antigen are generally based on Antigen exclusion and suppressive immunity leading to 'oral tolerance'(reviewed in [4]). In the case of allergic hypersensitivity to food antigen, oral tolerance is abrogated. Since costimulation is required for effective immunity, signals delivered by different costimulatory pathways –stimulatory or suppressive- may play a critical role in the balance between oral sensitization and tolerance induction.

The interaction between CD28 on T cells, and its two ligands B7-1 (CD80) and B7-2 (CD86) on APC is considered to be the master costimulatory pathway for optimal T cell responses [5]. In contrast to the stimulatory signals provided by CD28, interaction of CD80/CD86 with the CD28 homologue CTLA-4 provides signals that down-modulate T cell activation. Antagonizing CTLA-4 signaling enhances T cell proliferation [6], whereas cross-linking of CTLA-4 in vitro inhibits anti-CD3-induced T cell proliferation [7]. Furthermore, CTLA-4-deficient mice develop a fatal lymphoproliferative disease [8] showing a role for CTLA-4 in the establishment of peripheral self-tolerance as well as regulation of active immunity.

Although very little is known about the role of the CD28/CTLA-4-B7 pathway in food allergy, anti-CTLA-4 treatment has been shown to abrogate systemic hyporesponsiveness to orally administered ovalbumin (OVA) in oral tolerance models [9, 10]. This suggests that CTLA-4 signaling might be decisive in oral tolerance development. In addition, recent studies in murine models of allergic asthma (a comparable Th2-induced mucosal hypersensitivity response) suggest an indispensable role for the CTLA-4 signaling pathway in controlling the production of Th2 cytokines, and in the development of airway hyperresponsiveness. As such, inhibition of CD28-B7 interaction ameliorated [11], and even reversed asthmatic manifestations [12], whereas the blockade of CTLA-4 enhanced allergic sensitization [13]. In line with these observations, we hypothesized that CTLA-4 is involved in suppressing the induction and progression of sensitization to food proteins.

To test this hypothesis an established oral peanut sensitization model (with minor modifications) was used. In this model, exposure to peanut extract (PE) in the presence of the mucosal adjuvant cholera toxin (CT) induces sensitization to peanut, characterized by PE- and peanut allergen (*Ara h*)-specific IgE serum antibody and cytokine production, and subsequent clinical reactions [14]. In the present study mice were treated with the monoclonal

antibody (mAb) 4F-10 to inhibit the CTLA-4 signaling pathway, and the effects on parameters in an early and later phase of sensitization were explored. In agreement with our hypothesis, anti-CTLA-4 treatment resulted in enhanced sensitization to PE and the purified peanut allergens *Ara h 1*, *Ara h 3,* and *Ara h 6,* characterized by a profound elevation of specific-IgE production, and increased clinical reaction upon an oral challenge.

Additionally, the effect of anti-CTLA-4 treatment on oral exposure to PE without adjuvant, which results in unresponsiveness to peanut antigen (no PE- or allergen-specific IgE), was investigated. Despite the induction of Th2 cytokines, activation of APC, and an elevation in total serum IgE levels, anti-CTLA-4 treatment failed to induce PE-specific or *Ara h*-specific IgE antibodies or mast cell degranulation.

These results suggest that CTLA-4 signaling plays an important role in regulation of the intensity of hypersensitivity responses to food proteins, but is not decisive in preventing the induction of sensitization to dietary antigen. Moreover, current data challenge the pivotal role of a Th2 cytokine environment in the induction phase of food allergy.

Materials and Methods

Mice

Female, specific pathogen-free C3H/HeOuJ Ico mice, 4 wks of age were purchased from Charles River (Lyon, France) and were maintained under barrier conditions in filter-topped macrolon cages with wood chip bedding, at mean temperature of 23 ± 2°C, 50-55% relative humidity and a 12-h light/dark cycle. Drinking water and standard laboratory food pellets were provided *ad libitum*. The experiments were approved by the animal experiments committee of the faculty of veterinary medicine, Utrecht University.

Chemicals and reagents and monoclonal antibodies

Chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands) unless stated otherwise. Cells producing anti-CTLA-4 (4F-10) were obtained from American Type Culture Collection (ATCC; Manassas, VA) and antibodies were purified using thiophilic agarose (SeaKem, Danmark). Purified hamster IgG (Rockland Immunochemicals, Gilbertsville, PA) was used as an isotype control. For cytokine measurements, IL-4, IL-5, and IFN-γ capture and detecting antibodies and IL-10 ELISA kit were obtained from BD Pharmingen (Hamburg, Germany) and streptavidin-HRP from Sanquin (Amsterdam, The Netherlands). BD Pharmingen was the supplier of rat anti-mouse IgE and the Digoxigenin (DIG)-coupling kit was obtained from Boehringer Mannheim (Mannheim, Germany) and anti-DIG-peroxidase Fab fragments from Roche Diagnostics (Mannheim, Germany). Alkaline phosphatase (AP)-conjugated goat anti-mouse, human adsorbed IgG1, IgG2a, and AP-conjugated rat anti-mouse IgE were purchased from Southern Biotechnology Associates (Birmingham, AL). Campina Melkuni (Eindhoven, The Netherlands) was the supplier of milk powder used in blocking buffer. Mouse Ig reference serum with 2 μg/ml monoclonal DNP-specific IgE was obtained from ICN Biomedicals (Costa Mesa, CA). Medium that was used for the in vitro restimulation was complete RPMI 1640 with Glutamax-I (Life Technologies, Paisley, Scotland) supplemented with 10% FCS (ICN Pharmaceuticals, Costa Mesa, CA) and 2% penicillin-streptomycin.

Preparation of peanut extract and purification of allergens

Peanuts from the Runner cultivar (Cargill, Dawson, GA) were kindly provided by Imko Nut Products (Doetinchem, The Netherlands). The PE was prepared as described by Koppelman *et al.* [15]. Briefly, peanuts were ground and the protein was extracted by mixing 25 g ground peanut with 200 ml 20 mM Tris buffer (pH 7.2). After 2 h stirring at room temperature the aqueous fraction was collected by centrifugation (3000 *g* for 30 min) and subsequently centrifuged at 10000 *g* for 30 min to remove residual traces of fat and insoluble particles. The extract contained 30 mg/ml protein as determined by Bradford analysis with BSA as a standard. SDS-PAGE analysis confirmed the presence of proteins migrating at the same

molecular weights as the purified allergens *Ara h 1*, *Ara h 3*, and *Ara h 6* (data not shown). The allergens *Ara h 1* and *Ara h 3* were purified as described by Koppelman *et al.* [15] and *Ara h 6* was purified from a side-fraction.

Treatment protocol

Mice (n=6) were orally exposed to PE + CT, PE alone, or were left untreated. Oral exposure to PE was performed by intra-gastric dosing of 6 mg roasted PE on three consecutive days, followed by weekly dosing of 6 mg PE (4 wks). CT (10 µg) was coadministered on days 1, 2, 3, 8, 15, and 21. Equivalent groups (n=6) were additionally injected i.p. with 100 µg anti-CTLA-4 mAb on days 1, 3, 7, 14, and 20 during the oral dosing regime. PE-exposed mice were also treated with an appropriate isotype control, which had no effect on any of the measured parameters (data not shown). At day 30, mice received an oral challenge with 12 mg PE. Mice were killed by cervical dislocation on day 7 or 31 days after the onset of exposure.

Measurement of serum IgG1, IgG2a, and IgE antibodies

Blood was collected at weekly intervals and levels of PE and *Ara h*-specific antibodies were measured by ELISA (IgG1 and IgG2a) or sandwich ELISA (IgE). Plates (highbond 3590; Costar, Cambridge, MA) were coated overnight with 20 µg/ml PE or *Ara h* (for IgG1 and IgG2a detection) or with 1.5 µg/ml purified rat anti-mouse IgE (for antigen-specific and total serum IgE detection) in carbonate buffer (pH 9.6), followed by 1 h blocking (37°C) with PBS-Tween/3% milk powder. Each test serum was titrated starting at 1:8 or 1:16 dilution and incubated for 1 h (37°C). A pre-sera pool was used as reference value (dilution 1:4) for antigen-specific antibody levels and a standard curve of a reference serum (2 µg/ml IgE) was used to determine total serum IgE levels. For detection of IgG1 and IgG2a and total serum IgE antibodies, AP-conjugated antibodies were added (1 h at 37°C). Subsequently 1 mg/ml p-nitrophenylphosphate in diethanolamine buffer was used for the color reaction, which was stopped with a 10% EDTA solution and absorbance was measured at 405 nm using an ELISA reader ELX800 (BIOTEK Instruments, Winooski, VT). To measure antigen-specific IgE antibodies, serum was incubated for 2 h and subsequently a PE- or *Ara h*-digoxigenin (DIG) conjugate solution was added (1 h at 37°C). The coupling of DIG to PE or the *Ara h*'s was performed according to the manufacturer's instructions. Briefly, the coupled proteins were separated on a sephadex G-25 column and labeling efficiency was determined spectrophotometrically at 280 nm. After incubation (1 h at 37°C) with peroxidase-conjugated anti-DIG fragments, a TMB-substrate (0.1 mg/ml) solution was used and the color reaction was stopped with 2 M H_2SO_4. Absorbance was measured at 450 nm. The reciprocal of the furthest test serum dilution resulting in an extinction higher than the reference value was read as a titer.

Cell culture and cytokine measurement

Spleen and mesenteric lymph node (MLN) single cell suspensions (150 µl of $2.5x10^6$ cells/ml in complete RPMI 1640) were incubated in the presence or absence of PE (200 µg/ml) in 96-well plates for 96 h at 37 °C, 5% CO_2. After centrifugation for 10 min at 150 *g*, supernatant was collected and stored at -20°C until analysis.

In the culture supernatants, IFN-γ, IL-4, IL-5, and IL-10 were determined by sandwich ELISA. Plates (highbond 3590; Costar) were coated overnight with 1.5 µg/ml rat anti-mouse IL-4, IL-5, or IFN-γ, and the following day plates were blocked with PBS-Tween/3% milk powder for 4 h at room temperature. Samples and cytokine standards were added in several dilutions and incubated overnight at 4°C. Plates were incubated with 0.25 µg/ml rat anti-mouse IL-4, IL-5, or IFN-γ conjugate for 1 h at room temperature followed by streptavidin-HRP incubation for 45 min. Finally, TMB-substrate (0.1 mg/ml) was added and the color reaction was stopped with 2 M H_2SO_4. Absorbance was measured at 450 nm. The IL-10 ELISA was performed in accordance with the manufacturer's instructions.

Measurement of serum mouse mast cell protease-1

Blood was collected before, and 1 h after an oral challenge with PE at day 30 and serum levels of mouse mast cell protease-1 (mmcp-1) were determined with an ELISA kit (Moredun, Scotland). The ELISA was performed according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using SigmaStat statistical software package (SPSS, Chicago, IL). For serum antibody levels, cytokine levels and mmcp-1 serum levels, the differences between group means (log-transformed data) were determined by using one-way ANOVA with Bonferroni as *post hoc* test. Values of $p<0.05$ were considered statistically significant.

Results

Different effects of antagonizing CTLA-4 on PE-specific antibody levels in the absence or presence of an adjuvant

Mice were orally exposed to PE in the presence or absence of the mucosal adjuvant CT. Additionally, both PE- and PE + CT-exposed mice were treated with anti-CTLA-4 mAb or did not receive mAb treatment.

The group receiving 4 wks of oral treatment with PE + CT showed PE-specific serum IgG1, IgG2a, and IgE (Fig. 1). Blockade of CTLA-4 during the oral sensitization protocol increased ($p<0.01$) both Th2-related (IgG1 and IgE) and Th1-related (IgG2a) PE-specific antibody levels compared to PE + CT treatment group.

The group that was exposed to PE without CT produced low levels of PE-specific IgG1 and IgG2a and no IgE. In the similarly exposed group, blockade of CTLA-4 had no effect on antibody levels, demonstrating that hyporesponsiveness to peanut upon oral PE exposure without adjuvant is not abrogated by anti-CTLA-4 treatment.

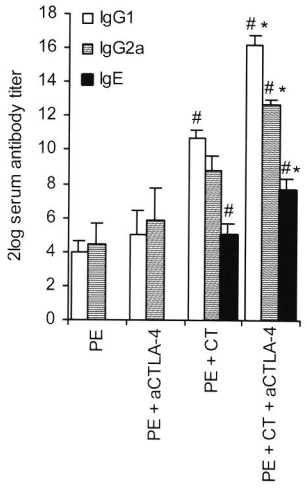

Figure 1. Effect of anti-CTLA-4 treatment on PE-specific antibody production. PE-specific Th1-associated (IgG2a) and Th2-associated (IgG1 and IgE) serum antibody levels were determined after 4 wks of oral PE exposure in the presence or absence of CT and with or without anti-CTLA-4 mAb (aCTLA-4) treatment. The data are presented as the mean 2log antibody titer ± SEM of 6 mice per group. *, Significantly different ($p<0.01$) from the corresponding non-CTLA-4-treated group. #, Significantly different ($p<0.01$) from the corresponding non-CT-exposed group.

Anti-CTLA-4 treatment increases total serum IgE levels

In contrast to PE-specific IgE levels, anti-CTLA-4 treatment clearly augmented total IgE production in both PE-exposed groups (irrespective of the presence of CT, see Fig. 2). Mice treated with anti-CTLA-4 without oral dosing of PE showed a similar significant elevation of total IgE serum levels compared with control mice that received no treatment at all. These data demonstrate a prominent role for T cells expressing CTLA-4 in regulating total circulating IgE levels.

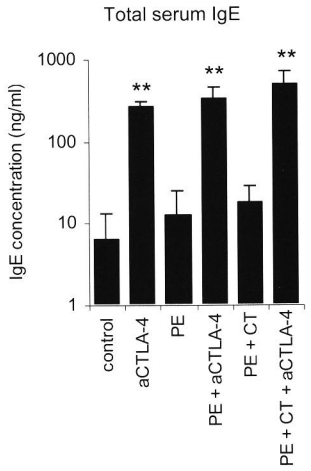

Figure 2. Effect of anti-CTLA-4 treatment on total serum IgE levels. Mice were orally exposed to PE with or without CT or were left untreated (control). Indicated groups received blocking anti-CTLA-4 mAb (aCTLA-4) during the oral exposure protocol. Total serum IgE levels were measured by ELISA after 4 wks of dosing. The data are presented on a log scale as the group mean ± SD of 6 mice per group. *, Significantly different (*$p<0.05$, **$p<0.01$) from corresponding non-anti-CTLA-4-treated group.

Anti-CTLA-4 treatment increases Ara h-specific IgE serum levels in sensitized mice

The remarkable differences between PE-specific IgE and total IgE levels following CTLA-4 blockade warranted further evaluation of antigen-specific IgE responses.

Peanut extract consists of different peanut proteins, and we have earlier demonstrated that it is possible to determine specific serum IgE antibodies against individual proteins from the extract [16]. Several peanut proteins are recognized by serum IgE of peanut allergic patients and these allergens are referred to as *Ara h 1-7* [17]. In the current study we used the purified allergens *Ara h 1*, *Ara h 3*, and the minor allergen *Ara h 6* to measure allergen-specific serum IgE responses upon oral PE exposure. Treatment with PE + CT for 4 wks induced low levels of allergen-specific IgE (Fig. 3). Blockade of CTLA-4 in this group resulted in a profound increase of allergen-specific IgE levels with the highest responses against *Ara h 1*, followed by *Ara h 3* and *Ara h 6*.

No allergen-specific IgE was found after oral exposure to PE alone. Treatment with anti-CTLA-4 mAb during PE exposure in the absence of adjuvant did not provoke IgE responses against *Ara h 1*, *Ara h 3,* or *Ara h 6*, confirming that CTLA-4 blockade does not overcome the IgE unresponsiveness to peanut allergens in mice that are orally exposed to PE without an adjuvant.

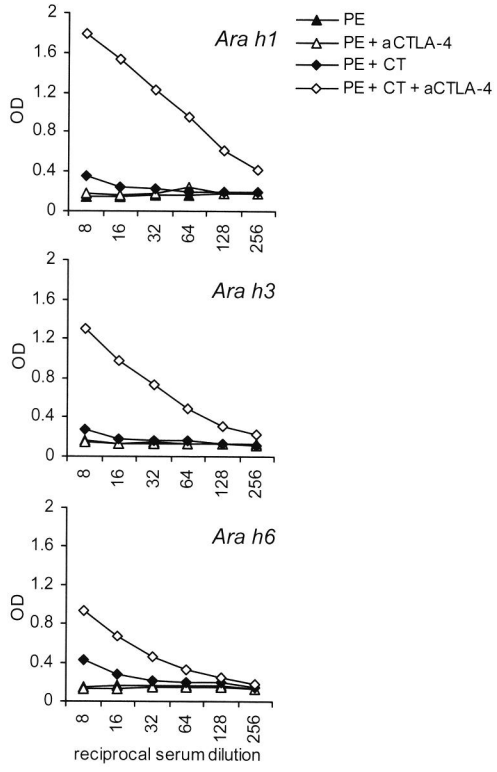

Figure 3. Effect of CTLA-4 blockade on allergen-specific IgE antibody responses. Serum IgE antibody levels against the purified peanut allergens *Ara h 1*, *Ara h 3*, and *Ara h 6* were determined after 4 wks of oral PE exposure in the presence or absence of CT. Indicated groups received blocking anti-CTLA-4 mAb (aCTLA-4) during the oral exposure protocol. *Ara h*-specific IgE antibody levels were measured in pooled sera (6 mice per group) using a sandwich ELISA.

Anti-CTLA-4 treatment enhances mast cell degranulation upon oral challenge with PE

Clinical symptoms in peanut allergic patients are caused by mast cell mediators that are released upon ingestion of peanut. In the current murine model, mast cell degranulation upon an oral challenge with PE was determined by measuring serum levels of the mast cell mediator mmcp-1 before and after challenge. Treatment with PE + CT induced a significant elevation of mmcp-1 serum levels after challenge compared with control and PE-treated mice (Fig. 4).

Anti-CTLA-4 treatment induced higher base levels of serum mmcp-1 before oral challenge compared with levels in non-anti-CTLA-4-exposed animals. Blockade of CTLA-4 only induced an increase in mmcp-1 serum concentration after oral challenge in the PE + CT treatment group, with mmcp-1 levels being four times higher than in the PE + CT treatment group. These results demonstrate that the challenge response to PE is exacerbated by anti-CTLA-4 treatment.

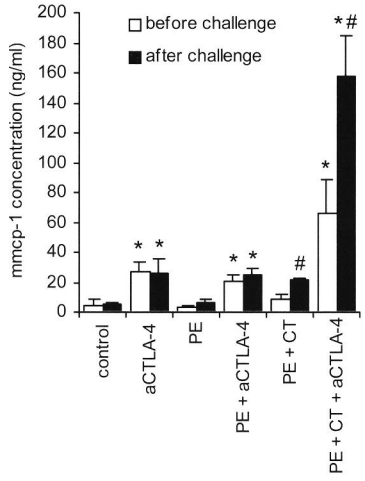

Figure 4. Effect of anti-CTLA-4 treatment on mmcp-1 serum levels upon an oral challenge with PE. Mice were orally exposed to PE with or without CT or were left untreated (control). Indicated groups received blocking anti-CTLA-4 mAb (aCTLA-4) during the oral exposure protocol. Four wks after the initial exposure all groups received an oral challenge with 12 mg PE. Levels of mmcp-1 were determined by ELISA in blood samples collected before and 1 h after the oral challenge. Levels are expressed as the group mean ± SD of 6 mice per group. *, Significantly different ($p<0.05$) from corresponding non-anti-CTLA-4-treated group. #, Significant difference ($p<0.05$) between levels before and 1 h after oral challenge.

Enhancement of Th2-related cytokine production upon anti-CTLA-4 treatment

The cytokine environment plays an important role in the development of sensitization to food proteins, including the production of antigen-specific antibodies. Therefore, the effect of CTLA-4 blockade on the production of IL-4, IL-5, IL-10, and IFN-γ was investigated, 4 wks after the onset of exposure to PE by culturing single-cell suspensions in the presence or absence of PE. Treatment with anti-CTLA-4 significantly enhanced Th2-associated (IL-4 and IL-5) and Tregulatory (Treg)-related (IL-10) cytokine production in both MLN and spleen independent of PE or CT administration (Fig. 5). Cells cultured in the presence or absence of PE produced comparable amounts of IL-4, IL-5, and IL-10 indicating that the stimulation of cytokine production was polyclonal. This implies that a Th2 cytokine environment is not the key factor in sensitization to PE.

Levels of the Th1-associated cytokine IFN-γ were also elevated in the anti-CTLA-4-treated groups (Fig. 5). However, co-administration of the adjuvant CT significantly elevated IFN-γ levels compared with levels in the non-CT-exposed groups. Furthermore, dosing with PE + CT induced antigen-specific IFN-γ illustrated by significantly higher levels in cultures restimulated with PE.

Since development of sensitization to food proteins occurs already in an early phase of exposure, we additionally examined the effect of CTLA-4 blockade early (7 days after the primary exposure to PE) in the response. Also in an early stage anti-CTLA-4 treatment strongly induced IL-5, IL-10, and IFN-γ cytokine production in cultures of both spleen and MLN compared to non-mAb injected groups (data not shown).

Conclusively, CTLA-4 blockade induces non-PE-specific IL-4, IL-5, and IL-10 production in both spleen and MLN, whereas the adjuvant CT seems to be the most important factor for the induction of IFN-γ production.

