Regulation of allergic sensitization and oral tolerance to peanut

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Regulation of allergic sensitization and oral tolerance to peanut

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"A little learning is a dangerous thing; drink deep, or taste not the Pierian spring: there shallow draughts intoxicate the brain, and drinking largely sobers us again."

Alexander Pope, 1709

For Michel & Isabela

Contents

CHAPTER 1

Introduction

The gut-associated lymphoid tissue (GALT) is the largest and most complex immune organ in the body. Being part of the main route of contact with the external environment, the mucosal immune system is constantly challenged with external stimuli, sometimes from pathogens (bacteria, viruses, fungi, protozoa) or toxic substances, but mostly from harmless food or commensal flora. The GALT must constantly avoid potentially harmful adverse responses to food and commensal flora while still effectively combating dangerous pathogens. The typical response to harmless antigens that appear in the gut is the induction of local and systemic immunological tolerance, known as oral tolerance. The lack of oral tolerance due to absence of optimal immunoregulation may be involved in causing food allergy.

Food allergy

1. Prevalence and risk factors

It is estimated that currently 8% of children and 3-4% of adult individuals are suffering from some kind of food allergy [1, 2]. Peanut allergy accounts for the majority of severe food-related allergic reactions, it is most commonly associated with anaphylaxis, and may have a fatal outcome. In addition, although most childhood food allergies generally resolve spontaneously with age, peanut allergy resolves in only 20% of cases, resulting in a life-long allergy with a high impact on daily life [3, 4]. The proportion of children that developed food allergies has increased in developed countries over the past several decades, but there are reports that prevelance has now stabilized [5-8].

Among many factors involved in the development of IgE-mediated allergic diseases, the most important factor seems to be genetic predisposition to allergies, which is termed atopy [9]. The presence of an atopic phenotype in one or two parents will substantially increase the likelihood of an atopic phenotype in the child. However, atopy itself does not determine if a child will become allergic and to which particular protein an allergic response will develop, implicating environmental factors in the outcome of an allergic disease. These environmental factors may include the age at which an antigen is introduced, the composition of the gut microflora, the infectional status of the gastrointestinal tract at the time of antigen introduction, the dietary composition, and formula versus breastfeeding [10].

2. From sensitization to clinical manifestations

Although 30% of the population believe to have food allergies, the actual prevalence of food allergy is in fact much lower [11]. This may be due to the misconception that abnormal reactions to foods are a result of allergic responses. Food allergies affect only susceptible individuals and are most commonly IgE-mediated (summarized in Fig. 1). IgE-mediated food allergic reactions, also referred to as Type-1 food allergies, are characterized by antigen-specific IgE dependent release of mast cells [2].

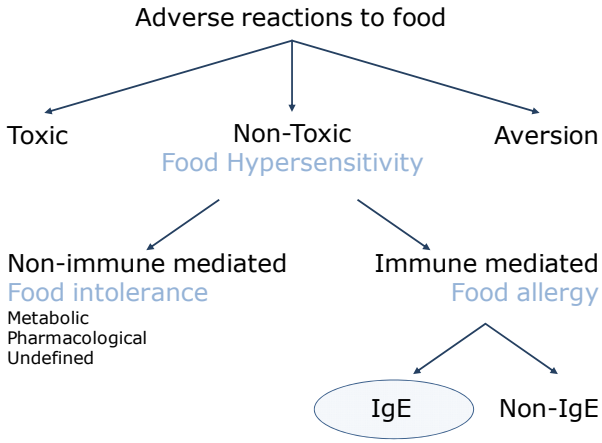

Figure 1.
Schematic presentation of the different adverse reactions to food
Adverse reactions to food include any abnormal reaction resulting from the ingestion of a food or food additive. It can be devided into toxic, non-toxic, and aversion. Aversion reactions are mainly due to a phycological dislike to the food, while toxic reactions are due to factors inherent in a food and will occur in any exposed individual when ingested at an appropriate dose. Non-toxic food reactions affect only susceptible individuals and are either non-immune mediated (caused by unique characteristics of the host, such as metabolic disorders) or immune mediated. Immune mediated food allergies are either IgE or non-IgE mediated reactions [2, 12].