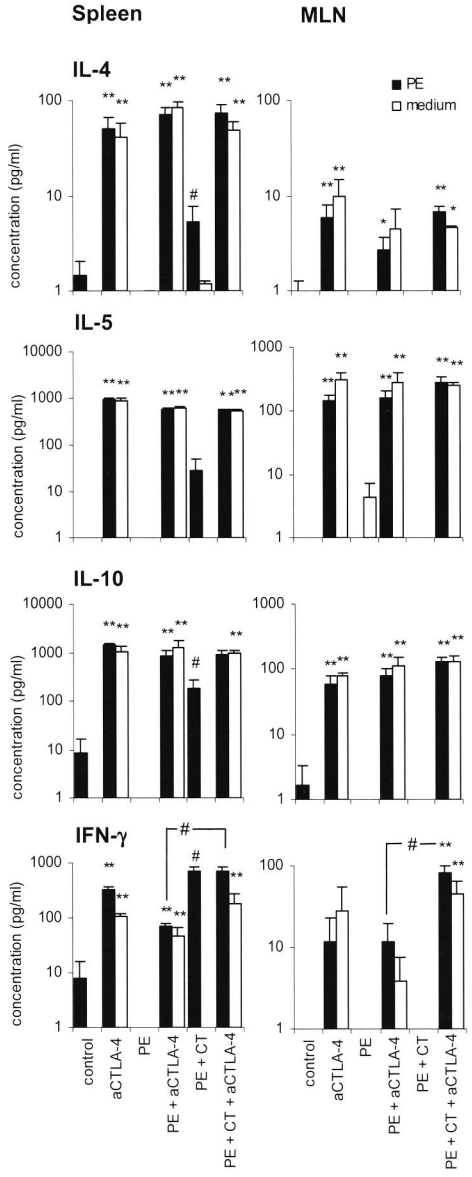

Figure 5. Effect of CTLA-4 blockade on cytokine levels in splenocyte and MLN cell culture supernatants. Mice were orally exposed to PE with or without CT or were left untreated (control). Indicated groups received blocking anti-CTLA-4 mAb (aCTLA-4) during the oral exposure protocol. Spleen and MLN cells were removed 4 wks after the initial oral exposure and were cultured ($2.5x10^6$ cells/ml) for 96 h in the presence or absence of PE (200 μg/ml). Culture supernatants were collected and cytokine levels of IL-4, IL-5, IL-10, and IFN-γ were determined by ELISA. Levels of cytokines induced by restimulation with an irrelevant protein BSA were similar to levels induced in cultures with medium alone (data not shown). Data are presented on a log scale as the group mean ± SEM of 6 mice per group. *, Significantly different (*$p<0.05$, **$p<0.01$) from the corresponding non-anti-CTLA-4-treated group. #, Significantly different ($p<0.05$) from the corresponding non-CT-treated group.

Discussion

In the current study, the involvement of CTLA-4 signaling in immune responses to oral food allergens was investigated and it was demonstrated that CTLA-4 regulates the intensity of a food allergic response but is not decisive in the induction of sensitization.

Blockade of functional CTLA-4 signaling during oral sensitization to PE + CT resulted in enhanced IgE responses against PE and the peanut allergens *Ara h 1*, *Ara h 3*, and *Ara h 6* leading to a more pronounced mast cell degranulation response upon oral challenge with PE. Disease exacerbation was accompanied by an increased production of Th2-associated cytokines (IL-4, IL-5, and IL-10) and the Th1-associated cytokine IFN-γ in both spleen and MLN.

CTLA-4 signaling thus seems to play a pivotal role in regulating the intensitiy of food allergic responses. Similarly, Hellings and colleagues [13] demonstrated an aggravation of Th2-mediated allergic airway disease during anti-CTLA-4 treatment. Furthermore, in humans, CTLA-4 promoter polymorphism has been linked to total serum IgE levels [18] and bronchial hyperresponsivity [19] in asthmatic patients, meaning CTLA-4 is implicated in regulating the intensity of allergic disease. Several non-mutually exclusive mechanisms can be proposed that may underlie regulation of an allergic response by CTLA-4 signaling. CTLA-4 engagement may have a direct suppressive effect on Th2 cells as shown by Oosterwegel and colleagues [20]. Furthermore, CTLA-4 is also expressed on B cells and CTLA-4 signaling on these cells has been shown to inhibit IL-4-driven isotype switching, thereby possibly preventing allergen-specific IgG1 and IgE production [21]. Thus, in the present study anti-CTLA-4 treatment may have directly affected T and B cell responses, resulting in increased Th2-associated responses against PE allergens and concomitant aggravated food allergic disease. CTLA-4 inhibition may additionally have an indirect effect via $CD4^+CD25^+$ Treg cells that downregulate immune responses in a polyclonal fashion. In the mouse intestine this population of Treg cells has been shown to constitutively expresses CTLA-4 and is able to control intestinal inflammation [22]. Cross-linking of CTLA-4 (resulting in CTLA-4 signaling) can induce TGF-β production by murine CD4+ T cells [23] and the suppressive effect of these Treg cells has been demonstrated to be TGF-β dependent [22]. The disruption of Treg cell function by anti-CTLA-4 treatment may have played an important role in the increased immune responses against peanut allergens that was observed in the present study.

Besides the role of CTLA-4 in regulating the intensity of an allergic response, it was investigated whether abrogation of CTLA-4 signaling also resulted in the induction of active immunity against oral antigen. It has been suggested that CTLA-4 is required for oral tolerance induction. This remains controversial however, since it has been reported that CTLA-4 blockade can abolish oral tolerance induced by oral exposure to OVA [9, 10, 24], while others have reported that CTLA-4 blockade failed to overcome development of oral

tolerance to OVA in a similar model [25]. In the present model, mice orally exposed to peanut without adjuvant do not develop PE-specific IgE and only very low IgG responses. We hypothesized that inhibition of CTLA-4 would abrogate this 'unresponsiveness' to peanut proteins and would induce a productive PE-specific humoral response. However, anti-CTLA-4 treatment failed to induce PE- or *Ara h*-specific IgE antibody responses following PE exposure, suggesting that CTLA-4 signaling is not decisive for the induction of sensitization to food proteins. Accordingly, in contrast to the severity of asthma, no significant association between incidence of asthma or atopy and the CTLA-4 polymorphic loci has been found in humans [18].

Since we demonstrated that an allergic response to food antigen is not initiated by CTLA-4 blockade, the question remains which factors are responsible for the development of sensitization to food proteins. In the present model, co-administration of the mucosal adjuvant CT turned out to be crucial for the induction of sensitization to PE. CT is thought to stimulate Th2-dependent immune responses to a bystander antigen by provoking Th2 cytokine production [26]. This phenomenon was also observed – to a much larger extent – upon anti-CTLA-4 treatment. Thus, our results suggest that excessive Th2 cytokine production per se is insufficient for the development of active immunity against food proteins and challenge the concept of Th2-bias as the key factor for food allergy [27].

For the induction of sensitization, a significant proportion of the intact protein has to be absorbed by the intestine and in food-allergic patients increased protein absorption has been reported. Thus an alternative explanation for the impact of CT may be the influence on (systemic) availability of bystander antigen for the immune system. The most prominent effect of CT in the present experiments was PE-induced IFN-γ production. One of the effects attributed to IFN-γ is the induction of changes in epithelial permeability and intestinal integrity [28]. Additionally, CT itself promotes DC activation [29, 30] and migration [31], which facilitates antigen presentation to the immune system.

Anti-CTLA-4 treatment (with or without PE) induced a profound increase in total serum IgE levels and polyclonal Th2 cytokine production. Blocking CTLA-4/B7 interaction promotes Th2 differentiation [20], which may explain the observed excessive Th2 cytokine production and IgE levels. It has also been shown that CTLA-4 plays a major role in downregulating autoaggressive T cells [32, 33] and that CTLA-4-deficient mice develop a fatal lymphoproliferative auto-immune disorder [8]. Concurring, in the present study, higher levels of IgG antibodies against ssDNA-specific were found in the serum of anti-CTLA-4 treated mice compared to control mice (data not shown) suggesting auto-antibody formation upon anti-CTLA-4 exposure.

The elevated levels of the mast cell mediator mmcp-1 that were observed upon anti-CTLA-4 treatment (in the absence of oral antigen) demonstrate that mast cells are also affected by anti-CTLA-4 treatment. The high production of IL-4 and IL-10 cytokines and serum IgE antibodies in anti-CTLA-4-treated mice may have enhanced mast cell

proliferation and differentiation as described by Lukacs and colleagues [34]. In addition, the CTLA-4 ligands CD80 and CD86 are expressed on mast cells [35]. It is unknown whether ligation of CTLA-4 with these molecules may have a direct suppressive effect on mast cells, but it might be another explanation for the observed mast cell degranulation upon anti-CTLA-4 treatment.

Taken together, these results suggest that in food allergy CTLA-4 signaling is involved in regulating the intensity of the hypersensitivity responses, but is not decisive for induction of sensitization to orally ingested antigen. Accordingly, CTLA-4 may represent a potential target for treatment of food allergic disorders. Furthermore, these findings imply that the initiation of food allergy requires another crucial factor in addition to a Th2 cytokine environment.

Acknowledgements

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Chapter 6

The role of $CD4^+CD25^+$ regulatory T cells in oral tolerance and allergic sensitization to peanut

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Submitted

Abstract

Naturally occurring $CD4^+CD25^+$ regulatory T cells (Tregs) play a critical role in the maintenance of self-tolerance and it has been suggested that these Tregs may also be involved in preventing allergic disease. The precise role of $CD4^+CD25^+$ T cells in the regulation of allergic immune responses to mucosal antigens remains to be elucidated.

In the present study it was investigated whether $CD4^+CD25^+$ T cells are involved in the induction of oral tolerance and whether they play a role in controlling allergic sensitization to food proteins. $CD4^+CD25^+$ T cells were depleted with PC61 monoclonal antibody prior to the induction of low dose oral tolerance to peanut extract (PE). In addition, $CD4^+CD25^+$ T cell depletion was performed during sensitization or before oral challenge, using a C3H/HeOuJ mouse model of allergic sensitization to peanut.

Oral tolerance to PE could not be induced in $CD4^+CD25^+$ T cell-depleted mice. However, $CD4^+CD25^+$ T cell depletion during long-term exposure to PE alone did not result in allergic sensitization. In sensitized mice, anti-CD25 treatment during oral exposure resulted in higher levels of PE-specific IgE and an increase in mast cell degranulation upon an oral challenge. In contrast, anti-CD25 treatment of PE-sensitized mice before oral challenges did not affect the level of mast cell degranulation.

$CD4^+CD25^+$ Tregs are involved in maintaining tolerance to oral antigens and regulate the intensity of a food allergic IgE response. Accordingly, $CD4^+CD25^+$ Tregs may represent a potential tool for the treatment of food allergic disorders.

Introduction

Food allergy has emerged as a major health problem in westernized countries, affecting up to 8% of children under 3 years of age and approximately 2% of the adult population [1] and at present no definitive therapy is available. Revealing the underlying mechanisms of sensitization to food proteins may contribute to the development of new therapeutic strategies.

Normally, oral ingestion of food proteins induces active inhibition of the immune response to these antigens, leading to a state of tolerance. Failure to induce oral tolerance or breakdown of oral tolerance may result in hypersensitivity responses to food antigens [2]. The balance between oral tolerance and active immunity is tightly regulated by different cells of the mucosal immune system and their interactions. Among others, several types of regulatory T cells (Tregs), such as NK T cells, TGF-β-producing Th3 cells, and IL-10-producing Tr1 cells [3] appear to be involved in modulating the immune response to harmless food antigens. Recently, another T cell subset has received attention as a possible mucosal immune regulator i.e. the naturally occurring $CD4^+CD25^+$ T cells, first described by Sakaguchi *et al* [4]. These T cells are characterized by an anergic phenotype, a constitutive high expression of the IL-2 receptor α chain, and the selective expression of the transcription factor forkhead box p3 (Foxp3). Although it was originally suggested that these cells were all thymus-derived, it has become apparent that they can also be generated in the periphery [5, 6]. Their immunosuppressive activity is likely mediated by direct cell-cell contact in a process that is dependent on signaling via CTLA-4, as well as cell-bound TGF-β (reviewed by von Boehmer, 2005) [7]. $CD4^+CD25^+$ Tregs play a critical role in the maintenance of self-tolerance and have been shown to prevent the development of organ-specific autoimmune disease and allograft rejection[8].

Currently, there is considerable interest in whether this subset of Tregs is also involved in prevention of sensitization to allergens or in controlling an established allergic sensitization. It has been demonstrated in mouse models that oral exposure to protein induces activation of functional $CD4^+CD25^+$ T cells in the gut draining lymph nodes [9, 10]. Furthermore, patients with X-linked autoimmunity-allergic dysregulation syndrome, which is caused by a *FOXP3* mutation, often develop atopic dermatitis, elevated serum IgE levels and food allergy [11]. Finally, it has recently been shown by Karlsson and colleagues that children who have outgrown their cow's milk allergy, develop a population of $CD4^+CD25^+$ T cells with regulatory function in the peripheral blood [12]. However, the exact role of $CD4^+CD25^+$ Tregs in controlling mucosal immune responses to food antigens remains to be elucidated.

Of all food hypersensitivities peanut allergy is the most serious in terms of severity and persistence. This prompted us to investigate the role of $CD4^+CD25^+$ T cells in the development of oral tolerance and sensitization to peanut proteins. For this purpose, an oral

tolerance model and an established model of peanut sensitization were used. In this long-term sensitization model, exposure to peanut extract (PE) plus the mucosal adjuvant cholera toxin (CT) induces PE-specific antibody levels and subsequent clinical reactions, whereas exposure to PE alone induces a state of non-responsiveness to peanut [13]. In these two different models, $CD4^+CD25^+$ T cells were depleted using a monoclonal antibody (mAb).

We show here that the induction of oral tolerance to PE is impaired in $CD4^+CD25^+$ T cell-depleted animals but that depletion of $CD4^+CD25^+$ T cells is not sufficient to induce allergic sensitization in animals exposed to PE in the absence of the adjuvant CT. Nevertheless, in sensitized mice, depletion of $CD4^+CD25^+$ T cells resulted in increased allergic responses to PE.

These results suggest that $CD4^+CD25^+$ T cells play an important role in both controlling tolerance induction and regulating the intensity of allergic responses to peanut, but are not exclusively decisive in preventing allergic sensitization.

Methods

Mice and reagents

Five-week-old female specific pathogen-free C3H/HeOuJ Ico mice purchased from Charles River (Lyon, France) were maintained under barrier conditions in filter-topped macrolon cages. Drinking water and peanut-free laboratory food pellets were provided *ad libitum*. The experiments were approved by the animal experiments committee of the faculty of veterinary medicine, Utrecht University.

Peanuts from the Runner cultivar (Cargill, Dawson, GA) were kindly provided by Imko Nut Products (Doetinchem, The Netherlands) and PE was prepared as previously described [13, 14]. Chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands), unless stated otherwise.

In vivo depletion of $CD4^+CD25^+$ T cells

$CD4^+CD25^+$ T cells were depleted *in vivo* by i.p. administration of purified rat anti-CD25 (IL-2Rα) mAb produced by hybridoma PC61 (kindly provided by Dr. G. Leclercq, University of Gent, Belgium). Control mice received a rat anti-mouse IgG antibody (Rockland Immunochemicals, Gilbertsville, PA), according to the same schedule.

It has been shown that PC61 selectively depletes $CD4^+CD25^+$ T cells *in vivo* and that the deletion is short-term and reversible [15, 16]. The efficacy of CD25 depletion in mesenteric lymph nodes (MLN) and spleen was confirmed by FACScan analysis (Becton Dickinson) using FITC-conjugated anti-CD25 mAb (7D4), PE-conjugated anti-CD4 mAb (L3T4) and Cy-conjugated anti-CD3ε mAb (145-2C11), all obtained from BD Pharmingen (Hamburg, Germany). Two days after anti-CD25 treatment (200 µg at day -2 and 0), a 85% reduction of $CD4^+CD25^+$ cells was found in the MLN and a 50% reduction in the spleen. Maximal depletion lasted for at least 7 days These results are consistent with previous observations using the same PC61 mAb [15, 16]. Further flow cytometric analysis confirmed that anti-CD25 treatment had no effect on B cell, cytotoxic T cell, $CD4^+CD25^-$ T cell, or dendritic cell (DC) percentages in both spleen and MLN.

Treatment protocols (see Fig. 1)

CD25 depletion before oral tolerance induction. Mice (n=5) were intragastrically exposed to 1 mg PE (to induce oral tolerance) or PBS on 3 consecutive days (days 1-3). Before oral exposure to PE, mice received an i.p. injection with anti-CD25 mAb, or rat anti-mouse IgG control antibody (RIgG, 200 µg at day -2 and 0). Mice were challenged i.p with 100 µg PE in alum (25 mg/ml) 21 and 28 days after the last oral exposure and mice were sacrificed at day 42.

CD25 depletion during long-term oral exposure. Mice (n=5-6) were orally exposed to PBS, PE alone (6 mg), or PE (6 mg) plus CT. Oral exposure was performed by

intragastric dosing on 3 consecutive days, followed by weekly dosing (days 8, 15, and 22). In the PE plus CT group, CT (10 µg) was coadministered on days 1, 2, 3, 8, 15, and 22 to induce sensitization. Mice were additionally injected i.p. with 200 µg anti-CD25 mAb or RIgG on days -2, 0, and 14 during the oral dosing regime. At day 31, mice received an oral challenge with 25 mg PE (without adjuvant) and were sacrificed 7 or 31 days after the onset of oral exposure.

CD25 depletion before oral challenge. Mice (n=8) were orally exposed to PBS, PE, or PE plus CT for 4 wk as described in the long-term oral exposure protocol. Subsequently, groups were injected i.p. with 200 µg anti-CD25 mAb or RIgG at days 28 and 30. Finally, mice were orally challenged with 30 mg PE at days 31 and 45 and were sacrificed at day 50.

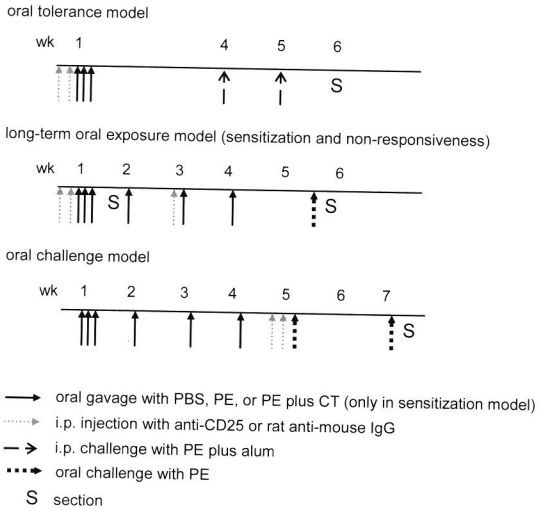

Figure 1. Treatment protocols

Measurement of serum IgG1, IgG2a, and IgE antibody levels

Blood samples were collected at various time points and stored at –20°C until analysis. Levels of PE-specific IgG1 and IgG2a and total IgE antibodies were determined by ELISA and levels of PE-specific IgE by sandwich ELISA as previously described [13]. Briefly, plates (highbond 3590; Costar, Cambridge, MA) were coated overnight with 20 µg/ml PE (for IgG1 and IgG2a detection) or with 1.5 µg/ml purified rat anti-mouse IgE (BD Pharmingen) in carbonate buffer (pH 9.6), followed by 1 h blocking with PBS-Tween/3% milk powder. Each test serum was titrated (at least 8 serial dilutions) starting at 1:8 or 1:16 dilution and incubated for 1 h. For PE-specific antibody levels, a titrated pre-sera pool was used as reference value (start dilution 1:4) and a standard curve of a reference serum (2 µg/ml IgE) was included to determine total serum IgE levels. For detection of IgG1 and IgG2a and total

serum IgE antibodies, alkaline phosphatase-conjugated IgG1, IgG2a (poylclonal goat anti-mouse) and IgE (rat anti-mouse, clone 23G3) antibodies (Southern Biotechnology Associates, Birmingham, AL) were added (1 h at 37°C). Subsequently 1 mg/ml p-nitrophenylphosphate in diethanolamine buffer was used for the color reaction, which was stopped with a 10% EDTA solution and absorbance was measured at 405 nm. To measure PE-specific IgE antibodies, serum was incubated for 2 h and subsequently a PE-digoxigenin (DIG) conjugate solution was added (1 h at 37°C). The coupling of DIG to PE was performed according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). After incubation (1 h at 37°C) with peroxidase-conjugated anti-DIG fragments (Roche Diagnostics, Mannheim, Germany), a tetramethylbenzidine substrate (0.1 mg/ml) solution was added and the color reaction was stopped with 2 M H_2SO_4. Absorbance was measured at 450 nm. The reciprocal of the highest test serum dilution resulting in an extinction higher than the reference value was read as a titer.

Cell culture and cytokine measurements

Spleen and MLN single cell suspensions ($3.75x10^5$ cells in 200 µl complete RPMI 1640 (Life Technologies, Paisley, Scotland) containing 10% FCS (ICN Pharmaceuticals, Costa Mesa, CA)) were incubated in the presence or absence of 200 µg/ml PE in 96-well plates for 96 h at 37°C, 5% CO_2. In the culture supernatants, levels of IFN-γ, IL-4, IL-5, IL-10, and IL-13 were determined by sandwich ELISA (sensitivity for IL-4 and IL-5; 0.5 pg/ml-250 pg/ml and for IFN-γ, IL-10, and IL-13; 4 pg/ml-1000 pg/ml). IFN-γ, IL-4, and IL-5 capture and biotin-conjugated antibodies were obtained from BD Pharmingen and the ELISAs were performed as previously described [13]. The IL-10 (BD Pharmingen) and IL-13 ELISA (Biosource, Camarillo, CA) were performed in accordance with the manufacturer's instructions.

Measurement of serum mouse mast cell protease-1 (mmcp-1)

Blood was collected before and 45 min after oral challenge with PE and serum levels of mmcp-1 were determined using an ELISA kit (Moredun, Scotland). The ELISA was performed according to the manufacturer's instructions.

Statistics

Data were analyzed using SigmaStat statistical software package (SPSS, Chicago, IL). The differences between group means were determined by using one-way ANOVA with Bonferroni as *post hoc* test. For cytokine levels and mmcp-1 serum levels, statistical analysis was performed following logarithmic transformation (to achieve normal distribution). A value of $p<0.05$ was considered statistically significant.

Results

Oral tolerance induction to PE is impaired in the absence of $CD4^+CD25^+$ T cells

In the low dose oral tolerance model, mice were exposed to 1 mg PE or PBS for 3 consecutive days. After 21 days, mice were immunized i.p. with PE plus alum twice within a 1-week interval and sacrificed two weeks later. In this model, oral pre-treatment with PE induces significantly lower levels of Th2-associated cytokines IL-4, IL-5, and IL-13 upon *in vitro* restimulation of splenocytes and lower serum levels of PE-specific IgG1 compared to the PBS-exposed controls. To determine whether oral tolerance to PE can be established in the absence of $CD4^+CD25^+$ T cells, $CD4^+CD25^+$ T cells were depleted before oral exposure to PE. In the anti-CD25-treated group, cytokine (Fig. 2A) and PE-specific IgG1 levels (Fig. 2B) were significantly higher than levels found in the PE-exposed, control antibody-treated group and comparable to those found in PBS-dosed control mice. These results show that suppression of systemic Th2-associated PE-specific responses, achieved by oral dosing of PE, cannot be established after $CD4^+CD25^+$ T cell depletion.

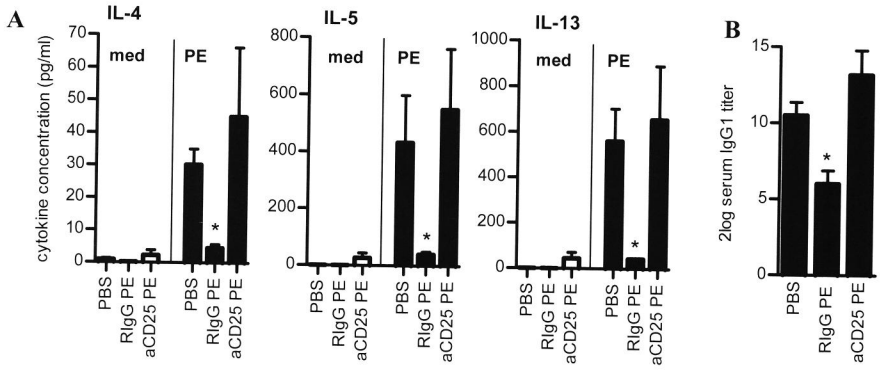

Figure 2. Effect of anti-CD25 treatment on the induction of oral tolerance to PE. (A) Cytokine levels in splenocyte culture supernatants. Cell suspensions were cultured in the presence (black bars) or absence (white bars) of PE. Data are presented as the group mean ± SEM of 5 mice per group. (B) PE-specific serum IgG1 antibodies. The data are presented as the mean ^{2}log antibody titer ± SEM of 5 mice per group.*, Significantly different ($p<0.05$) from the PBS-exposed group. Data are representative of two independent experiments.

Depletion of $CD4^+CD25^+$ T cells enhances PE-specific antibody levels, cytokine levels and challenge response in sensitized mice, but does not abrogate non-responsiveness to PE

Since our data indicate that induction of oral tolerance is impaired in the absence of $CD4^+CD25^+$ T cells, we next investigated whether $CD4^+CD25^+$ T cell depletion results in allergic sensitization to orally administered proteins. To address this question we used repeated oral exposure to PE without adjuvant, which normally induces non-responsiveness. In addition, the effect of $CD4^+CD25^+$ T cell depletion during an ongoing peanut allergic response (oral exposure to PE plus CT) was investigated. Hence, mice were orally exposed for 4 weeks to PBS, PE alone, or PE plus CT and groups were treated with anti-CD25 depleting mAb or isotype control during the oral dosing regime.

Groups of mice were sacrificed 7 days after the first exposure in order to determine early cytokine responses. Splenic single cell suspensions were cultured in the presence or absence of PE and cytokine production was measured in the supernatant. In agreement with earlier studies, only PE plus CT exposure resulted in significantly increased IL-4, IL-13, IL-10, and IFN-γ cytokine levels upon restimulation with PE (Fig. 3).

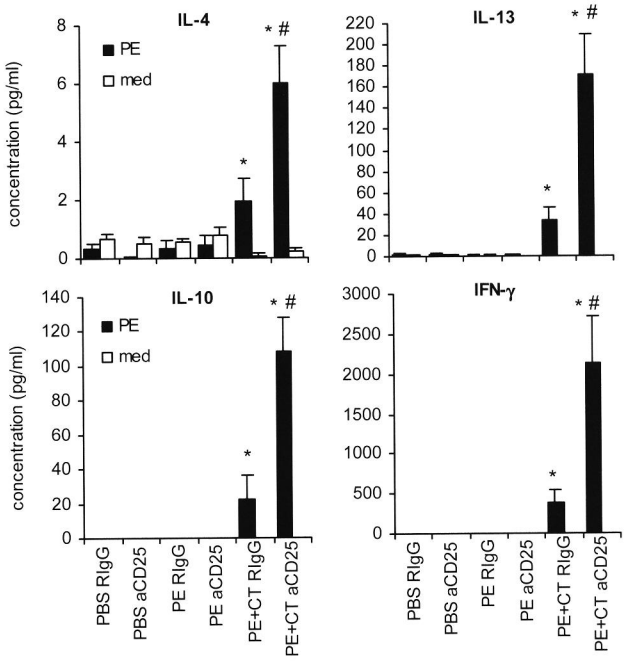

Figure 3. Effect of anti-CD25 treatment on cytokine levels in splenocyte culture supernatants. Spleens were removed 7 days after the initial oral exposure and cells were cultured in the presence (black bars) or absence (white bars) of PE. Data (representative of two independent experiments) are presented as the group mean ± SEM of 5 mice per group. #, Significantly different ($p<0.05$) from the corresponding control antibody (RIgG)-treated group. *, Significantly different ($p<0.05$) from the corresponding non-CT-treated group.

CD25 depletion before oral exposure to PE plus CT resulted in a further increase in cytokine production. This elevation appeared to be PE-specific since no cytokine production was observed in cultures with medium alone. Cell cultures derived from PBS or PE-exposed groups showed no cytokine production and this was not altered by anti-CD25 treatment.

Accordingly, mice exposed to PE plus CT for 4 wk developed PE-specific IgG1, IgG2a, and IgE serum antibodies (Fig. 4A). In this group, depletion of $CD4^+CD25^+$ T cells during sensitization resulted in significantly higher levels of both Th2-associated (IgG1 and IgE) and Th1-associated (IgG2a) antibodies.

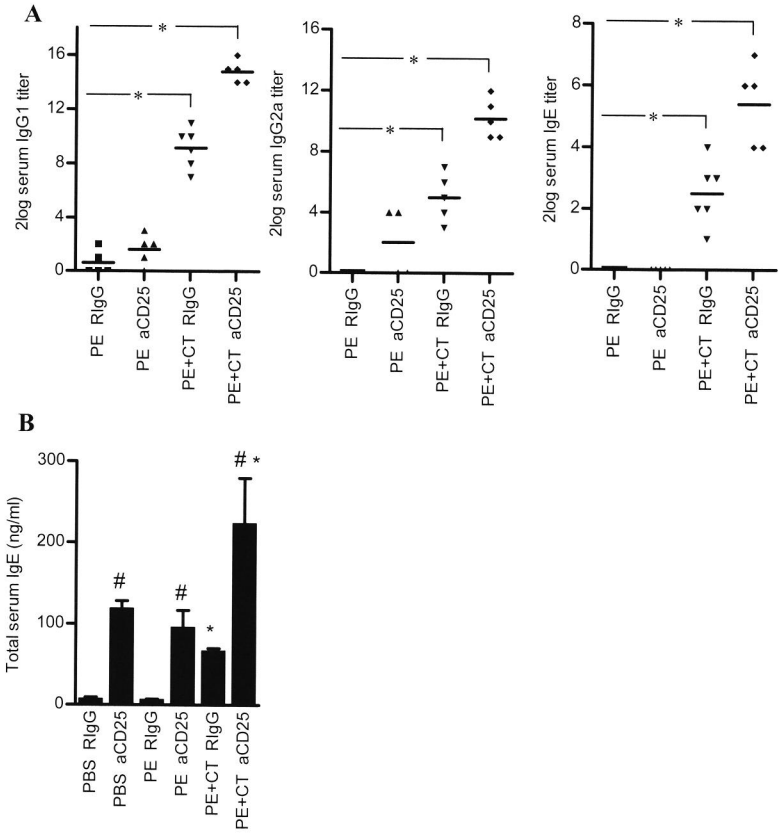

Figure 4. Effect of anti-CD25 treatment on antibody production. (A) PE-specific Th1-associated (IgG2a) and Th2-associated (IgG1 and IgE) serum antibody levels. The data are presented as the mean ^{2}log antibody titer of 5-6 mice per group (symbols indicate individual animals). *, Significantly different ($p<0.05$) from groups covered by the horizontal line. (B) Total IgE serum levels. The data (representative of two independent experiments) are presented as the group mean ± SEM of 5 mice per group. #, Significantly different ($p<0.01$) from the corresponding control antibody (RIgG)-treated group. *, Significantly different ($p<0.05$) from the corresponding non-CT-exposed group.

Mice exposed to PE alone showed low levels of PE-specific IgG1 and IgG2a and no IgE. In this exposure group, anti-CD25 treatment had no effect on the antibody levels confirming that the non-responsiveness to peanut induced by oral exposure to PE without adjuvant is not abrogated after $CD4^{+}CD25^{+}$ T cell depletion. Interestingly, in contrast to PE-specific IgE levels, anti-CD25 treatment clearly augmented total IgE production in all exposure groups (Fig. 4B).

Anti-CD25 treatment also increased the production of IL-4, IL-13, and IFN-γ in spleen cultures, irrespective of the oral exposure (PBS, PE, or PE plus CT) and culture conditions (with or without PE restimulation) (Fig 5). However, cytokine levels in PE-restimulated cultures from PE plus CT-exposed mice were markedly elevated when compared to cultures from PBS- or PE-exposed mice. In RIgG-treated groups significant cytokine production was only found in PE-restimulated spleen cultures of the PE plus CT-exposed group.

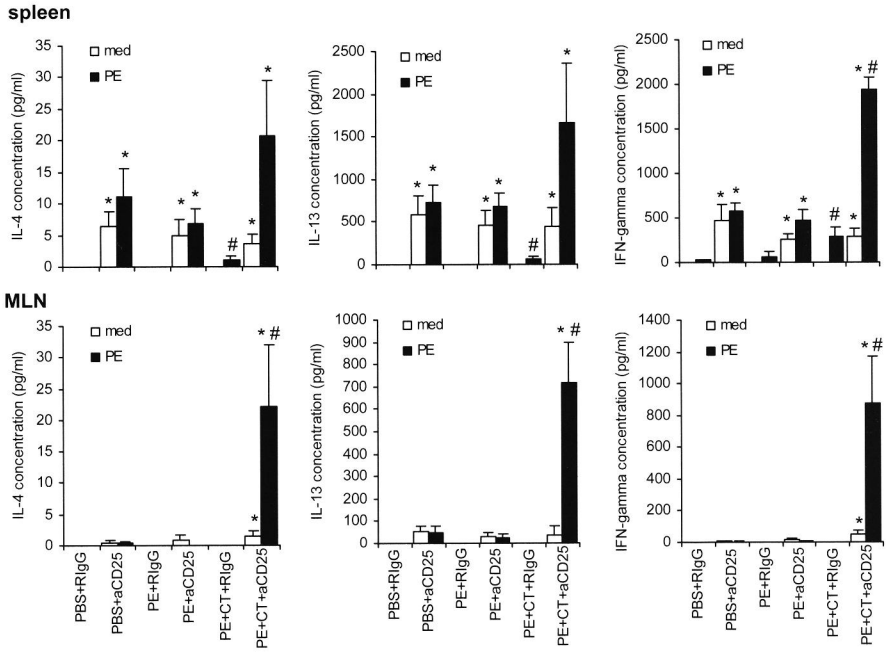

Figure 5. Cytokine levels in the spleen and MLN, 4 wk after the initial oral exposure. Mice were orally exposed to PBS, or PE with or without CT. Indicated groups received depleting anti-CD25 mAb or RIgG during the oral exposure protocol. Spleen and MLN single cell suspensions were cultured for 96 h in the presence (black bars) or absence (white bars) of PE. Data are presented as the group mean ± SEM of 5-6 mice per group. *, Significantly different ($p<0.05$) from the corresponding RIgG-treated group. #, Significantly different ($p<0.05$) from the corresponding non-CT-treated group.

In MLN cell cultures of PBS- or PE-exposed mice, anti-CD25 treatment only slightly increased cytokine production compared to RIgG-treatment, irrespective of antigen restimulation. In contrast, cytokine levels in MLN cultures from PE plus CT-exposed mice were significantly increased when cells were restimulated with PE.

After the 4 week oral exposure regime, all mice received an oral challenge with PE and the serum concentration of mmcp-1 45 minutes after challenge was used as a read-out for the challenge response. In accordance with PE-specific IgE levels, mast cell degranulation upon an oral challenge was only found in the PE plus CT-exposed mice (Fig. 6). In this group, treatment with anti-CD25 during sensitization resulted in a higher levels of mmcp-1 than treatment with the isotype control. Exposure to PE alone induced no significant mast cell degranulation compared to the control group, irrespective of anti-CD25 treatment.

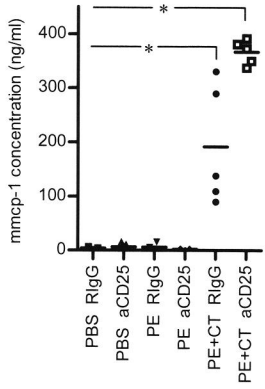

Figure 6. Mmcp-1 serum levels upon an oral challenge with PE. Levels of mmcp-1 were determined in blood samples collected 45 min after the oral challenge. The data (representative of two independent experiments) are presented as the group mean (symbols indicate individual animals) of 5-6 mice per group. *, Significantly different ($p<0.05$) from groups covered by the horizontal line.

Depletion of $CD4^+CD25^+$ T cells before oral challenge does not result in an exacerbated challenge response

It has been hypothesized that Tregs may directly or indirectly affect effector cells such as mast cells and basophils [17, 18]. Therefore, it was examined whether depletion of $CD4^+CD25^+$ T cells before oral challenges would also affect the challenge response. For this purpose mice were orally exposed for 4 weeks to PBS, or PE with or without CT. Subsequently, mice were treated with anti-CD25 mAb or isotype control and orally challenged with PE, 1 day and 2 weeks after the last antibody treatment. Only mice exposed to PE plus CT showed a significant increase in mmcp-1 serum levels after the first and second oral challenge (Fig. 7). Anti-CD25 treatment before the oral challenges did not affect the extent of mast cell degranulation in any of the exposure groups suggesting that $CD4^+CD25^+$ T cells are not directly involved in controlling the challenge response to peanut.

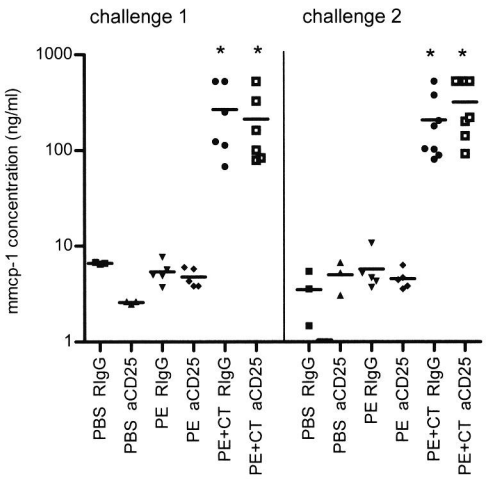

Figure 7. Mmcp-1 serum levels upon oral challenges with PE 1 day and 2 weeks after $CD4^+CD25^+$ T cell depletion. Levels of mmcp-1 were determined in blood samples collected 45 min after the oral challenge. The data are presented on a log scale as the group mean (symbols indicate individual animals) of 5-8 mice per group. *, Significantly different ($p<0.01$) from all non-CT-exposed group.

Discussion

$CD4^+CD25^+$ T cells have been described to be important in maintaining self-tolerance [19] but they also appear to play a role in the induction of tolerance to exogenous antigens [4]. It has been shown that oral exposure to ovalbumin (OVA) in OVA TCR transgenic mice induces proliferation of $CD4^+CD25^+$ T cells that are able to suppress proliferation of $CD4^+CD25^-$ T cells *in vitro* [9, 10] and to transfer tolerance to wildtype mice [9, 20]. However, it remains to be elucidated whether $CD4^+CD25^+$ T cells are involved in controlling tolerance induction to orally administered antigen in non-transgenic mice. In the present study we have demonstrated that $CD4^+CD25^+$ T cells are crucial for the induction of oral tolerance to PE. In $CD4^+CD25^+$ T cell-depleted animals, PE-specific IgG1 antibodies and PE-induced cytokine production were no longer suppressed by oral pre-treatment with a low dose of PE. In line with our results, $CD4^+CD25^+$ T cells were recently shown to be involved in the induction of low dose oral tolerance to OVA [21].

While it is clear that Th2 responses play a pivotal role in the development of allergic responses it remains unclear which mechanisms are involved in controlling and/or preventing allergic sensitization. Failure to induce or breakdown of oral tolerance may be one of the processes that precede the induction of allergic sensitization. Therefore, we additionally examined the role of $CD4^+CD25^+$ T cells in the regulation of allergic sensitization in an established model of peanut hypersensitivity [13]. In the present study we demonstrate that deletion of $CD4^+CD25^+$ T cells during allergic sensitization to peanut (exposure to PE in the presence of the adjuvant CT) results in increased PE-induced cytokine production, PE-specific IgE levels and challenge responses. These data indicate that $CD4^+CD25^+$ T cells are involved in regulating the intensity of allergic sensitization to dietary peanut. In support of a role for $CD4^+CD25^+$ T cells in controlling Th2 responses it has been shown that infusion of mouse $CD4^+CD25^+$ T cells prevents high serum IgE levels in transgenic mice [22] and that $CD4^+CD25^+$ T cells suppress the *in vitro* differentiation of Th2 cells [23]. In accordance, it has been found that $CD25^+$ T cells control allergen-specific T cell responses in healthy individuals [24, 25]. Furthermore, Karlsson and colleagues have recently demonstrated that when allergy to cow's milk is outgrown, this is associated with the development of $CD4^+CD25^+$ T cells that are capable of suppressing allergen-specific effector cells [12]. Conclusively, $CD4^+CD25^+$ T cells seem to play an important role in controlling the intensity of allergic sensitization to peanut proteins.

Data presented in the current manuscript additionally revealed that mast cell degranulation upon oral challenge was not affected when $CD4^+CD25^+$ T cells were depleted before oral challenge, whereas depletion during sensitization did enhance the challenge response. Hence, $CD4^+CD25^+$ T cells appear to be mainly involved in the induction phase of an allergic response and do not play a major role in the challenge response, when sensitization has already been established.

There has been some debate on the mechanism of suppression by $CD4^+CD25^+$ T cells. *In vitro* studies have shown that $CD4^+CD25^+$ T cells exert their regulatory activity on other T cells via a cell-contact dependent mechanism mediated by either CTLA-4 signaling[26] or membrane-bound TGF-β [27]. *In vivo* studies have additionally shown that certain forms of immunity can be suppressed by IL-10 secreting $CD4^+CD25^+$ T cells [28, 29]. Our unpublished data suggest that in the present sensitization model, neutralization of TGF-β and/or IL-10 does not have a major influence on PE-induced immune responses. However, in a previous study we have shown that blockade of the CTLA-4 signaling pathway results in enhanced allergic sensitization to peanut [13]. Hence, in the present model, the suppressive function of $CD4^+CD25^+$ T cells may be mainly mediated by CTLA-4-dependent interaction of these Tregs with other T cells or antigen presenting cells (APC). It is likely however that *in vivo* $CD4^+CD25^+$ T cells suppress immunity by several distinct mechanisms that may depend on the microenvironment in which these cells are activated and further research is needed to delineate the mechanisms of $CD4^+CD25^+$ T cell-mediated suppression upon oral antigen administration.

Interestingly, although $CD4^+CD25^+$ T cells appear essential for the induction of oral tolerance to PE, our data show that depletion of $CD4^+CD25^+$ T cells is not sufficient to induce allergic sensitization after long-term exposure to PE without adjuvant. Data on the early phase of sensitization (day 7) demonstrated that anti-CD25 treatment increased cytokine production solely in sensitized mice and also IgE production and challenge responses were only affected in sensitized mice. These results suggest that in the absence of $CD4^+CD25^+$ T cells, an additional stimulus (such as the adjuvant activity of CT) is required to induce allergic sensitization to an orally administered protein. We speculate that in the absence of $CD4^+CD25^+$ T cells, other local regulatory factors in the gut such as specialized subsets of DCs and other subsets of Tregs create a suppressive environment, which prevents the development and function of effector T cells and subsequent allergic sensitization to oral antigens. The local suppression in the gut of orally induced responses may then coexist with impaired peripheral tolerance to the same antigen. This is confirmed by the observation that despite the failure to induce PE-specific IgE levels, total IgE levels were increased in PE plus anti-CD25-treated mice compared to PE-treated mice, which proposes a more extensive regulation of immune responses to orally administered antigens than to systemic (presumably self-) antigens. In line with these results, humans that have a mutation in the *FOXP3* gene do not always develop food allergy [30], which supports the necessity for an additional trigger for the induction of sensitization.

In conclusion, the data presented in this manuscript provide new insights into the role of $CD4^+CD25^+$ T cells in the regulation of immune responses to orally administered proteins. The results suggest that $CD4^+CD25^+$ T cells are crucial for the induction of tolerance to peanut proteins. Furthermore, although $CD4^+CD25^+$ T cells appear not to be exclusively decisive in the prevention of allergic sensitization, they are involved in

regulating hypersensitivity responses to peanut especially in an early stage of sensitization. Accordingly $CD4^+CD25^+$ T cells may represent a potential tool for the treatment of food allergic disorders or even for the re-establishment of tolerance.

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Chapter 7

The role of plasmacytoid dendritic cells in sensitization and tolerance induction to orally administered peanut proteins

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Submitted

Abstract

Dendritic cells (DCs) are thought to play a key role in controling both tolerance and immunity and an emerging concept is that different types of DCs may have unique functions in regulating this balance between tolerance and immunity. Plasmacytoid DCs (pDCs) represent a unique subset of DCs associated with tolerogenic capacity and the induction of regulatory T cells. Furthermore, they have been implicated in preventing immune responses to harmless inhaled antigens. The involvement of pDCs in responses to orally administered antigens remains to be elucidated. Unraveling the mechanisms underlying the induction of oral tolerance versus sensitization may contribute to the development of new therapeutic strategies for severe food allergies such as peanut allergy. In the present study we have investigated the role of pDCs in the induction of oral tolerance and sensitization to peanut. pDCs were depleted *in vivo* using the 120G8 monoclonal antibody before low dose oral tolerance induction and during allergic sensitization to peanut extract (PE).

pDC depletion induced non-specific cytokine production in the spleen and increased levels of total IgG2a and mainly IgG1 and IgE antibodies. However, the induction of oral tolerance was not impaired in pDC-depleted mice. Additionally, pDC depletion did not affect allergic sensitization and challenge responses to peanut.

The presented data suggest that pDCs do not play a crucial role in the induction of oral tolerance and in the regulation of allergic responses to food proteins. Under non-inflammatory conditions pDCs seem to be mainly involved in maintaining immune homeostasis.

Introduction

The intestine encounters more antigen than any part of the body and the gut immune system faces the challenging task to discriminate between potential harmful and harmless antigens, such as food proteins. The default immune response to orally administered proteins is the induction of oral tolerance, a state of systemic antigen-specific hyporesponsiveness [1]. A failure of oral tolerance induction or a breakdown of its maintenance may induce hypersensitivity responses to food proteins leading to food allergic disease [2]. IgE-mediated reactions are the basis of most allergic responses to food and can be divided in a sensitization phase where, due to the presence of Th2 cytokines, IgE responses to the food are developed and an elicitation phase where, upon ingestion of the offending food, cross-linking of IgE antibodies on mast cells induces the release of mediators such as histamine leading to clinical allergic reactions. Of all food allergies, peanut allergy is the most serious due to its persistence and high risk of severe anaphylaxis. To date no treatment is available for food-allergic patients and a better understanding of the mechanisms underlying allergic sensitization to food antigens may contribute to the development of therapeutic strategies.

Dendritic cells (DCs) are now recognized to play a central role in the generation of immune responses in the intestine and they have been shown to contribute to both tolerogenic and immunogenic responses to mucosally derived antigens [3, 4]. Intestinal DCs constitutively migrate from the lamina propria (LP) and/or Peyer's Patches (PPs) to T cell zones in the PPs or mesenteric lymph nodes (MLNs) [5]. In the presence of inflammatory stimuli DCs undergo a maturation process leading to the upregulation of MHC class II and costimulatory molecules, secretion of proinflammatory cytokines and activation of naïve T cells [6]. Under steady-state conditions however, DCs possess an immature phenotype and induce tolerance by the induction of antigen-specific T cell anergy and the generation of regulatory T cells [7]. In addition, certain DC subpopulations such as the $CD11c^{+}CD8\alpha^{+}$ subtype and the recently identified plasmacytoid DC (pDC) have been suggested to be specialized in tolerance induction [8, 9]. pDCs represent a specialized DC population with an immature phenotype that produces high levels of IFN-α in response to viral stimulation. Human and mouse pDCs share most of their morphological, phenotypic and functional features and can be distinguished from other DC subtypes by the expression of several pDC-restricted surface antigens (reviewed in Colonna *et al.* [10]). pDCs, particularly in their immature state, or in certain anatomic localizations such as the MLN [11], are believed to exert tolerogenic functions via the induction of anergic regulatory T cells [12]. Intriguingly, de Heer and colleagues have recently demonstrated that depletion of pDCs in mice during inhalation of normally inert OVA leads to the development of allergic asthma, whereas adoptive transfer of OVA-pulsed pDCs before sensitization prevented asthma induction [13]. These results suggest that pDCs play an important role in maintaining tolerance and preventing allergic sensitization to inhaled harmless antigens. In the intestine, pDCs are

found in the MLN, PP [11, 14], and LP [15] where they also have been associated with regulatory and homeostatic functions. However, the precise role of mucosal pDCs in responses to oral proteins remains to be elucidated. Regarding the results found in the asthma model we hypothesized that pDCs are involved in inducing and maintaining oral tolerance and preventing sensitization to dietary proteins.

In the present study we investigated the involvement of pDCs in the induction of oral tolerance and development of sensitization to orally administered peanut proteins. For this purpose pDCs were depleted using the 120G8 monoclonal antibody (mAb) in both an oral tolerance model and an established model of peanut hypersensitivity. We show here that although *in vivo* pDC depletion leads to a disturbed immune homeostasis, it does not affect oral tolerance induction and allergic sensitization to peanut antigens.

Methods

Mice

Female specific pathogen-free C3H/HeOuJ Ico mice (4-5 wk of age) purchased from Charles River (Lyon, France), were maintained under barrier conditions in filter-topped macrolon cages. Drinking water and peanut-free laboratory food pellets were provided *ad libitum*. The experiments were approved by the animal experiments committee of the faculty of Veterinary medicine, Utrecht University.

Peanuts and chemicals

Peanuts from the Runner cultivar (Cargill, Dawson, GA) were kindly provided by Imko Nut Products (Doetinchem, The Netherlands) and PE was prepared as previously described [16, 17]. The extract contained 30 mg/ml protein as determined by Bradford analysis (Pierce, Rockford, IL) with BSA as a standard.

Chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands), unless stated otherwise.

In vivo plasmacytoid dendritic cell depletion and flow cytometric analysis

For the depletion of pDCs the 120G8 rat anti-mouse mAb, which has been shown to selectively deplete pDCs *in vivo* [14], was used. The cell line producing 120G8 was kindly provided by C. Asselin-Paturel, Schering-Plough, Dardilly, France. Purified rat anti-mouse IgG antibody (Rockland Immunochemicals, Gilbertsville, PA) was used as an isotype control in all studies. To investigate efficiency of pDC depletion, flow cytometric analysis was performed. All staining reactions were performed at 4°C and all antibodies were obtained from BD Pharmingen (Hamburg, Germany). Briefly, spleen and MLN single cell suspensions were incubated with CD16/CD32 Fc block (2.4G2) to reduce non-specific binding and after washing cells were stained with anti-B220-FITC (RA3-6B2), anti-Ly6C(Gr-1)-PE (RB6-8C5), anti-CD11c-APC (HL3), anti-120G8-biotin, or MHCII-biotin (NIMR-4) followed by Streptavidin-PerCp incubation.

Anti-pDC treatment (2 or 3 i.p. injections on consecutive days) resulted in a 50% reduction of $CD11c^{intermediate}B220^{high}Ly6C^{high}120G8^{high}$ cells (formerly defined as mouse pDC) in both spleen and MLN without affecting $CD11c^{hi}B220^{low}$ cells (conventional DCs), T cells ($CD3\varepsilon^{+}$), or B cells ($CD19^{+}$). No difference in depletion was found following 50 or 100 μg 120G8 exposure. Depletion was maximal 24 hrs after the last anti-pDC treatment and 7 days after the last anti-pDC injection pDC percentages were back at control levels.

Oral treatment protocols (see Fig. 1)

Oral tolerance induction to PE. Mice (n=6) were intragastrically exposed to 1 mg PE (tolerance induction) or PBS on 3 consecutive days (day 1-3). Before oral exposures, mice received an i.p. injection with anti-pDC or rat anti-mouse IgG control antibody (both 100 µg) on days -2, -1, and 0. Mice were challenged i.p with 100 µg PE in alum (25 mg/ml), 14 and 21 days after the last oral exposure and mice were sacrificed on day 35.

Long-term oral exposure to PE. Mice (n= 4-6) were orally exposed to PBS, PE, or PE plus cholera toxin (CT). Oral exposure was performed by intragastric dosing of 6 mg PE on 3 consecutive days (days 1-3), followed by weekly dosing (days 8, 15, 22, and 29). In the PE plus CT group, 10 µg CT (List Biological Laboraties, CA) was coadminstered on days 1, 2, 3, 8, 15, 22, and 29 to induce sensitization [17]. During oral exposure, mice were injected i.p. with anti-pDC or rat anti-mouse IgG control antibody (both 100 µg) on days -2, -1, 0, 6, 7, 13, and 14. At day 38, mice received a double oral challenge with 30 mg PE. Mice were sacrificed on day 7, or day 39.

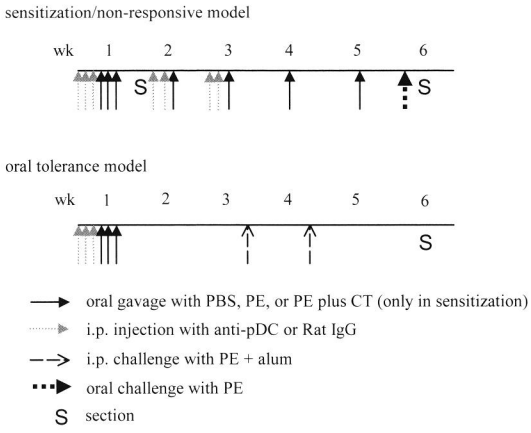

Figure 1. Schematic overview of the oral exposure models.

Measurement of PE-specific serum IgG1, IgG2a, and IgE antibody levels

Blood samples were collected at various time points and stored at –20°C until analysis. Levels of PE-specific antibodies were determined by ELISA (IgG1 and IgG2a) or sandwich ELISA (IgE) as previously described [17]. Briefly, Plates (highbond 3590; Costar, Cambridge, MA) were coated overnight with 20 µg/ml PE (for IgG1 and IgG2a detection), or with 1.5 µg/ml purified rat anti-mouse IgE (clone R35-72, BD Pharmingen) in carbonate buffer (pH 9.6), followed by 1 h blocking with PBS-Tween/3% milk powder. Each test serum was titrated starting at 1:8 or 1:16 dilution and incubated for 1 h. A pre-sera pool was used as reference value (dilution 1:4). For detection of IgG1 and IgG2a, polyclonal goat anti mouse alkaline phosphatase (AP)-conjugated antibodies (Southern Biotechnology Associates, Birmingham, AL) were added (1 h at room temperature). Subsequently 1 mg/ml p-

nitrophenylphosphate in diethanolamine buffer was used for the color reaction, which was stopped with a 10% EDTA solution and absorbance was measured at 405 nm. To measure antigen-specific IgE antibodies, serum was incubated for 2 h and subsequently a PE-digoxigenin (DIG) conjugate solution was added (1 h at room temperature). The coupling of DIG to PE was performed according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). After incubation (1 h at room temperature) with peroxidase-conjugated anti-DIG fragments (Roche Diagnostics, Mannheim, Germany), a tetramethylbenzidine substrate (0.1 mg/ml) solution was used and the color reaction was stopped with 2 M H_2SO_4. Absorbance was measured at 450 nm. The reciprocal of the furthest test serum dilution resulting in an extinction higher than the reference value was read as a titer.

Measurement of total IgG1, IgG2a, and IgE antibodies

Plates (highbond 3590; Costar) were coated overnight with 1.5 µg/ml purified polyclonal goat anti-mouse IgG1, IgG2a (Southern Biotechnology Associates), or purified rat anti-mouse IgE (clone R35-72, BD Pharmingen) in carbonate buffer (pH 9.6), followed by 1 h blocking with PBS-Tween/3% milk powder. Each test serum was measured at several dilutions. Standard curves of purified mouse IgG1 (clone A111-3), IgG2a (clone G155-178) and IgE (clone C38-2) obtained from BD Pharmingen were used to calculate the antibody concentration. For detection AP-conjugated goat anti-mouse IgG1 and IgG2a, and rat anti-mouse IgE antibodies (all Southern Biotechnology Associates) were added (1 h at 37°C). Subsequently 1 mg/ml p-nitrophenylphosphate in diethanolamine buffer was used for the color reaction, which was stopped with a 10% EDTA solution and absorbance was measured at 405 nm.

Cell culture and cytokine measurements

Spleen and MLN single cell suspensions ($3.75x10^5$ cells in 200 µl complete RPMI 1640 (Life Technologies, Paisley, Scotland) containing 10% FCS (ICN Pharmaceuticals, Costa Mesa, CA)) were incubated in the presence or absence of 200 µg/ml PE in 96-well plates for 96 h at 37°C, 5% CO_2. After centrifugation for 10 min at 150 *g*, supernatant was collected and stored at -20°C until analysis.

In the culture supernatants, levels of IFN-γ, IL-5, and IL-13 were determined by sandwich ELISA. IFN-γ and IL-5 capture and biotin-conjugated antibodies were obtained from BD Pharmingen and the ELISAs were performed as previously described [17]. The IL-13 ELISA (Biosource, Camarillo, CA) was performed in accordance with the manufacturer's instructions.

Measurement of serum mouse mast cell protease-1 (mmcp-1)

Blood was collected 45 min after the second oral challenge with PE and serum levels of mmcp-1 were determined using an ELISA kit (Moredun, Scotland). The ELISA was performed according to the manufacturer's instructions.

Statistics

Data were analyzed using SigmaStat statistical software package (SPSS, Chicago, IL). The differences between group means were determined by using one-way ANOVA with Bonferroni as *post hoc* test. In the oral tolerance study, the differences between PBS and PE-exposed group means were determined by using independent samples *t* test procedure. For cytokine levels and mmcp-1 serum levels, statistical analysis was performed following logarithmic transformation (to achieve normal distribution). A value of $p<0.05$ was considered statistically significant.

Results

Effects of pDC-depletion on the induction of oral tolerance

To assess the role of pDCs in the induction of oral tolerance, mice were exposed to low doses of PE on 3 consecutive days and were treated with either anti-pDC mAb or rat IgG (RIgG) before oral exposure. 14 and 21 days after the last oral exposure, mice were challenged i.p. with PE plus alum and sacrificed 11 days after the last challenge. Antibody production and spleen cytokine production were used as a readout of responsiveness to challenge. As expected, control (RIgG-treated) mice pre-treated with PE exhibited lower levels of PE-specific serum IgG1 (Fig. 2A) compared to PBS-exposed mice as well as decreased production of Th2 cytokines IL-5 and IL-13 (Fig. 2B) following *ex vivo* PE restimulation. Treatment with anti-pDC before oral exposure to PE failed to prevent the suppression of PE-specific IgG1 and Th2 cytokines.

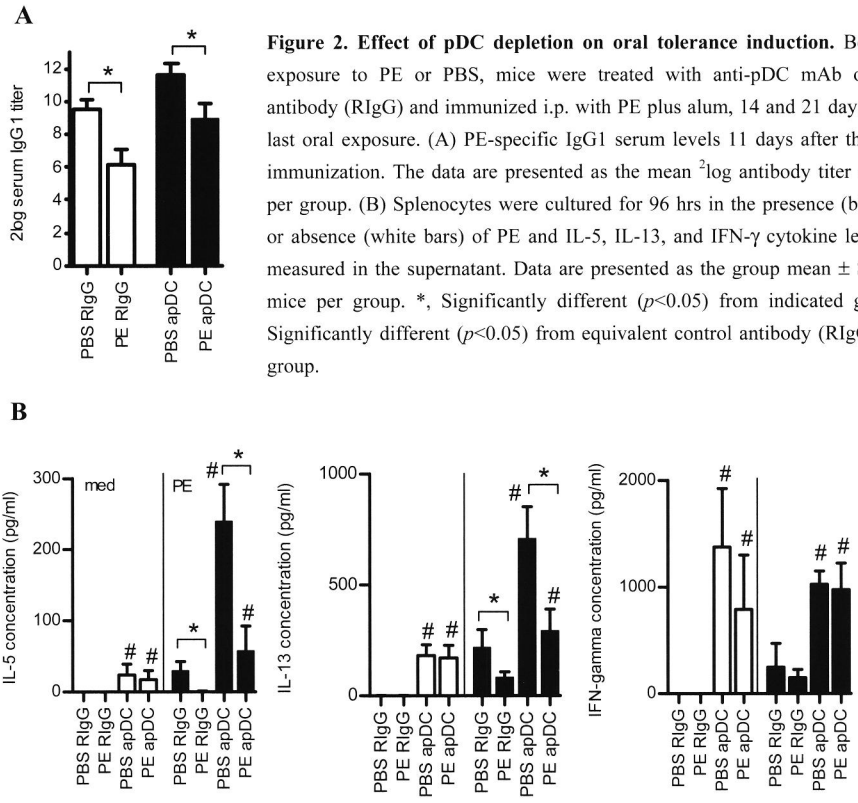

Figure 2. Effect of pDC depletion on oral tolerance induction. Before oral exposure to PE or PBS, mice were treated with anti-pDC mAb or control antibody (RIgG) and immunized i.p. with PE plus alum, 14 and 21 days after the last oral exposure. (A) PE-specific IgG1 serum levels 11 days after the last i.p. immunization. The data are presented as the mean ^{2}log antibody titer of 6 mice per group. (B) Splenocytes were cultured for 96 hrs in the presence (black bars) or absence (white bars) of PE and IL-5, IL-13, and IFN-γ cytokine levels were measured in the supernatant. Data are presented as the group mean ± SEM of 6 mice per group. *, Significantly different ($p<0.05$) from indicated groups. #, Significantly different ($p<0.05$) from equivalent control antibody (RIgG)-treated group.

However, in anti-pDC-treated groups, cytokine levels in PE-restimulated cultures were higher than levels found in cultures of rat IgG-treated mice and anti-pDC treatment also induced cytokine production in non-restimulated cultures irrespective of the oral pre-treatment. Notably, the Th1-associated cytokine IFN-γ was not suppressed following oral PE pre-exposure in both anti-pDC- and RIgG-treated groups. These data suggest that pDC-depletion has no effect on the establishment of oral tolerance, despite an increase in non-specific cytokine production.

Effects of anti-pDC treatment on allergic responses to PE

To investigate the role of pDCs in preventing and/or controlling allergic sensitization to peanut pDCs were depleted during the first 3 wk of oral exposure (see Fig. 1). We addressed the questions whether depletion of pDCs affects non-responsiveness to peanut (exposure to PE alone) and additionally whether it influences allergic responses to peanut (exposure to PE plus CT). Mice were sacrificed at day 7 to determine early cytokine production in spleen cultures. In control antibody-treated groups significant cytokine production was only found in PE-restimulated cultures of the PE plus CT-exposed group (Fig. 3). In contrast, significant IL-5, IL-13, and IFN-γ cytokine production was found in all cultures of anti-pDC-treated groups, irrespective of oral treatment or PE restimulation, indicating that pDC-depletion results in increased non-antigen specific cytokine production in the spleen. However, in the anti-pDC-treated PE exposure groups (with or without CT) PE restimulation induced significantly higher cytokine levels than levels found in non-restimulated cultures suggesting that pDC-depletion may additionally lead to enhanced antigen-specific cytokine responses.

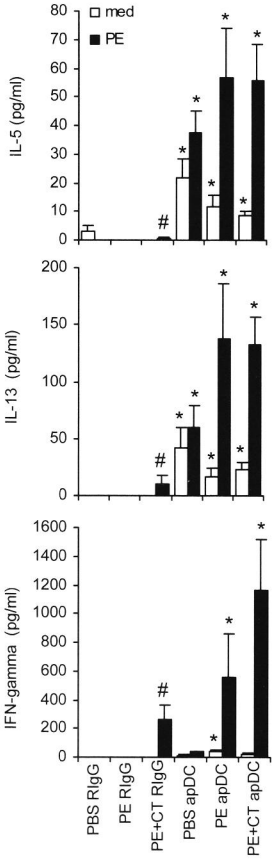

Figure 3. Effect of pDC depletion on early cytokine production. Mice were orally exposed on 3 consecutive days to PBS, PE, or PE plus CT. Indicated groups were treated with either anti-pDC mAb or RIgG before oral exposure. At day 7 splenocytes were cultured in the presence (black bars) or absence (white bars) of PE and IL-5, IL-13, and IFN-γ cytokine levels were measured in the supernatant. Data are presented as the group mean ± SEM of 6 mice per group. *, Significantly different ($p<0.05$) from equivalent control antibody (RIgG)-treated group. #, Significantly different ($p<0.05$) from equivalent non-CT-treated group.

In order to examine the effect of pDC depletion on allergic sensitization to peanut (exposure to PE plus CT), PE-specific serum antibody levels were measured during (day 21) and after (day 39) the oral dosing regime and mast cell degranulation was determined following an oral challenge with PE at day 38. As expected, exposure to PE alone led to hyporesponsiveness to peanut characterized by low PE-specific IgG1 and IgG2a levels (Fig. 4A) and the absence of PE-specific IgE and challenge effects (Fig. 4B).

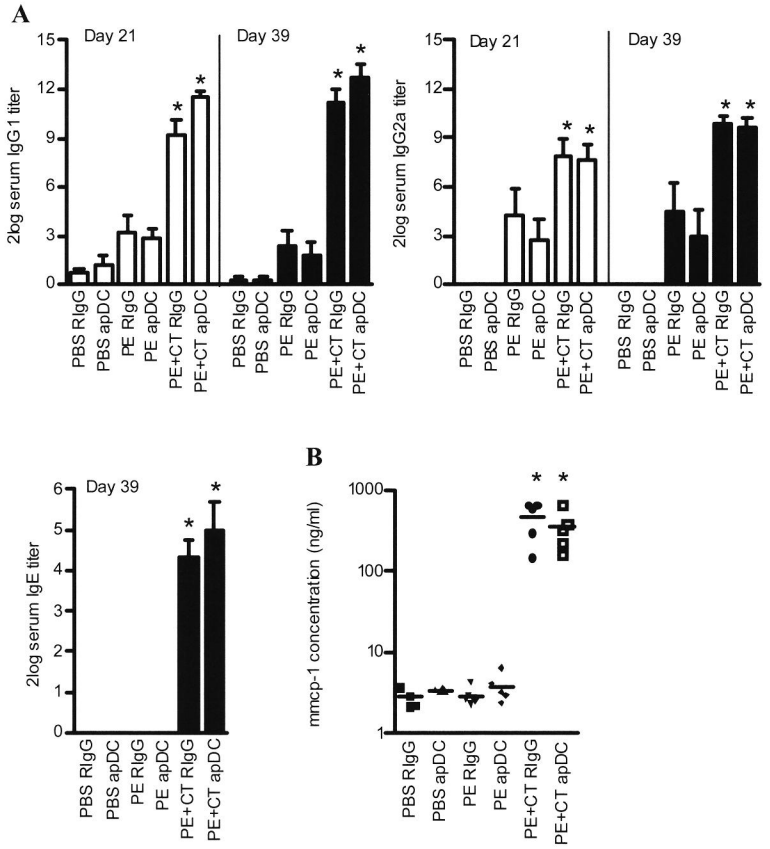

Figure 4. Effect of pDC depletion on PE-specific antibody and PE-induced challenge responses. After 5 wk of oral exposure to PBS, PE, or PE + CT, mice received an oral challenge with PE and were sacrificed a day later. Indicated groups were treated with either anti-pDC mAb or RIgG during the first three weeks of oral exposure. PE-specific serum antibody levels (A) were determined during and at the end of the oral exposure protocol and mmcp-1 levels (B) were measured 45 min after the last oral challenge. Antibody data are presented as the mean ^{2}log antibody titer of 6 mice per group and mmcp-1 values (ng/ml) are presented on a log scale (symbols indicate individual animals). *, Significantly different ($p<0.05$) from the equivalent PBS-exposed control group.

In agreement with the findings from the previous study on the induction of oral tolerance, pDC depletion did not affect the hyporesponsiveness to peanut observed after long-term oral exposure to harmless PE antigens.

Exposure to PE in the presence of CT induced allergic sensitization as demonstrated by high PE-specific IgG1, IgG2a, and IgE serum levels (Fig. 4A) and elevated mmcp-1 levels (Fig. 4B) upon an oral challenge with PE. In this oral exposure group also no differences in PE-specific antibody levels or challenge responses were found between anti-pDC-treated and the RIgG-treated mice.

Effects of pDC depletion on general immune homeostasis

One might argue that the absence of an effect on PE-specific antibodies in pDC-depleted animals might be due to insufficient (50%) depletion. However, anti-pDC mAb treatment dramatically increased total serum IgG1, IgG2a, and IgE serum antibody levels compared to levels found in RIgG-treated groups (Fig. 5), indicating that 50% depletion of pDCs is sufficient to significantly affect serum antibody levels. Interestingly, anti-pDC mAb treatment induced a larger increase in IgG1 antibodies (12.9-fold increase) than IgG2a antibodies (2.8-fold increase), suggesting that pDC depletion leads to type 2 antibody-bias. Co-administration of CT also significantly enhanced total IgG1 and IgE levels (to a smaller extent than anti-pDC treatment), but had no effect on total IgG2a levels confirming its type 2-skewing effect. These results imply that pDCs play an important role in controlling systemic immune homeostasis.

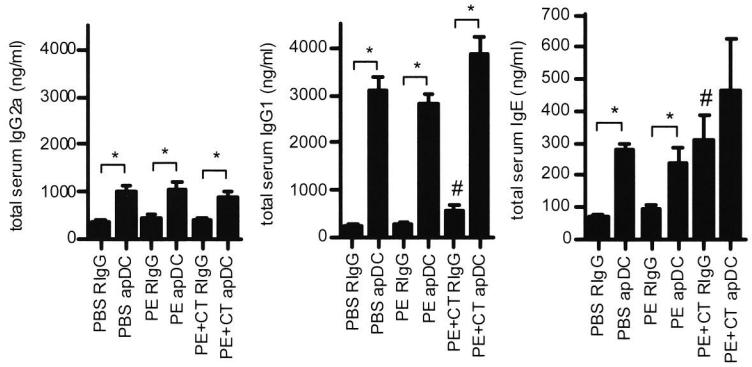

Figure 5. Effect of pDC depletion on total serum antibody levels. Mice were exposed for 5 wk to PBS, PE, or PE + CT. Indicated groups were treated with either anti-pDC mAb or RIgG during the first three weeks of oral exposure. Endserum was used to determine total serum IgG1, IgG2a, and IgE levels. Data are presented as the group mean ± SEM of 6 mice per group. *, Significantly different ($p<0.05$) from equivalent control antibody (RIgG)-treated group. #, Significantly different ($p<0.05$) from equivalent non-CT-treated group.

To further explore the effect of pDC depletion on immune homeostasis, cytokine production was determined in both spleen and MLN cultures at the end of the long-term oral exposure model (day 39). In RIgG-treated groups significant cytokine production was only found in PE-restimulated spleen (IL-13 and IFN-γ) and MLN (IFN-γ) cultures of the PE plus CT-exposed group (Fig. 6). In contrast, high levels of Th2 and Th1 cytokines were found in spleen cell cultures of all anti-pDC-treated groups. In these groups no differences in cytokine levels were found between PE-restimulated or non-restimulated cultures or between the different oral exposure groups. Intriguingly, in the MLN the effect of anti-pDC treatment on cytokine production was more restricted compared to the general effects found in the spleen; although in both PBS and PE exposure groups a small increase in (non-specific) cytokine production was found following pDC depletion, far higher levels of IL-5, IL-13, and IFN-γ were found in MLN cultures of PE + CT-exposed mice, irrespective of PE-restimulation. This might be an indication of additional suppression mechanisms in the MLN.

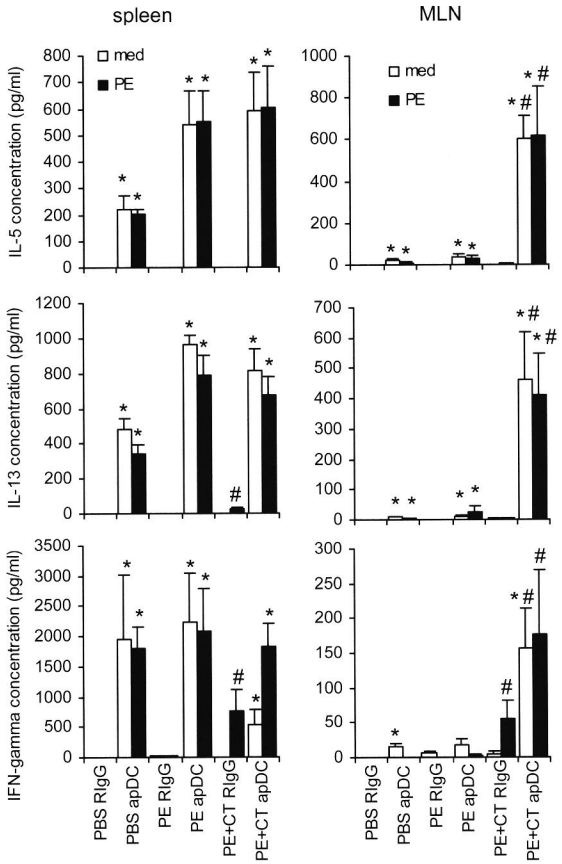

Figure 4. Effect of pDC depletion on spleen and MLN cytokine production. Mice were exposed for 5 wk to PBS, PE, or PE + CT. Indicated groups were treated with either anti-pDC mAb or RIgG during the first three weeks of oral exposure. After 5 wk, spleen and MLN single cell suspensions were cultured for 96 hrs in the presence (black bars) or absence (white bars) of PE and IL-5, IL-13, and IFN-γ cytokine levels were measured in the supernatant. Data are presented as the group mean ± SEM of 6 mice per group. *, Significantly different ($p<0.05$) from equivalent control antibody (RIgG)-treated group. #, Significantly different ($p<0.05$) from equivalent non-CT-treated group.

Discussion

DCs are thought to play an essential role in both tolerogenic and immunogenic responses to oral antigens. Although controversial, it has been suggested that particular functions of DCs such as the induction of tolerance versus immunity might be exerted by specialized subsets of DCs. In the current study the involvement of a special subpopulation of DCs, the pDC, in responses to orally administered peanut proteins was investigated by depleting pDCs *in vivo*. Since pDCs have been described to exert tolerogenic and regulatory functions we hypothesized that pDC depletion would lead to a failure of oral tolerance induction and to the development of allergic sensitization to harmless peanut proteins. However in the present study no abrogation of oral tolerance was observed following pDC depletion. This is in agreement with data obtained in Fms-like tyrosine kinase 3 ligand (flt3L)-deficient mice that have virtually no pDCs. In these knock-out mice, normal intranasal tolerance could be induced, suggesting that pDCs are not essential for mucosal tolerance induction [18].

In line with our results on oral tolerance, pDC depletion did not affect hyporesponsiveness to PE (no PE-specific IgE, PE-induced cytokines or mast cell degranulation upon oral PE challenge) induced following long-term oral exposure to PE without adjuvant In contrast, de Heer and colleagues have shown that pDC depletion results in allergic airway responses to normally inert OVA [13]. A possible explanation for these different observations may be a difference in immune environment and immune regulation between the lung and the intestine. DCs in the intestines seem to be generally tolerogenic due to the unique immune environment. For instance, although present in all lymphoid organs, myeloid DCs (mDCs) from the PP and MLN seem to have the unique ability to produce high levels of IL-10 rather than IL-12 and polarize naïve T cells to a regulatory phenotype [19, 20]. Since innocuous soluble peanut proteins are normally not capable of inducing DC activation, the absence of activated mDCs in PE-exposed pDC-depleted animals may have prevented the breakdown of tolerance to peanut. Accordingly, our data show differences in non-specific cytokine production between the spleen and MLN of pDC-depleted mice. Whereas cytokine levels in spleen cultures of pDC-depleted mice were increased independent of the oral treatment, cytokine levels in the MLN were only elevated in the sensitized group. Together these data support the idea that the gut immune environment is highly suppressive and that oral tolerance is maintained via a variety of mechanisms that act simultaneously, and that may be mediated by different DC subpopulations [21].

In the current study allergic sensitization to peanut induced by oral exposure to PE in the presence of the mucosal adjuvant CT was also not affected by pDC depletion. Consistent with this finding no difference in priming of T cell responses to inhaled antigen, or induction of allergic responses was found between wildtype and flt3L-knockout mice [18]. In addition, Oriss *et al.* [22] have recently investigated the dynamics of DC phenotypes in

airway inflammation and tolerance. Intranasal exposure of OVA in the presence of CT resulted in a low increase in the number of pDCs, but a substantial increase of mDCs (also expressing higher levels of costimulatory molecules) in the draining lymph nodes. Mice treated with OVA alone (induction of tolerance) had similar proportions of pDCs and mDCs as naïve mice. These results suggest that the influx of mDCs bearing costimulatory molecules is responsible for the induction of allergic sensitization to OVA. In our peanut sensitization model, mDC activation by CT might also be decisive for the induction of sensitization since the number of pDCs available did not affect the allergic responses to peanut.

One could argue that the incomplete pDC depletion (50% depletion in both spleen and MLN) in the presented studies may have been responsible for the absence of significant effect on responses to orally administered peanut. However, the general immune activation (including increased serum antibody levels and cytokine production) observed following pDC depletion suggests that depletion was sufficient to significantly affect systemic and gut immune responses. Nevertheless we cannot rule out the possibility that in the complete absence of pDCs more vigorous effects would have been observed.

Together, we speculate that in non-inflammatory conditions intestinal pDCs are mainly involved in maintaining immune homeostasis and do not play a dominant role in antigen presentation of orally administered proteins compared to conventional mDCs. This hypothesis is supported by recent data demonstrating that pDCs have poor endocytotic activity [23, 24] and are less efficient in presenting exogeneous antigen compared to mDCs [24, 25]. However, they may modulate the function of other DC subsets by cytokine secretion and this may be an explanation for the increased total serum antibody levels (including type 2 antibody skewing) and enhanced splenic cytokine production observed in pDC-depleted mice.

In conclusion, the current studies demonstrate that pDCs are involved in maintaining systemic immune homeostasis. In addition, the results suggest that pDCs do not play a crucial role in regulating tolerogenic and sensitization responses to orally administered proteins indicating that oral tolerance is probably maintained by a number of DC subpopulations.

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Chapter 8

General discussion

The gastrointestinal tract is constantly bombarded with a plethora of antigens and the gut mucosal epithelium represents the primary site for antigen entry. Consequently, the immune system of the gut, which is the largest immunologic organ in the body, faces the daunting task of distinguishing harmless dietary proteins and commensal bacteria from potentially dangerous pathogens.

The normal immune response to dietary proteins is the induction of oral tolerance, which refers to a state of systemic unresponsiveness following oral administration of a specific antigen. However, a small number of food proteins are associated with the generation of unwarranted allergic responses in susceptible individuals. It is clear that characteristics of both the food and the individual affect the development of food allergy, but the underlying mechanisms that control oral tolerance versus oral sensitization remain to be elucidated (chapter 1). Further understanding of these mechanisms may be of key importance for the development of new therapeutic strategies and for the prediction of allergenic potential of food products.

Animal models may provide a useful tool to study the biology of allergic responses *in vivo* and to assess allergenicity of (novel) foods. They offer the ability to investigate the onset of sensitization, which is not possible in humans for obvious ethical reasons. In the present thesis an oral mouse model of peanut hypersensitivity was adapted to study peanut extract (PE)- and allergen-specific responses (chapter 2 and 3). Additionally, the popliteal lymph node assay was used to examine the intrinsic adjuvant capacity of purified allergens versus a whole protein extract (chapter 3). In chapter 4-7 initiating mechanisms underlying allergic sensitization versus oral tolerance were explored, including the involvement of the CD28/CTLA-4-CD80/CD86 signaling pathway (chapter 4 and 5), $CD4^+$ $CD25^+$ regulatory T cells (Tregs) (chapter 6), and plasmacytoid dendritic cells (DCs) (chapter 7). For this purpose both the peanut hypersensitivity model as well as a newly developed model of low dose oral tolerance to peanut were used.

1. Prediction of protein allergenicity

In recent years, with the increasing prevalence of food allergy and growing reports on anaphylaxis, there has been increased consumers pressure on the food industry to identify and label foods containing (low) levels of allergens. In this context genetically modified (GM) foods present a new issue. GM plants are produced by altering the DNA of the plant genome through the introduction, removal, or rearrangement of DNA. The resulting GM crops offer, among others, improved disease resistance or higher yields. However, one of the main health concerns regarding these novel foods is their potential allergenicity [1].

The allergy risk to consumers of GM crops may be placed in one of three different categories [2]:

a) The transfer of a known allergen or a cross-reactive allergen into a food crop
b) Changing the expression level of endogenous allergens
c) Creating a new, unknown allergen by expression of novel proteins

In 2001, a decision tree approach has been proposed by the FAO/WHO for the assessment of protein allergenicity (see Fig. 1) [3]. However, for the implementation of this step-by-step approach, further research is warranted including (a) elucidation of the relationships between protein structure and function and sensitizing activity, (b) improved understanding of the possible relationship between resistance of proteins to digestion and sensitizing potential and (c) the development of animal models [4]. In chapter 2 and 3 of this thesis, different aspects related with these research questions were investigated using *in vivo* models and will be discussed in the following paragraphs.

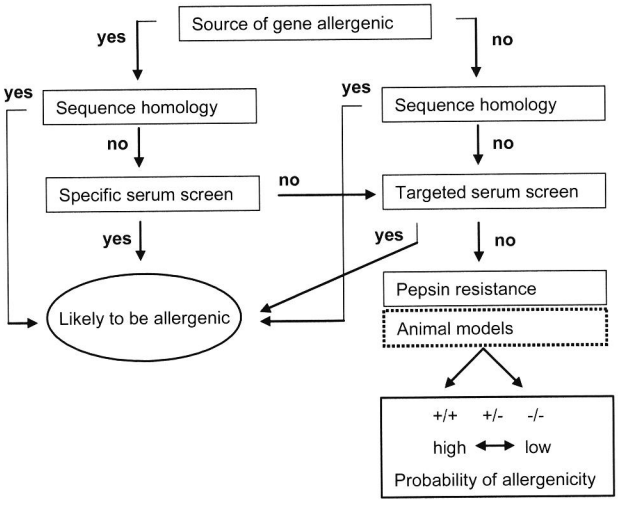

Figure 1. The decision tree as proposed by the FAO/WHO (2001).

1.1 Characteristics of (peanut) allergens

Among thousands of food proteins, only a relatively small number are involved in IgE-dependent allergic reactions and the question remains: why are these proteins allergenic? To date, the characteristic structural and biochemical properties that render a protein allergenic are still unknown. One of the properties that is considered to be shared by many food allergens is stability to digestion [5]. However in a study carried out by Fu *et al.* no clear relationship was found between digestibility measured *in vitro* and protein allergenicity [6]. From this study it was concluded that results on digestion stability obtained with *in vitro*

assays are highly influenced by the assay conditions used. Similarly, contradictive results have been found regarding the *in vitro* resistance to pepsin digestion of the major peanut allergens *Ara h 1* and *Ara h 2* [6-8]. In the peanut hypersensitivity model described in this thesis, oral administration of purified peanut allergens (*Ara h 1-3* and *Ara h 6*) with or without the presence of a food matrix resulted in allergen-specific IgE levels (chapter 2, 3), indicating that these proteins are stable enough *in vivo* to reach the immune system in a relatively intact form (i.e. still containing intact IgE epitopes).

Another condition for a protein to become a sensitizing allergen is the ability to stimulate the immune system. In chapter 3 we clearly show that individual major peanut allergens possess little intrinsic immune stimulating properties, suggesting that activation of the immune system by allergens depends on additional, non-inherent factors. These adjuvating factors may be present in the food itself and it has been hypothesized that the food matrix may influence immune responses to orally administered proteins [9]. Results from the popliteal lymph node assay (PLNA) study (chapter 3) indicate that the food matrix is able to adjuvate immune responses to individual allergens present in the food extract. This is an important finding, indicating that research on food allergenicity should not be restricted to purified proteins. Although lipids and oils present in highly allergenic foods, such as peanuts and tree nuts, seem likely candidates responsible for the matrix adjuvant effect, we found no evidence that the presence of fat influences the immune response to orally administered or subcutaneously injected peanut allergens (chapter 3). Further studies are warranted to identify possible immune stimulating compounds present in the food body.

Together our data demonstrate that *in vivo* models provide an important tool to study characteristics of allergenic foods and proteins.

1.2 Development of predictive animal models

The proposed *in vitro* screening models (such as the serum screen tests) mainly tackle the problem of potential **allergenic activity**, i.e. the propensity of a substance to provoke allergic reactions in sensitized allergic patients. The term **allergenicity** describes the likelihood of inducing *de novo* sensitization in a non-allergic individual. Therefore allergenicity testing requires models of allergic sensitization. In this context *in vivo* animal models seem of crucial importance as they may provide a direct and holistic assessment of the sensitizing potential of proteins. To date there is no animal model to predict the allergenic potential of novel food proteins. Although newly developed swine and dog models more closely mimic human clinical manifestations and the human digestive system [10, 11], current research is focusing on Th2-predisposed rodent models, which are more practical to study hazard identification [12]. Several issues have been pointed out that have to be taken into account when developing an animal model including route of exposure, the presence of adjuvants, and form of the test material.

Route of exposure

In the context of food allergy, the oral route is obviously the most relevant route of exposure. However, as in humans, oral exposure to soluble proteins normally leads to antigen-specific tolerance instead of sensitization. This phenomenon may be circumvented by the use of a susceptible strain and/or the use of an adjuvant. In addition, systemic exposure to food proteins has been proposed as a screening method for allergenic potential [13]. It has been shown that i.p. injection of purified allergen results in the induction of antigen-specific IgE responses, whereas non-allergens elicited antigen-specific IgG responses [14, 15]. Unfortunately, very limited data are available. Results described in chapter 3 on the intrinsic immune stimulating capacity of purified allergens do not support the underlying idea of systemic exposure, *i.e.* allergenicity is strongly related to overall immunogenicity. Furthermore, (additional) oral testing will reckon with digestion, natural barriers such as the mucosal epithelial layer, and the presence of natural adjuvants. In chapter 2 of this thesis marked differences in allergen-specific responses were found following either oral or parenteral exposure to a peanut extract (PE). Although similar PE-specific IgE levels were found in both exposure groups, allergen-specific IgE levels were higher in the orally exposed group, suggesting that the use of the oral route is more adequate in identifying allergenic proteins. Comparable results were found in the Brown Norway (BN) rat upon oral and i.p. exposure to PE without the presence of an adjuvant (Knippels and van Wijk, unpublished data). Oral exposure induced high levels of *Ara h 1*-, *Ara h 2*-, and *Ara h 3*-specific antibodies, whereas after i.p. injection antibodies were mainly directed against *Ara h 2* and to a much lower extent towards *Ara h 1* and *Ara h 3*. Together, these data suggest that the route of antigen delivery profoundly affects the nature of an immune response. The oral route remains the favored route of exposure for the assessment of protein allergenicity, while systemic exposure models should only be employed as a method for hazard identification.

Obviously, the idea that oral exposure is most appropriate for allergenic potential testing is based on the assumption that most GM products are used for food production. However, GM products may also cause respiratory sensitization by pollen production or during food processing. Therefore, other exposure and sensitization scenarios may be considered in allergenicity assessment [16].

Besides allergenicity testing, animal models may also be used to assess allergenic activity i.e. is a protein able to elicit an allergic reaction in a sensitized animal? For this purpose parenteral or sub/epicutaneous [17] sensitization followed by oral challenge(s) may be considered in order to obtain the most apparent clinical responses.

Use of an adjuvant

The use of an adjuvant in oral screening models has been a major issue of debate. Adjuvants are used for their non-specific immunoenhancing effects but they can also influence the

quality of an immune response (for example Th2-skewing). The biggest concern is that adjuvants may modify the inherent properties of proteins to induce IgE, resulting in false positives. The BN rat food allergy model is the most well-known screening model that does not rely on the use of an adjuvant. In this model, daily gavage of proteins such as OVA results in antigen-specific IgE responses and clinical symptoms following oral challenge [18]. Although this model seems promising, published data are limited to a small number of allergens, and attempts to assess relative allergenicity of known allergens and non-allergens have not yielded consistent data yet.

Other rodent models of food allergy do depend on co-administration of an adjuvant, and cholera toxin (CT) is the most common adjuvant used for oral sensitization. We and others have shown that allergen-specific IgE responses are induced following oral purified allergen or whole extract exposure in the presence of CT [19-21], whereas in the same model oral protein exposure in the absence of CT leads to oral tolerance (this thesis). The effect of CT has been shown to be dose-dependent [22] and although oral sensitization by CT co-administration has been demonstrated in different mouse strains (C3H/HeJ, C3H/HeOuJ, Balb/c), the magnitude of the sensitization response, and particularly the challenge responses seem to be strain-dependent [21, 23]. Interestingly, immunoblotting has revealed that in the C3H/HeJ mouse oral exposure to PE in the presence of CT results in IgE responses against the same proteins that are recognized by serum IgE of peanut-allergic patients [19]. However, for the future use of this model as a screening tool the issue of false positives should be addressed by including negative controls (non-allergenic proteins). Gaudry and colleagues have tested several allergens and non-allergens in the same model and have shown that mice and man similarly respond to major and non-allergens [24]. These preliminary results suggest that CT merely facilitates the development of allergic responses to co-administered proteins, but does not confer sensitizing potential on non-allergenic proteins.

Finally, one should realize that in real-life situations adjuvants may also play a role in the sensitization process. Intestinal inflammation or infection has been shown to enhance gut permeability leading to an increased epithelial passage of intact antigen [25]. Furthermore, infections may provide adjuvant or 'danger' signals that may trigger initiation of allergic sensitization to food proteins [26]. Defining the factor(s) that may act as initiators of food allergic sensitization in susceptible individuals is one of the challenging topics of future research.

Use of purified allergens versus an extract.

In this thesis we show that it is possible to measure IgE responses against a total protein extract as well as against individual allergens following oral exposure to a whole extract (chapter 2). This provides an excellent tool to study overall allergenicity of the food and individual protein allergenicity at the same time. In addition we revealed that the food

matrix may adjuvate responses to individual proteins (chapter 3) although this was not confirmed in the oral exposure model. Together, in our opinion it would be more appropriate to assess the overall ability of a food extract to induce allergic reactions than studying purified proteins. Not only would extracts more closely mimic human exposure by preserving glycosilation and/or binding to natural adjuvants found in the crop of interest, polysaccharide- and protein-protein interactions have also been found to influence allergen digestion [27, 28]. Additionally, considering the possibility that the insertion of a foreign protein may induce an upregulation of allergen expression, it may be advisable to compare whole tissue extracts from the wildtype crop with tissue extracts from the GM crop in both the sensitization and elicitation phase. This is of particular importance when known allergenic foods such as soy are bioengineered.

1.3 Animal models; conclusions

Although the risk assessment process is currently far from complete and GM crops are already extensively consumed, to date no biotech proteins in foods have been documented to cause allergic reactions [29]. However, in the future, production and consumption of GM crops will increase and the risks of food allergic reactions and allergic sensitization should be minimized. The transfer of a known allergen or cross-reactive allergen represents the highest risk, but also seems to be most easily obviated. A category that represents a lower risk is the expression of novel proteins that may become allergenic in man [2]. Although the risk seems to be relatively low, assessment of these novel proteins is most difficult and challenging and requires *in vivo* animal models of allergic sensitization. Our results, together with findings from other groups [19, 24] imply that the C3H/He(Ou)J mouse model is a promising model to study sensitizing potential of food proteins. However, it is unlikely that a single animal model will be sufficient to address all issues concerning prediction of allergenicity to humans and therefore the development of diverse animal models is warranted. These animal models may additionally be useful to study the effects of food processing on allergenicity, and to test recombinant hypoallergenic proteins or hypoallergenic foods such as child formulas. Animal models also provide excellent tools to unravel the underlying mechanisms of food allergy. Investigation of basic mechanisms is crucial for the understanding of what makes a protein allergenic and what makes an individual susceptible. Finally, animal models are essential for the development of new therapeutic strategies.

2. Initiating mechanisms involved in tolerance versus allergic sensitization to oral proteins

Orally administered antigen can induce either immune tolerance or priming both locally and systemically. Factors that influence the outcome of an immune response to orally administered antigens include among others (1) antigen availability, (2) the immune environment, (3) type of the antigen presenting DC, (4) DC maturation and activation, (5) the level and form of costimulation, and (6) the actions of Tregs and their cytokines (for an overview of the factors see figure 2). However, how the mucosal immune system 'decides' whether to induce tolerance or priming when exposed to fed antigens remains largely unclear. In the present thesis we investigated the effects of several of the mentioned factors on (a) oral tolerance induction (oral exposure to PE alone, followed by i.p. challenge with PE plus alum to measure tolerance induction), (b) initiation of sensitization to harmless food proteins (long-term oral exposure to PE alone), (c) established hypersensitivity responses to food (long-term exposure to PE in the presence of CT).

2.1 Antigen dose/availability

The most important condition for the initiation of immune responses (either tolerogenic or sensitizing) to oral antigens is the capture of antigen by antigen presenting cells (APC). Under the condition that antigen is available, antigen dose may effect tolerance versus sensitization as well as the form, location, and/or magnitude of the tolerance or sensitization response.

Soluble food proteins are known to pass the epithelial barrier and to be taken up by lamina propria DCs. These DCs migrate to T cell areas in the Peyer's Patches (PPs) and/or mesenteric lymph nodes (MLNs) where they present the antigen to naïve T cells. In addition, under both tolerogenic and immunogenic conditions, free antigen can be found in the bloodstream and on APCs from the spleen and peripheral lymph nodes within an hour after feeding [30]. This may be one of the mechanisms by which peripheral tolerance to harmless oral antigen is ensured in addition to local tolerance. Nevertheless, presentation of fed antigens to T cells is more efficient and long-lasting in the MLNs than in the peripheral lymphoid organs [31] and high doses of peripheral antigen might even favor priming instead of tolerance.

In the present thesis intragastric dosing of a peanut protein extract was used for all oral exposures. Intragastric administration of protein leads to antigen presentation in both the gut and periphery [32] and, dependent on the presence of an adjuvant, results in either systemic tolerance or systemic sensitization (this thesis). In chapter 2 we show that sensitization responses are dose-dependent. This also accounted for challenge responses (unpublished data). Induction of oral tolerance (reduction of PE-specific/allergen-specific

serum antibody responses and PE-induced cytokine production in the spleen following i.p. immunization) was achieved with both 1 mg and 6 mg PE dosing. Strid and colleagues exposed BALB/c mice (single intragastric feed) to PE at doses varying from 0.02 to 100 mg and immunized the mice 7 and 28 days later with 100 µg PE in complete Freund's adjuvant. Interestingly, tolerance induction was only found in the high dose group, but not in the 20 mg- and 2 mg-exposed groups and dosing of 0.2 and 0.02 mg PE even induced sensitization [33]. The discrepancy in results may be explained by the use of another mouse strain and different dosing and immunization protocols. Nevertheless these results illustrate the possible effects of the antigen dose on tolerance versus sensitization and emphasize the influence of the treatment protocol on the outcome of oral tolerance. Antigen dose has also been demonstrated to affect the mechanism of tolerance induction (deletion, anergy or active suppression) [34]. Since clonal deletion by apoptosis has only been demonstrated for $CD4^+$ T lymphocytes of TCR transgenic mice following high dose feeding [35], it seems most likely that in the present oral tolerance model tolerance is induced by clonal anergy and/or active suppression.

2.2 T cell responses

Th1 versus Th2

In 1986 Mosmann and colleagues provided evidence that $CD4^+$ T cell clones can be assigned in two different subsets on the basis of distinct non-overlapping cytokine secretion patterns [36]. The so-called Th1 cells are characterized by the secretion of IL-2 and IFN-γ and are associated with cell-mediated immunity, whereas Th2 cells are characterized by IL-4, IL-5, and IL-13 secretion and are involved in the induction of humoral immunity. Various lines of evidence indicate that Th2-type cells play a central role in orchestrating the response seen in allergic sensitization. Accordingly, in the food hypersensitivity model described in the present thesis, Th2 cytokine production was only found in PE-restimulated spleen cell cultures of (IgE) sensitized animals. However, the same applied to the production of IFN-γ (among others chapter 2). This seemed remarkable since it is generally assumed that Th1 and Th2 cytokines are mutually inhibitory at the T cell level [37]. Concordantly, peanut-allergic children display a high Th2-polarization to peanut antigens compared to Th1-polarization of non-allergic and tolerant children [38]. However, recent data have challenged the absolute Th1-Th2 dichotomy in allergic responses [39]. DCs have been shown to prime a Th2 response in the presence of a pre-existing antigen-specific Th1 environment [40]. Moreover, instead of attenuating Th2 responses, Th1 responses have been shown to worsen ongoing allergic responses [41]. Furthermore, it has been demonstrated more than a decade ago that IFN-γ treatment abrogates the induction of oral tolerance [42]. Hence, pro-inflammatory Th1 cells might even contribute to allergic disease [43]. In line with these results, we hypothesized that IFN-γ may contribute to the initiation of sensitization to orally

administered proteins. Yet, blockade of IFN-γ with a monoclonal antibody did not affect hypersensitivity responses to peanut nor the induction of oral tolerance (unpublished data) suggesting that in the current models IFN-γ does not play a major role in either tolerance or sensitization induction to PE. In conclusion, although the effect of Th1 cytokines on allergic responses remains to be established, it seems unlikely that pure Th1 responses are effective in regulating the development of allergic diseases.

Regulatory T cells

Over the past decade, the Th1/Th2 universe has been expanded with $CD4^+$ "regulatory" T cells (see chapter 1). The term regulatory T cell (Treg) refers to cells that actively control or suppress the function of other cells, generally in an inhibitory fashion. Oral administration of soluble proteins is associated with the generation of antigen-specific TGF-β-producing (Th3) and IL-10-producing (Tr1) $CD4^+$ T cells and both cells have been implicated in regulating tolerance and preventing sensitization to innocuous antigens [44, 45]. Regardless of their dependence on antigen stimulation for their development, Tr1 and Th3 cell functions are mediated by bystander suppression. The focus in the present thesis was on the subset of CD25-expressing, naturally occurring Tregs. These cells have emerged as key players in the development of tolerance to auto-antigens as well as foreign antigens [46] but their role in (tolerance) responses to foreign mucosal antigens remains to be elucidated. In chapter 6 we show that in the absence of $CD4^+CD25^+$ T cells the induction of oral tolerance is impaired. Furthermore, $CD4^+CD25^+$ T cells seem to regulate established food allergic responses since $CD4^+CD25^+$ T cell depletion during sensitization resulted in enhanced PE-specific IgE responses and PE-induced challenge responses. Several mechanisms of action have been proposed for $CD4^+CD25^+$ Treg function including cell-cell contact through CTLA-4 as well as secretion of the suppressive cytokines TGF-β and/or IL-10 [47]. Whereas, in the present models, *in vivo* blockade of IL-10 and/or TGF-β had no effect on tolerance or sensitization responses to PE (unpublished results), inhibition of CTLA-4 signaling resulted in abrogation of oral tolerance (unpublished data) and increased PE-specific allergic responses (chapter 5). These results suggest that ($CD4^+CD25^+$ Treg-associated) CTLA-4 signaling plays a major role in the establishment of oral tolerance and in dampening allergic responses to PE. However, since we provided only circumstantial evidence, adoptive transfer and *in vitro* studies are required to confirm (or contradict) these results. Also the role of TGF-β and IL-10 in tolerance deserves further attention since these cytokines have been implicated in Treg function as well as Treg development. Revealing their specific role(s) *in vivo* will be complicated due to the plethora of determining factors such as location, timing, cell type that is producing the cytokine, mutual effects, active and inactive forms (TGF-β), the presence of numerous target cells, and their role in homeostasis.

Another interesting subset of $CD4^+$ Tregs is represented by the NK T cell that shares properties of NK cells and conventional T cells and is associated with CD1d-restricted responses. NK T cells rapidly produce high levels of IFN-γ and/or IL-4 upon TCR stimulation. This may allow NK T cells to regulate adaptive immune responses and Th1/Th2 immune deviation. However, data on the roles of NK T cells in both tolerogenic and allergic responses are contradictive. Activation of NK T cells has been shown to inhibit Th2 cell differentiation, IgE production [48] and allergic airway inflammation [49], whereas others have demonstrated an essential role for NK T cells in the production of IL-4 and IL-13 and in the development of allergic asthma [50]. Whereas stimulation of NK T cells has been shown to abrogate oral tolerance induction [51], NK T cells seem to be required for the induction of oral tolerance to nickel [52]. In our model, treatment of mice during oral sensitization with α-galactosylceramide (α-GalCer) - a potent stimulator of NK T cells - resulted in increased PE-specific antibody and PE-induced cytokine responses, indicating that NK T cells may be capable of intensifying allergic sensitization (data not shown).

Although it is known that NK T cells are activated by glycolipid presented on CD1d expressed by DCs, the physiological ligand(s) of NK T cells have not yet been identified. It is most likely that NK T cells are triggered by ligand(s) from endogenous origin, but it has additionally been suggested that allergens (glycoproteins that often bind lipids) might be able to activate NK T cells directly [53], possibly via CD1d expressed on intestinal epithelial cells [54].

Together, still very little is known about NK T cells and their regulatory properties. The unique features of NK T cells (including the link between innate and adaptive immunity and their apparently combined effector and regulatory functions) make them interesting study objects in the context of allergy and make them attractive (and at the same time risky) targets for immunotherapy.

T cell activation and induction of sensitization

An intriguing finding described in this thesis with respect to cytokine production is that disturbed immune homeostasis leading to enhanced (Th2) cytokine production and total serum IgE antibody levels is insufficient to induce sensitization to orally administered PE. In chapter 5 we show that blockade of CTLA-4 spectacularly enhanced Th2 cytokine production in both spleen and MLN. However, whereas CTLA-4 blockade increased PE-specific responses in sensitized mice (exposed to PE + CT) no PE-specific responses were induced in the PE-exposed group and the non-responsiveness to harmless PE antigen was not abrogated. Similar results were found following $CD4^+CD25^+$ T cell depletion (chapter 6) and pDC depletion (chapter 7). These results suggest that the (Th2) cytokine environment can amplify but not initiate allergic responses to orally administered antigens.

The classic pathway of Th2-skewing during naïve T cell activation depends on IL-4. The main producer of IL-4 is the Th2 cell itself and activated Th2 cells have been

demonstrated to exert a paracrine effect on the differentiation of naïve T cells (even in a Th1 environment) [55]. Furthermore, IL-4 produced by effector Th2 cells facilitates Th2-priming leading to polysensitization [56], likely mediated by affecting DC function [57]. However, high levels of IL-4 in both spleen and MLN of anti-CTLA-4-treated mice did not result in Th2-priming to orally administered peanut (chapter 5).

One could argue that the antigen availability is simply too low to induce T cell priming. But, although antigen dose may be a limiting factor, the fact that the same dose of PE is capable of inducing systemic tolerance indicates that antigen should be available for presentation to T cells.

Another reason might be that the suppressive environment of the GALT created by local cytokines and regulatory cells is so strong that it is able to compensate for disturbed homeostasis. Several observations in this thesis support this assumption. First, depletion of $CD4^+CD25^+$ Tregs abrogated the induction of systemic oral tolerance but did not lead to sensitization to oral PE, indicating that local suppression in the gut of orally induced responses may co-exist with impaired peripheral tolerance to the same antigen (chapter 6). Second, although pDC depletion induced increased non-specific cytokine production in the spleen of naïve, tolerant, and sensitized mice, MLN cytokines were only enhanced in sensitized mice (chapter 7). Third, in contrast to the lack of effect on responses against oral antigen, anti-CTLA-4 treatment did increase antigen-specific antibody responses following i.p. antigen exposure without adjuvant (unpublished data), confirming that compensatory suppressive mechanisms may be active in the GALT. These results support the idea that the gut immune environment is highly suppressive and that oral tolerance is maintained via a variety of mechanisms that act simultaneously. Hence the suppressive environment seems to play an important role in controlling responses to harmless luminal antigens but this may not be a direct effect on T cells. Therefore it seems useful to 'take a step back' and shift focus to the cells that control naïve T cell differentiation and activation; the DCs.

2.3 DCs as initiators of the response

DC maturation and activation

It is now generally accepted that in the absence of external stimuli, DCs exist in a resting state in which they have only limited ability to prime naïve T cells. Innate immune stimuli such as proinflammatory cytokines and toll like receptor (TLR)-signaling are able to induce mature and activated DCs capable of T cell priming. This concept is well-established for Th1-mediated responses, but the pathways and events that govern the ability to instruct Th2 cell differentiation remain largely unknown. It has been suggested that Th2 responses reflect a default pathway that occurs in the absence of inflammatory stimuli and the subsequent production of IL-12. However, recent evidence supports the idea that DC-driven Th2 responses are not the product of default programming, even though DCs remain largely

quiescent following exposure to Th2 stimuli [58, 59]. Instead, resting DCs are thought to promote Treg generation and tolerance induction [60]. The crucial importance of DCs in the initiation of Th2-associated allergic responses is illustrated by the fact that DCs isolated from spleen and PPs of food allergic mice induce allergen-specific IgE responses in naïve recipients in the absence of previous immunization or subsequent allergen challenge [61].

We hypothesized that a lack of DC maturation might have been the reason for the failure to induce oral sensitization in an environment with highly activated T cells and high levels of Th2 cytokines. We set up an experiment in which mice were injected with FGK (a CD40 agonist that induces DC maturation by inducing upregulation of CD80, CD86, and CD54 expression [62]). In agreement with observations from other studies [63-65], CD40 ligation alone did not affect non-responsiveness to oral antigen. But, most importantly, the combination of FGK and anti-CTLA-4 treatment was also insufficient to induce allergic sensitization to PE (unpublished results) indicating that other signals are involved in the decision process of Th2 sensitization versus tolerance.

Interestingly, it has been recently proposed that fully mature but non-activated DCs can induce tolerance instead of immunity [66]. Although the terms 'maturation' and 'activation' have been used interchangeably they may represent two distinct processes. Maturation is a process of DC differentiation that confers the capacity to stimulate naïve T cells, which is required for both tolerance induction and priming. However, the outcome of T cell stimulation as tolerance or immunity may depend on whether DCs have been activated. Recent studies suggest that direct recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) such as TLRs and C-type lectins is critical for priming and that inflammatory mediators such as cytokines are unable to initiate adaptive immune responses [67, 68]. Although PAMPs have long been related solely with Th1 responses, more and more evidence points to a role for PAMPs in driving Th2-differentiation by DCs (reviewed by Eisenbarth *et al.* [69] and Pulendran [68]). For instance, several TLRs such as TLR4 and TLR2 have been associated with promoting Th2 responses and allergic disease. Various signaling pathways (including MyD88-dependent and independent pathways) have been implicated in these responses, depending on PAMP doses and site of exposure [69]. One of the mechanisms by which Th2 differentiation may be induced is the differential expression of the ligands Jagged and Delta on DCs [70]. These ligands interact with the Notch family of receptors on T cells. Known Th2 stimuli such as prostaglandin (among others secreted by pathogenic yeasts [71]) and CT have been shown to induce the expression of Jagged 1, and Jagged 1 interactions with Notch 1 induce early IL-4 cytokine production in naïve T cells [70]. Since instruction of Th2 differentiation by Notch 1 is independent of IL-4 receptor signaling, Notch 1 signaling (and not IL-4 from the environment) might be the crucial trigger for early IL-4 production. This early IL-4 production could subsequently serve to augment the Th2 response in an auto/paracrine fashion.

Notably, on the other hand PAMPs seem also capable of counter-regulating allergic responses by changing DC functions [72], which will be further discussed in paragraph 4.

Considering these new insights it is tempting to speculate that in the highly tolerogenic/suppressive immune environment of the gut, allergic sensitization to oral antigens requires DC activation induced by 'innate' (PRR-associated or other) signaling pathways, leading to Th2-generating capacity. It is even not unlikely that certain allergens may be able to stimulate PRRs themselves [53]. This would imply that in susceptible individuals the initiation of allergic sensitization may be dependent on the presence (or absence) of innate immune stimuli and signaling pathways and would have important implications for the development of therapeutic strategies.

Together, DCs (like every cell of the immune system) integrate all kinds of environmental stimuli before 'instructing' the quality and quantity of an immune response. It seems likely that responses are initiated in a threshold-depending fashion. All the incoming signals together may either contribute or negatively affect reaching of this threshold. Setting of these thresholds is probably also influenced by environmental factors and genetic factors (atopy), which emphasizes the complexity of the 'decision' made by DCs.

DC subsets and the microenvironment

Phenotypic characterization of DCs has revealed a number of different subsets that may perform specialized functions. Different subpopulations of DCs have been shown to induce different types of Th responses (including immunity versus tolerance [73] and Th1 versus Th2 [74]). The GALT has been reported to host several DCs with unique features [75, 76]. However, to date, analysis of DC subsets failed to identify a particular lineage unique to the GALT that may account for the propensity of these tissues to generate tolerogenic responses. Recently, the population of pDC has gained attention, with regard to their tolerogenic potential. pDCs are present in all lymphoid organs, but pDCs from MLNs have been demonstrated to be far more efficient than spleen pDCs in supporting Tr1-like cell differentiation [77]. In chapter 7 of this thesis we aimed to unravel the involvement of pDCs in responses to oral antigens by depleting this DC subset *in vivo* before the induction of oral tolerance and during sensitization. Although pDC depletion resulted in disturbed systemic immune homeostasis (increased cytokine production and elevated total serum antibody levels), the effects on immune homeostasis in the MLNs were less profound. Moreover, pDC depletion did not impair the induction of oral tolerance and had no effect on allergic responses to peanut. These results confirm that the immune environment of GALT is highly suppressive and indicate that oral tolerance is induced and maintained by different DC subsets. Therefore, one may speculate that the absence or presence of DC maturation/activation signals is of higher importance in the initiation of oral tolerance versus sensitization than the presence of 'specialized' subsets of DCs. This assumption is

supported by several observations. First, the 'default' effect of DC subsets on Th polarization can be overridden by factors such as the nature of the maturation stimulus and the dose of antigen [78, 79]. Second, there are indications that DC subsets can develop into another subset *in vivo* depending on environmental conditions [80], illustrating the plasticity of DCs. Third, certain DC subsets may represent a different maturation stage of the same DC lineage [81]. Fourth, DCs with high tolerogenic potential, such as the pDCs, can become immunogenic upon stimulation with appropriate stimuli [82]. Finally, DCs in distinct microenvironments have been shown to induce different Th responses [76, 83]. Together these data indicate that different niches exist in lymphoid organs, which give rise to a diversity of (plastic) DCs arising from a small number of precursor progenitors.

Hence, although specialized subsets of DCs may exert distinct functions in a particular condition, DCs are not likely inherently tolerogenic. This also seems to apply to intestinal DCs, which have been described as highly tolerogenic. Like in other organs, intestinal DCs seem to integrate environmental signals to both regulating T cell responses as well as maintaining local immune homeostasis. In turn, the behavior of intestinal DCs is controlled by an intimate interplay with other local cell types (such as epithelial cells and mesenchymal cells) and their secretion products. Given the plasticity of DCs it is reasonable to think that precursor DCs are modified (among others by IL-10 and TGF-β) after their arrival in the intestinal tissue instead of being derived from a distinct lineage. Finally it is important to realize that danger signals released by local pathogens can effectively overcome the default tolerogenic status of the intestine.

2.4 Costimulation

Engagement of costimulatory molecules during DC-T cell interaction is crucial for the generation of effector T cells. Costimulation may usually involve the interactions of several pairs of receptors and ligands expressed by DCs and T cells (reviewed by Croft [84]). Two major molecular families of costimulatory pathways have been described (see paragraph 6.2.1 of chapter 1); the immunoglobulin superfamily (including the CD28-B7 pathway) and the tumor necrosis factor receptor superfamily (including the CD40-CD40L pathway).

CD40 is expressed on DCs and B cells, while CD40 ligand (CD40L) is expressed by activated T cells. Costimulation through CD40 may represent an early event in the interactions between DCs and T cells as it results in the upregulation of MHCII and costimulatory molecules. Initially it was thought that CD40-CD40L interactions would be particularly important in Th1 responses [85] but recent data suggest that CD40 ligation is also critical for the development of Th2 responses [85, 86]. Consistent with these results, *in vivo* blockade of CD40L during sensitization to PE partially prevented the induction of PE-specific IgE responses (data not shown).

In chapter 4 we show that CD28-B7 interaction is indispensable for allergic responses to peanut induced by oral PE exposure in the presence of CT. This is in agreement with data obtained by others in allergic asthma models [87-89]. As both B7-1 (CD80) and B7-2 (CD86) can bind to CD28, we additionally explored the individual roles of these molecules in allergic responses to peanut. For the optimal generation of PE-specific IgE, CD86 interaction was crucial whereas CD80 only played a complementary role. This supports the idea that different costimulatory molecules may favor one type of responses (Th1 versus Th2).

Together, initiation of allergic responses to oral antigens crucially depends on costimulation. It remains to be established whether these pathways are also indispensable for the induction of challenge responses in allergic animals, which was not the case in a mouse model of allergic asthma [89].

Immature DCs, which are known to induce tolerance, express very low levels of costimulatory molecules, whereas mature DCs, which are associated with effector responses, express high levels of costimulatory molecules. Therefore it was long thought that the presence (or absence) of costimulation is decisive in the induction of tolerance versus immunity. However, recent evidence suggests that costimulation may play an active role in tolerance induction [90].

For instance, it has been shown that mucosally induced systemic T cell unresponsiveness to OVA requires CD40L-CD40 interactions [91]. However, for this study $CD40L^{-/-}$ mice were used, which display multiple functional defects including a failure to form germinal centers [89]. We (unpublished data) and others [92] have demonstrated that blockade of CD40L-CD40 interaction with anti-CD40L mAb does not impair the induction of oral tolerance.

With respect to tolerance, the B7 pathway may be more relevant. That is, both CD80 and CD86 can bind the CD28 homologue CTLA-4, which induces signals that suppress T cell activation. Since both B7 and CTLA-4 are upregulated by activated T cells, the postulated function of CTLA-4-B7 interaction was to downregulate effector responses. In a resting state, T cells do not express B7 or CTLA-4. However, resting DCs have been found to express low levels of CD80 and CD86. CTLA-4 has a much higher affinity for both B7 receptors than CD28 and it has been proposed that, when expressed in low levels, CD80 and CD86 might actually engage CTLA-4, thereby contributing to oral tolerance. In chapter 4 we demonstrate that CD80, but not CD86 interaction is involved in the induction of oral tolerance to peanut implying that CD80 and CD86 appear to have distinct as well as overlapping roles in regulating responses to orally administered antigens. Since anti-CTLA-4 treatment also abrogated oral tolerance (unpublished data) it seems likely that the CTLA-4-CD80 pathway contributes to oral tolerance induction. This is consistent with data showing that CD80 has a much higher CTLA-4 binding affinity and avidity than CD86 [93].

As CTLA-4 is constitutively expressed only on $CD4^+CD25^+$ Tregs, these cells appeared to be likely candidates providing CTLA-4 receptor binding under non-inflammatory conditions. Indeed, depletion of $CD4^+CD25^+$ Tregs also impaired the induction of tolerance (chapter 6). In line with these results, it has been shown *in vitro* that CTLA-4 utilizes CD80 and requires $CD4^+CD25^+$ Tregs for the inhibition of human T cell proliferation [94].

In addition to their involvement in the induction of oral tolerance, 'coinhibitors' are presumably required to maintain tolerance and to dampen immune responses. In the peanut hypersensitivity model, blockade of CTLA-4 signaling resulted in increased food allergic responses (chapter 5). Similar results were found following $CD4^+CD25^+$ T cell depletion (chapter 6) indicating that Tregs are involved in controlling the intensity of allergic responses, possibly via CTLA-4 signaling. Accordingly, in humans, polymorphisms in the CTLA-4 gene are associated with the severity of allergic responses [95].

Long-term CTLA-4 blockade also had profound effects on immune homeostasis and induced autoimmune responses (chapter 5). This is in agreement with other studies that revealed a crucial role for CTLA-4 in preventing autoimmune disease [96, 97]. Hence, CTLA-4 plays a key role as a negative regulator of T cell activation, leading to downregulation of T cell responses and to the preservation of T cell homeostasis, mucosal and peripheral tolerance to both self and non-self antigens. Potential inhibitory mechanisms include CTLA-4-initiated negative signaling, competition for B7 ligand [98] and B7-induced negative signaling (reverse signaling) [99]. Notably, although most research has focused on B7 present on APCs, B7 expressed on T cells may also be involved in CTLA-4-mediated regulation [47].

In the past decade, the B7 family has been extended with newly identified costimulatory and coinhibitory molecules. Some of them also have seemingly opposing functions. For example signaling via the interaction of ICOS and ICOS-L can induce effector as well as Treg responses [100]. Collectively, these data illustrate the complexity of costimulatory and coinhibitory pathways in different stages of the immune response. The functional outcome likely depends on the context, such as expression levels of molecules involved, the presence of danger signals, and antigen dose.

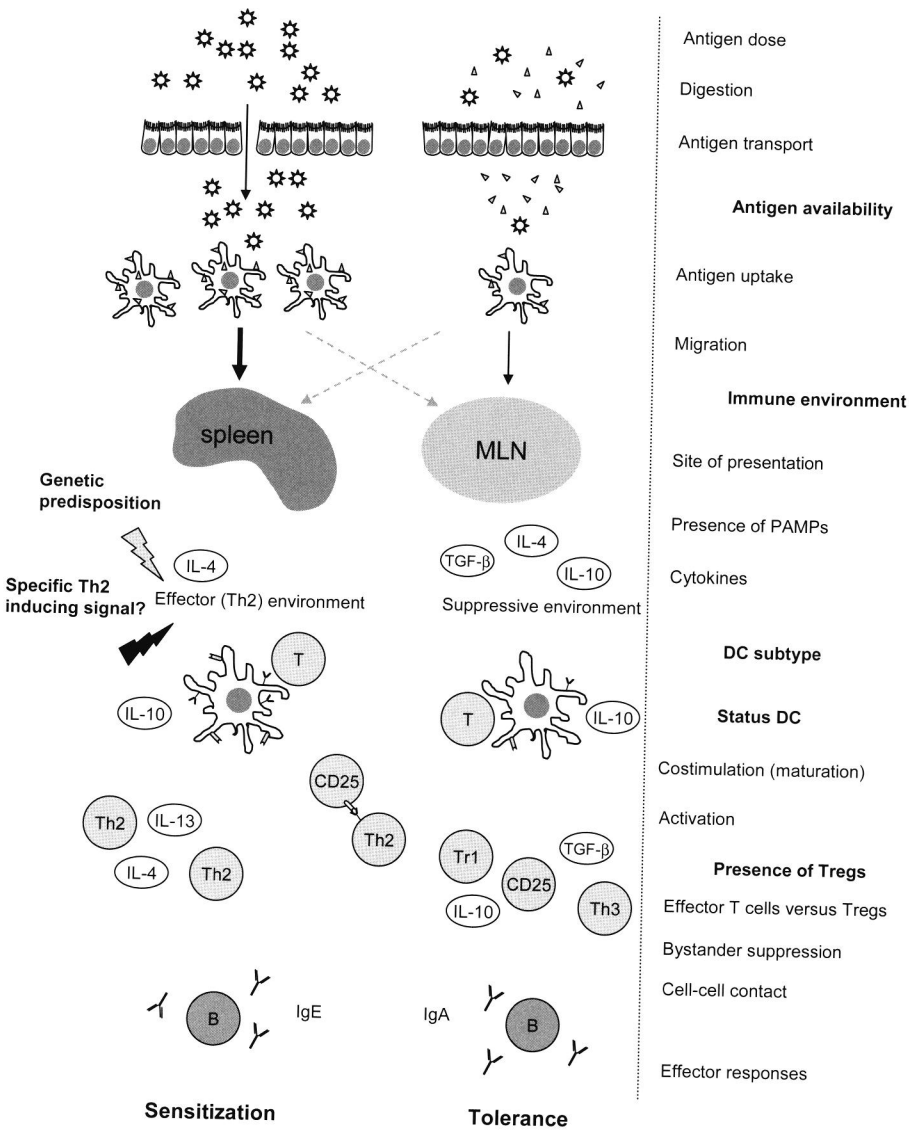

Figure 2. Factors that influence the outcome of an immune response to orally administered antigens (tolerance versus Th2 sensitization). These factors are discussed in detail in paragraph 8 of chapter 1 and paragraph 2 of this chapter.

2.5 Is CT hiding the answers?

The experiments described in chapter 5-7 demonstrated that interference with different mechanisms of regulation had profound effects on immune homeostasis, oral tolerance, and/or the intensity of sensitization responses but was never sufficient to induce allergic sensitization to harmless peanut proteins. In contrast, CT co-administration induced PE- and allergen-specific IgE responses and associated challenge reactions in 90-100% of the animals. Although the mechanisms underlying the adjuvant effect of CT were not subject of investigation in the present thesis, valuable lessons may be learned from the fact that CT on its own is able to create the unique set of conditions that favor Th2 sensitization to oral proteins.

CT is a bacterial endotoxin produced by *Vibrio cholerae* and is composed of two distinct subunits. The pentameric B subunit (CTB) is responsible for binding to the GM1 ganglioside receptor, present on virtually all mammalian cells. The A subunit has ADP-ribosyltransferase activity and is required for optimal adjuvant activity. Although the cellular mechanisms involved in CT-mediated promotion of Th2 cell development remain largely unknown, several factors have been identified that may contribute to the exceptional mucosal adjuvant properties of CT. First, CT has been reported to enhance antigen availability by increasing intestinal permeability [101]. However, when we measured gut permeability 2 hours or 2 days after CT administration by the use of Ussing chambers, no increase in permeability was found compared to control mice (unpublished data). Furthermore, after long-term oral exposure followed by oral challenge, increased gut permeability was observed in the PE plus CT, but not in the PBS plus CT group, suggesting that the allergic response and not CT is responsible for changes in barrier integrity in sensitized mice. Nevertheless, CT may increase antigen availability by enhancing transport of antigen over the epithelial barrier or by enhancing the uptake of antigen by DCs [102]. Second, CT seems to affect both DC recruitment as well as migration. CT has been shown to mobilize DCs near the intestinal epithelium [103] and to induce migration of DCs from the intestinal epithelium and supepithelial dome to the MLNs [103] and PPs [104]. Remarkably, the DC subset that seems mainly affected by CT is the $CD11c^{+}CD8\alpha^{-}$ subset, which has been implicated with Th2 responses in other studies [74, 105]. Third, CT induces *in vitro* and *in vivo* DC maturation accompanied by upregulation of costimulatory molecules such as CD80 and CD86 [102, 103, 106]. Fourth, CT-primed DCs promote Th2 polarization [107]. This is the most intriguing feature of CT with respect to the previous discussion about activation of DCs and Th2 skewing. Factors that have been implicated with the induction of Th2-polarizing capacity include inhibition of IL-12 by enhancing cAMP and upregulation of the receptor Jagged 1 [70], but the cellular mechanisms remain to be elucidated. Finally, in paragraph 2.3 of this chapter it is proposed that innate signals may be required for full activation of DCs, also in Th2 associated responses. Interestingly, Kawamura and colleagues have demonstrated that CT requires signaling through GM1 gangliosides to exert mucosal

adjuvant properties *in vivo* [102]. GM1 signaling results in NF-κB activation and may represent the bridge between innate immunity and adaptive immunity induced by CT co-exposure.

Together, these data clearly illustrate that lessons may be learned from CT regarding initiation of Th2 responses. Knowledge can be even further extended by examining the distinct effects of the two subunits of CT. The CT B subunit, which lacks adjuvant capacity, has been shown to be a potent immune modulator [108] and a potent inducer of (oral) tolerance [109]. Therefore, CT and CTB represent a unique pair of modulating proteins that differ with regard to enzymatic activity, but share GM1 receptor binding ability, allowing them to be exploited for mechanistic studies aiming at unraveling the regulatory elements that control mucosal tolerance versus sensitization.

3. Effector responses and clinical symptoms

In the peanut allergy model described in this thesis, PE-specific serum IgE antibody titers as well as serum levels of mast cell protease-1 (mmcp-1), which is released by mucosal mast cells upon oral challenge with PE, were used as measures for allergic sensitization. The challenge response nicely correlated with PE-specific IgE levels and was shown to be dose-dependent. Despite the evidence of mast cell degranulation we never observed clinical symptoms following oral challenge, in contrast to i.p. or subcutaneous challenge (unpublished data). Bashir and colleagues [23] have shown that the C3H/HeOuJ mouse is less likely to develop clinical symptoms than the C3H/HeJ mouse strain (see chapter 1). Although the present C3H/HeOuJ model has been shown to be suited to study mechanisms of tolerance versus sensitization (this thesis), the more susceptible C3H/HeJ strain may be preferred for the investigation of therapeutic strategies. Furthermore, when mechanisms of oral challenge responses or modulation of clinical symptoms are subject of study, one might consider sensitizing i.p. (with or without an adjuvant) since this route represents a more effective and faster sensitization method than oral exposure.

4. Future research; therapeutic strategies

Nature begins with the cause and ends with the experience; we need to operate in reverse.
Leonardo da Vinci

Incomplete understanding of the initiating factors and mechanisms underlying allergic sensitization has hampered the development of preventive and therapeutic strategies for allergic disease. Therefore, also in the future fundamental research will remain essential to develop a successful remedy.

Primary prevention aims to decrease the risk of allergy development in susceptible (atopic) individuals. It has been proposed that there is a 'critical window' in early life during which it may be possible to 'adjust' the developing immune system [110]. In this context the concept of probiotics has advanced rapidly over the last decade. The intestinal microflora has been shown to play a crucial role in the development of mucosal tolerance and a lack of microbial stimuli has been associated with increased allergy incidence (see paragraph 1.4.1, chapter 1). Probiotic therapy is based on the manipulation and/or stabilization of the gut flora by daily intake of live microbial supplements, which may have beneficial immunomodulating effects. Although some beneficial effects of probiotics on gut health have been demonstrated, so far the effect on food allergy incidence seems to be minimal and while the commercial world has already embraced the idea of probiotics, the scientific understanding of the interaction between intestinal microbiota and mucosal immunity is still in its infancy.

Nevertheless, as described in paragraph 2.3 of this chapter, innate stimuli in general may have a huge impact on shaping immune responses. In line with these thoughts, Li and collegues have shown that in the C3H/HeJ model of peanut allergy, immunotherapy with recombinant peanut allergens was only successful and persistent when heat-killed bacteria were co-adminestered with the allergens [111, 112]. In addition, several TLR ligands appear to have preventive as well as therapeutic effects in animal models of allergic disease [113] and engagement of certain TLR ligands on DCs has been shown to induce a potent negative signal that prevents Th2 development [114]. Intriguingly, it was recently demonstrated that TLR9 stimulation on pDCs leads to a reduction in the expression of the high-affinity IgE receptor, while crosslinking of this FcεRI receptor induces downregulation of TLR9 [72]. These results imply mechanisms by which innate and IgE-dependent immune responses counter-regulate one another and therefore the innate immune system represents an interesting tool for the modulation of allergic responses.

The ultimate goal of allergy therapy is allergen desensitization, i.e. re-establishment of non-responsiveness. Classic injection immunotherapy, which is used successfully in the treatment of allergic diseases such as rhinitis, is currently not recommended for food allergy

because of the side effects [115]. Reversal of an established Th2 response seems hard, but is clinically most relevant. The predominant role of DCs in activating and controlling adaptive immune responses makes them an interesting target for immunotherapy (reviewed by Kuipers and Lambrecht [116]). Strategies may include counter-balancing Th2 responses by Th1-inducing DCs or direct suppression of Th2 responses, possibly by the use of innate stimuli. Also costimulatory molecules have been proposed as potential targets for the treatment of allergic disease [117]. However, I believe that due to the complexity of costimulatory (and coinhibitory) pathways, their omnipresence, and their function in the generation and survival of T cells (including Tregs), the development of selective treatments with acceptable side-effects will be extremely difficult (discussed in chapter 4).

Finally, Th2 responses might be suppressed and/or counter-balanced via the induction of Tregs. In chapter 6 of this thesis we show that $CD4^+CD25^+$ Tregs control the intensity of allergic responses. Furthermore, it has been demonstrated that children who have outgrown their cow's milk allergy develop a population of $CD4^+CD25^+$ T cells with regulatory function in the peripheral blood [118]. Although Tregs may represent promising tools and targets for modulation of (food) allergic responses, extended knowledge on the specific signals that induce the differentiation of $CD4^+CD25^+$ Tregs and other Tregs such as Th3 and Tr1 cells *in vivo* remain to be elucidated. In addition, the development of *in vivo* strategies or *in vitro* culture conditions to proliferate and expand (antigen-specific) Tregs would greatly facilitate the use of these cells in novel therapeutic avenues.

Science moves, but slowly slowly, creeping on from point to point.

Alfred, Lord Tennyson

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Onze darmen worden dagelijks blootgesteld aan enorme hoeveelheden ongevaarlijke lichaamsvreemde stoffen zoals voedingscomponenten en commensale bacteriën en hun producten. Tegelijkertijd zijn de darmen de belangrijkste toegangspoort tot het lichaam voor pathogene micro-organismen. Het immuunsysteem van de darm heeft de moeilijke taak om onderscheid te maken tussen ongevaarlijke en potentieel gevaarlijke stoffen en een bijpassende immunologische reactie te initiëren. Voedseleiwitten worden opgenomen via het darmepitheel en normaal gesproken worden immunologische reacties tegen deze eiwitten zoveel mogelijk onderdrukt. Dit gedogen wordt immunologische tolerantie genoemd, en in principe vindt tolerantie inductie plaats tegen alle eiwitten die via de orale route het lichaam binnenkomen. Wanneer er iets fout gaat bij het induceren van de tolerantie, of wanneer de tolerantie wordt doorbroken, treedt er sensibilisatie op en kunnen er overgevoeligheidsreacties tegen voedseleiwitten ontstaan. Deze heftige immunologische reacties kunnen leiden tot voedselallergie.

Voedselallergie

De meest voorkomende vorm van voedselovergevoeligheid is IgE-gemedieerde voedselallergie. Bij deze vorm van allergie maakt het lichaam IgE antilichamen die specifiek zijn voor bepaalde eiwitten (de allergenen) uit het opgenomen voedsel. De IgE antilichamen circuleren in het bloed en binden aan mestcellen op verschillende plaatsen in het lichaam (bijvoorbeeld in de huid en in de darmen). De allergische reactie wordt veroorzaakt doordat na consumptie het voedselallergeen herkend wordt door mestcel-gebonden IgE wat direct leidt tot afgifte van mediatoren zoals histamine en enzymen die verantwoordelijk zijn voor de allergische symptomen. De allergische symptomen ontstaan meestal binnen een uur (soms zelfs seconden) na consumptie en kunnen variëren van jeukklachten in de mond tot huidreacties, maagdarmklachten, luchtwegklachten en in het ernstigste geval een levensbedreigende anafylactische shock. Geschat wordt dat IgE-gemedieerde voedselallergie voorkomt bij 6-8% van alle kinderen en bij 2% van de volwassen bevolking. De meeste kinderen groeien over hun voedselallergie heen maar soms is de allergie blijvend. Een aantal voedselgroepen worden met name geassocieerd met voedselallergie: koemelk, eieren, soja, granen, vis en schaaldieren, noten en pinda's.

Pinda is een van de meest allergene voedselsoorten. Kleine hoeveelheden pinda kunnen al ernstige reacties veroorzaken in allergische patiënten en pinda-allergie blijft meestal levenslang. Het is niet bekend waarom pinda zo ontzettend allergeen is. Op dit moment zijn er geen therapieën die pinda- of andere voedselallergieën kunnen genezen of een allergische reactie kunnen voorkomen. Vermijden van het voedsel is de enige oplossing. In het geval van pinda wordt dit bemoeilijkt doordat pinda-eiwitten in allerlei voedingsmiddelen voorkomen. Het aantal mensen dat lijdt aan pinda-allergie lijkt de laatste jaren te zijn toegenomen.

Voedselallergenen

De meeste voedselallergenen zijn in water oplosbare (opslag) eiwitten die in substantiële hoeveelheden in een voedingsmiddel voorkomen. Hoewel biochemische analyses geen eenduidige algemene kenmerken van voedselallergenen hebben opgeleverd zijn er wel een aantal biochemische eigenschappen die terug te vinden zijn bij een groot aantal voedselallergenen. De meeste allergene eiwitten zijn geglycosyleerd (gebonden suikerstructuren) en dat lijkt een belangrijke rol te spelen bij de herkenning door IgE antilichamen. Verder zijn veel allergenen relatief stabiel wat zich uit in ongevoeligheid voor digestie en processing, zoals verhitting. Deze eigenschap zorgt ervoor dat een hoge hoeveelheid intact eiwit kan worden aangeboden aan het immuunsysteem.

Een voedingsmiddel kan meerdere allergenen bevatten en van veel voedingsmiddelen zijn de individuele allergenen geïdentificeerd. De allergenen uit pinda hebben de namen *Ara h 1* tot en met *Ara h 9* gekregen.

Voedsel bestaat natuurlijk niet alleen uit eiwitten en er is weinig bekend over de invloed van andere voedingscomponenten zoals vetten, suikers en micronutriënten (tezamen voedingsmatrix genoemd) op de allergeniciteit van eiwitten. Het is mogelijk dat bepaalde voedingsstoffen een immuunstimulerende werking hebben en daardoor de kans op een overgevoeligheidsreactie tegen eiwitten vergroten. Bovendien zou de aanwezigheid van bepaalde voedingstoffen de digestie en/of immuunpresentatie van eiwitten kunnen beïnvloeden.

Het voorspellen van allergeniciteit

Omdat allergenen geen duidelijke unieke eigenschappen hebben is het moeilijk om de allergeniciteit van een eiwit te voorspellen. Met de opkomst van genetisch gemodificeerd voedsel is het voorspellen van allergeniciteit een steeds belangrijker issue geworden. Voedingsgewassen worden genetisch gemodificeerd om een grotere voedselopbrengst te verkrijgen of om ze ongevoeliger te maken voor ziektes. De vervaardiging van genetisch gemodificeerd voedsel zou gepaard kunnen gaan met het inbouwen van nieuwe allergenen. Er zijn een aantal testen voorgesteld om de potentiële allergeniciteit van eiwitten te identificeren: het testen van homologie met bestaande allergenen, specifieke serum screening (het meten van IgE binding aan het eiwit door gebruik te maken van positief humaan serum) en stabiliteitsassays waarbij digestie door maagsappen *in vitro* wordt gesimuleerd. Echter, om het sensibiliserend vermogen definitief vast te stellen lijkt het gebruik van *in vivo* modellen onontbeerlijk. Zo'n model moet in staat zijn immunogeniciteit van allergeniciteit te onderscheiden en moet uiteindelijk een relatieve allergeniciteitsschaal (die overeenkomt met de humane situatie) opleveren. In de afgelopen jaren zijn verschillende diermodellen ontwikkeld, gebruikmakend van de muis, rat, cavia, hond en het varken, maar tot nu toe is er nog geen gevalideerd model voor het voorspellen van allergenciteit van eiwitten. Bij het ontwikkelen van deze modellen moet rekening gehouden

worden met de keuze van de stam, de route van blootstelling, het gebruik van een adjuvant en het gebruik van gezuiverde eiwitten of gehele extracten.

Dit proefschrift

Allergieonderzoek in mensen wordt beperkt omdat het sensibilisatieproces niet bestudeerd kan worden (als mensen last krijgen van voedselallergie heeft de sensibilisatie al plaatsgevonden) en omdat de organen waar de sensibilisatie plaatsvindt (immuunsysteem van de darm) vrijwel onbereikbaar zijn voor metingen. Daarom hebben we in dit project verschillende muizenmodellen opgezet die het mogelijk maken om het proces van tolerantie en sensibilisatie te bestuderen en die uiteindelijk ook van pas kunnen komen bij het voorspellen van de allergenicteit van eiwitten. We hebben gekozen voor pinda als modelallergeen, omdat dit het meest heftigste allergeen is dat bekend is. Verder hebben we gebruik gemaakt van de gezuiverde pinda-allergenen *Ara h 1*, *Ara h 2*, *Ara h 3* en *Ara h 6* die in mensen voor de meeste overgevoeligheidsreacties zorgen.

Wanneer muizen oraal worden blootgesteld aan voedingseiwitten worden ze net als mensen tolerant. Het tot stand komen van tolerantie kan doorbroken worden door het meegeven van een immuunstimulerend stofje (adjuvant), zoals cholera toxine (CT). Door muizen wekelijks oraal bloot te stellen aan een pinda-extract (PE) in combinatie met CT ontwikkelden ze uiteindelijk een allergie tegen pinda, gekenmerkt door PE-specifieke IgE antilichamen in het bloed en mestcel reacties. Met behulp van dit model hebben we gekeken naar immuunresponsen tegen PE en tegen individuele pinda-allergenen en hebben we de invloed van de voedingsmatrix bestudeerd.

De mechanismen die ten grondslag liggen aan de inductie van tolerantie versus sensibilisatie tegen voedingseiwitten zijn grotendeels onbekend. Het verkrijgen van meer kennis van mechanismen is belangrijk voor het ontwikkelen van therapeutische strategieën tegen voedselallergie. Om die mechanismen te bestuderen hebben we gebruik gemaakt van 1. het pinda-allergie model (langdurige orale blootstelling aan pinda plus CT), 2. een pinda-ongevoeligheids model (langdurige orale blootstelling aan pinda zonder adjuvant) en 3. een oraal tolerantie model (orale voorbehandeling met pinda gevolgd door een challenge in de buikholte met PE plus adjuvant om de tolerantie aan te tonen). De rol van verschillende cellen en moleculen in tolerantie en sensibilisatie werd onderzocht door gebruik te maken van specifieke monoklonale antilichamen die de werking van moleculen kunnen stilleggen tijdens de orale blootstelling of bepaalde celtypen kunnen depleteren.

In **hoofdstuk 2** wordt het muizenmodel voor pinda-allergie beschreven. Na dosering van PE plus CT werden PE-specifieke maar ook allergeen-specifieke T cel en B cel responsen gemeten. Zowel tegen het gehele extract als tegen de individuele allergenen werden type 1 (IFN-γ en IgG2a) en type 2 (IL-4, IL-5, IgG1 en IgE) imuunresponsen gevonden. Allergische responsen worden vooral geassocieerd met type 2 imuunresponsen en type 1

responsen zouden in staat zijn om type 2 responsen tegen te gaan. De resultaten in hoofdstuk 1 suggereren echter dat reponsen tegen voedselallergenen niet geheel gepolariseerd zijn naar type 2. Bovendien, omdat de dieren met de hoogste allergische (IgE) responsen ook de hoogste productie van IFN-γ en IgG2a lieten zien, lijkt het erop dat de specifieke type 1 responsen niet in staat zijn om de allergische respons te verminderen. Dit is belangrijk om te weten aangezien type 1-inducerende therapieën zijn voorgesteld voor de behandeling van allergie.

Verder hebben we in dit hoofdstuk orale blootstelling vergeleken met parenterale blootstelling. Beide blootstellingroutes gaven (vergelijkbare) hoge IgE responsen tegen PE. De IgE responsen tegen de individuele allergenen (na blootstelling aan het gehele extract) lagen echter veel hoger na orale dan na parenterale toediening wat suggereert dat de orale toedieningroute geschikter is voor de identificatie van allergenen in een voedingsextract.

In **hoofdstuk 3** hebben we naar eigenschappen van de gezuiverde pinda-allergenen *Ara h 1*, *Ara h 2*, *Ara h 3* en *Ara h 6* gekeken al dan niet in combinatie met de voedingsmatrix. Om de immunogeniciteit te bepalen werd gebruik gemaakt van de popliteale lymfklier test. In deze test werden de allergenen of het hele PE in de voetzool van een muis gespoten en na 7 dagen werd de immuunrespons in de drainerende lymfklier uitgelezen. De pinda-allergenen bleken van zichzelf niet immunogeen, maar na blootstelling aan PE (allergenen in combinatie met de voedingsmatrix) werden er wel allergeen-geïnduceerde cytokine responsen gemeten. De voedingsmatrix lijkt dus een adjuvant werking te hebben op immuunresponsen tegen eiwitten, waarschijnlijk door het activeren van dendritische cellen (DC). Het maakte niet uit of we een vet of ontvet extract gebruikten wat suggereert dat vetten niet de belangrijkste adjuverende component zijn. Deze resultaten geven aan dat bij het voorspellen van de immunogeniciteit of allergeniciteit van een eiwit, de interacties met andere voedingscomponenten zeker ook aandacht verdienen.

Vervolgens hebben we de gezuiverde allergenen ook getest in het allergiemodel (orale blootstelling in combinatie met CT). Tegen alle allergenen werden IgE responsen gevonden en de aanwezigheid van de voedingsmatrix had hier geen effect op. Dit betekent dat de geteste pinda-allergenen in ieder geval stabiel genoeg zijn om uiteindelijk een IgE respons op te wekken en dat ze hiervoor geen beschermende voedingsmatrix nodig hebben.

Voor de optimale activering van T cellen zijn, naast de herkenning van het peptide-MHCII complex, specifieke signalen nodig die worden geleverd door de antigeen presenterende cel (APC) in de vorm van receptor-ligand interacties (costimulatie) en/of oplosbare signaal moleculen (zoals cytokinen). Een van de bekendste costimulatoire interaties is het CD28/CTLA-4-CD80/CD86 cluster. Binding van CD28 (op T cellen) aan CD80 of CD86 (op APC) leidt tot activering van de T cel, terwijl binding van CTLA-4 (op geactiveerde T cellen) aan CD80 of CD86 leidt tot suppressie van de T cel.

In **hoofdstuk 4** hebben we de betrokkenheid van costimulatie in allergische sensibilisatie tegen pinda onderzocht. Allereerst werden CD28 interacties geblokkeerd tijdens de sensibilisatiefase met behulp van CTLA-4Ig. In muizen behandeld met CTLA-4Ig werden geen pinda geïnduceerde cytokines, PE-specifieke antilichamen of challengereacties gevonden. Dit betekent dat CD28 costimulatie essentieel is voor de inductie van pindaallergie. Aangezien CD28 zowel aan CD80 als aan CD86 kan binden hebben we vervolgens de relatieve betrokkenheid van CD80 en CD86 onderzocht met behulp van specifieke blokkerende antilichamen. Voor een optimale PE-specifieke IgE respons bleek vooral CD86 en in mindere mate CD80 verantwoordelijk.

Omdat CD80 en CD86 interacties met CTLA-4 juist voor een onderdrukking van de respons zouden kunnen zorgen werd vervolgens de rol van CD80 en CD86 in de inductie van orale tolerantie bestudeerd. Blokkade van CD80 zorgde voor het doorbreken van tolerantie terwijl CD86 blokkade geen effect had op orale tolerantie-inductie.

Deze resultaten laten zien dat de CD28/CTLA-4-CD80/CD86 costimulatie cluster een belangrijke rol speelt bij de inductie van zowel sensibilisatie als tolerantie tegen pinda-eiwitten. CD86 lijkt de belangrijkste rol te spelen in de activering van de allergierespons (door te binden aan CD28) terwijl CD80 met name betrokken lijkt bij de onderdrukkende respons (door te binden aan CTLA-4).

CTLA-4 speelt een belangrijke rol bij de inductie en instandhouding van tolerantie tegen lichaamseigen eiwitten en waarschijnlijk ook tegen lichaamsvreemde eiwitten zoals allergenen. Om de rol van CTLA-4 in allergie te bestuderen werd gedurende orale sensibilisatie tegen pinda CTLA-4 geblokkeerd met een specifiek antilichaam, zoals beschreven in **hoofdstuk 5**. CTLA-4 blokkade zorgde voor een enorme verhoging van de allergische respons tegen pinda. CTLA-4 lijkt dus normaal gesproken betrokken bij het reguleren van de intensiteit van een allergische respons. Vervolgens werd gekeken of CTLA-4 blokkade ook kan leiden tot de inductie van allergie in het pinda-ongevoeligheids model (langdurige blootstelling aan PE alleen). Ondanks het enorme stimulerende effect van de anti-CTLA-4 behandeling op Th2 cytokineproductie en totale serum IgE waarden, werd er geen PE-specifiek IgE gevonden. CTLA-4 is dus niet beslissend bij de inductie van sensibilisatie tegen pinda. Bovendien leidt de aanwezigheid van grote hoeveelheden Th2 cytokinen in de mesenteriale lymfklieren niet automatisch tot allergische sensibilisatie tegen oraal toegediende eiwitten.

CTLA-4 wordt constitutief tot expressie gebracht door een bepaalde groep T cellen: de $CD4^+CD25^+$ T cellen. Deze cellen worden gekenmerkt door een hoge expressie van de IL-2 receptor (CD25) en van de transcriptiefactor *foxp3*. $CD4^+CD25^+$ T cellen kunnen geactiveerde T cellen deactiveren en worden daarom regulatoire T cellen genoemd. Het is bekend dat deze cellen een belangrijke rol spelen bij het onderdrukken van responsen tegen

lichaamseigen eiwitten, maar er is minder bekend over de rol van deze cellen in responsen tegen lichaamsvreemde eiwitten. Daarom hebben we in **hoofdstuk 6** gekeken naar de rol van $CD4^+CD25^+$ T cellen in de inductie van orale tolerantie en sensibilisatie tegen pinda. Het bleek niet mogelijk om in de afwezigheid van deze cellen orale tolerantie tegen pinda te induceren. Bovendien leidde depletie van de $CD4^+CD25^+$ T cellen voor en tijdens orale sensibilisatie tot verhoogde PE-geïnduceerde cytokineproductie, PE-specifieke IgE responsen en mestcel reacties na orale challenge. $CD4^+CD25^+$ T cellen spelen dus een belangrijke rol in het onder controle houden van allergische responsen. Omdat na 7 dagen al een duidelijk effect was te zien van $CD4^+CD25^+$ T cel depletie op PE-geïnduceerde cytokineresponsen lijken deze T cellen al in een vroeg stadium van sensibilisatie een rol te spelen. Verder bleek de verhoogde challengereactie het gevolg te zijn van de toename in PE-specifiek IgE aangezien depletie van $CD4^+CD25^+$ T cellen voor de challengereactie (en dus na sensibilisatie) geen effect had op de mate van mestcel degeanulatie. Wanneer $CD4^+CD25^+$ T cellen gedepleteerd werden gedurende blootstelling aan PE alleen werd er geen allergie inductie tegen pinda gevonden. Depletie van $CD4^+CD25^+$ T cellen leidt dus tot een verhoging maar niet tot een initiatie van allergische sensibilisatie.

Ondanks de verstoring van immuunregulatie na CTLA-4 blokkade (hoofdstuk 5) en na $CD4^+CD25^+$ T cel depletie (hoofdstuk 6) werd er in beide gevallen geen inductie van allergische respons tegen pinda gevonden. Een reden hiervoor zou de afwezigheid van geactiveerde DCs kunnen zijn. Het immuunsysteem van de darm is onder normale omstandigheden vooral gericht op het onderdrukken van immuunresponsen. Voor de initiatie van een effectorrespons zijn geactiveerde DCs nodig die gekenmerkt worden door hoge levels van costimulatie-expressie en de productie van stimulerende cytokinen. In de darm komen verschillende typen DCs voor en hoewel al deze DCs in een niet-geactiveerde staat tolerantie induceren worden bepaalde groepen DCs met name geassocieerd met tolerantie-inductie. Een van deze DCs is de plasmacytoide DC (pDC). In de longen leidt depletie van pDCs tot astmatische reacties tegen normaal ongevaarlijk eiwit. In **hoofdstuk 7** is het effect van pDC-depletie op orale tolerantie en sensibilisatie tegen pinda beschreven. pDC depletie had geen effect op de inductie van orale tolerantie tegen pinda en ook niet op PE-specifieke allergische responsen. Wel werden er na pDC-depletie verhoogde cytokineproductie en verhoogde hoeveelheden antilichamen gevonden. pDCs lijken dus niet cruciaal voor het induceren van tolerantie maar wel voor het behouden van immuunhomeostase.

De bevindingen in dit proefschrift worden bediscussieerd in **hoofdstuk 8**

Curriculum Vitae

Femke van Wijk was born in Roosendaal en Nispen on 8 December 1977. In 1996 she graduated *cum laude* from the Gertrudis lyceum in Roosendaal. In the same year she began her study Fundamental Biomedical Sciences at the Utrecht University. During her undergraduate period she completed research projects at the department of comparative endocrinology under the supervision of Dr. Maarten Blom and Prof. Dr. Henk Goos and at the department of experimental immunology at TNO under the supervision of Dr. Léon Knippels. After a 6 month project on molecular immunotoxicology at Faculté de Pharmacie Paris XI in Paris, supervised by Dr. Fanny Boilève and Prof. Dr. Marc Pallardy, she received her Master's degree *cum laude* in September 2001. She started working as a junior researcher at the department of immunotoxicology at the Institute for Risk Assessment Sciences (IRAS), Utrecht University. In February 2002 she began her PhD program at the IRAS in collaboration with TNO under the supervision of Prof. Dr. Willem Seinen, Dr. Léon Knippels, and Dr. Raymond Pieters. She investigated mechanisms underlying oral tolerance and allergic sensitization to peanut proteins, which resulted in this thesis. During her PhD period she was trained in the field of Immunity & Infection at the Eijkman Graduate School.

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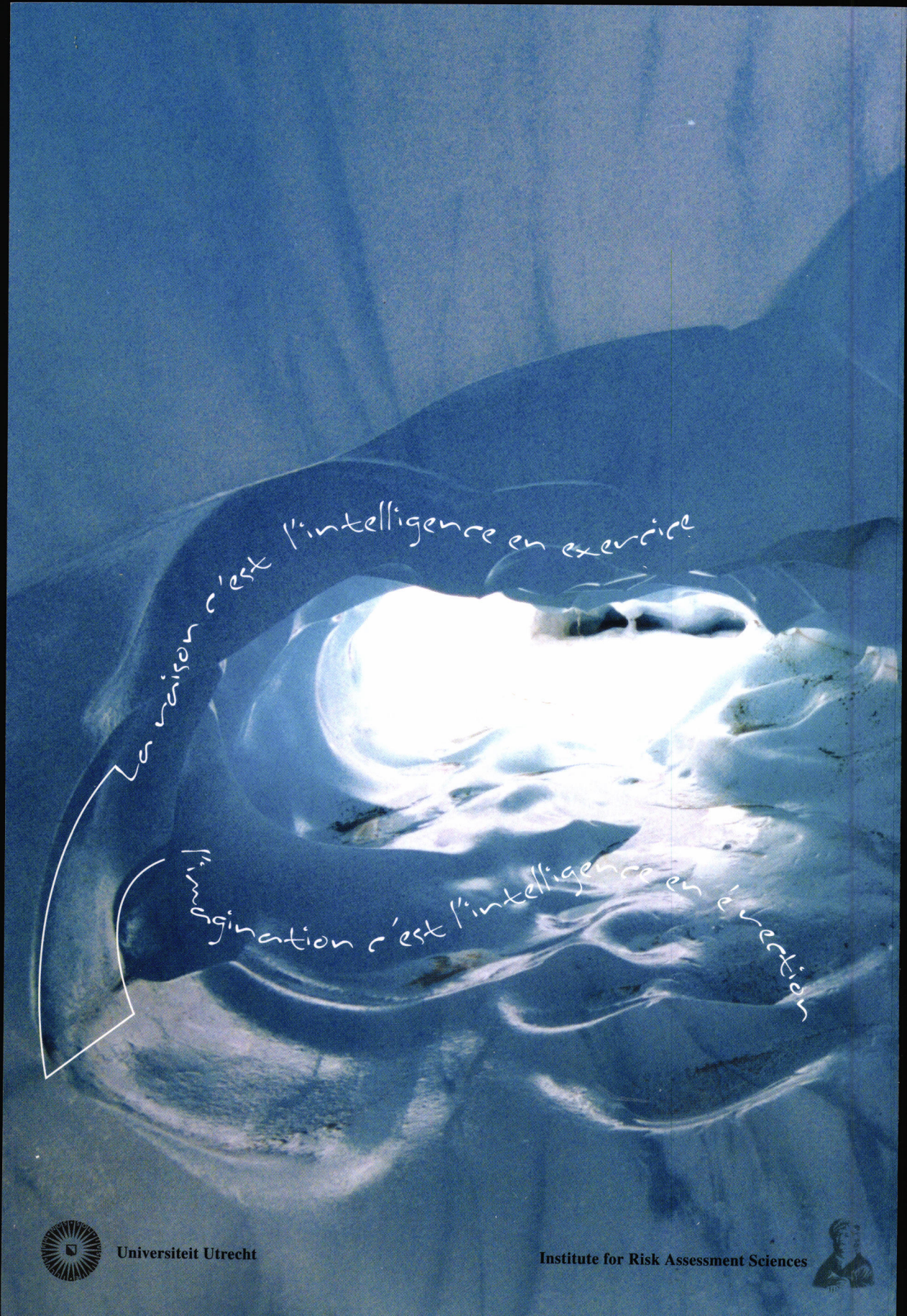

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