After ingestion, food antigens which cross the intestinal barrier or are sampled by mucosal dendritic cells (DCs), come into contact with the intestinal immune system (Fig. 2). Antigen presenting cells (APCs) capture these antigens and present them on their surface in the context of major histocompatibility complex class II (MHCII) molecules which are then recognized by specific T cell receptors on naïve T cells. In combination with co-stimulatory molecules and the presence of IL-4 and IL-10, naïve T cells differentiate into effector Thelper (Th) 2 cells. A Th2-type response is predominant in food allergic reactions and the accompanying cytokine profile includes IL-4, IL-5, IL-10, and IL-13. This cytokine cocktail encourages B cells to develop into plasma cells which produce allergen-specific IgE and IgG antibodies. Secreted antigen-specific IgE antibodies are distributed systemically and bind to the high affinity receptor FcεRI on mast cells and basophils. Upon the next exposure, the food allergen will bind to and cross-link IgE antibodies on the surface of mast cells, provoking degranulation and release of mediators such as histamine, leukotrienes, cytokines, and proteases. The release of mediators will lead to a variety of cutaneous (urticaria, angiodema, eczema), gastrointestinal (nausea, vomiting, abdominal pain,

diarrhoea), respiratory (cough, wheeze), and systemic (hypotension) symptoms within minutes to hours after ingestion of the food. As previously stated, these anaphylactic reactions are life-threatening and are the leading cause of anaphylactic reactions treated in emergency departments in Westernized countries [13].

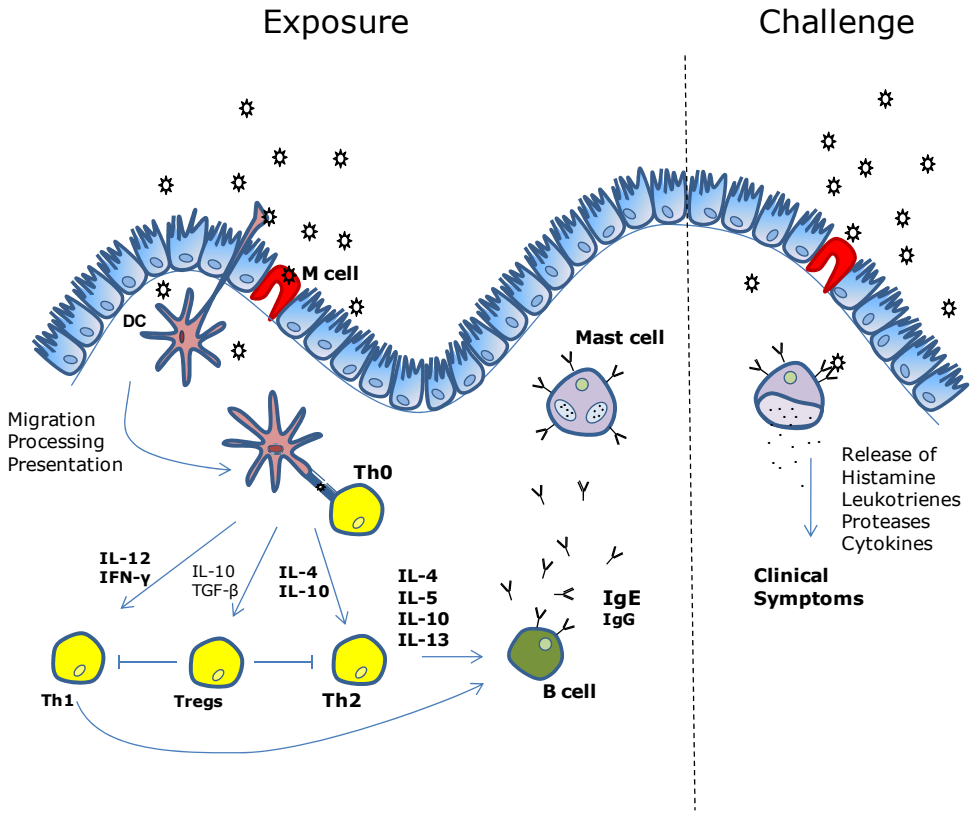

Figure 2.
IgE-mediated hypersensitivity
During sensitization, allergens cross the mucosal barrier or are sampled by mucosal DCs and are presented by APCs to naïve T cells. Following appropriate stimuli by APCs and cytokines in the local environment, naïve T cells develop into Th2 cells. Th2 cells produce cytokines such as IL-4 and IL-13 and stimulate B cells to produce allergen-specific IgE antibodies which in turn bind to mast cells. Following re-exposure to the allergen, the membrane-bound IgE molecules are cross-linked by interaction with the antigen. Cross-linking of the IgE molecules stimulates the release of preformed mediators in mast cells. These mediators initiate a cascade of events leading to clinical symptoms.

3 The hygiene hypothesis

The reason why food allergies had been increasing in developed countries is not known, but for almost 20 years the hypothesis described by Strachan, now popularly referred to as the 'hygiene hypothesis', has been invoked to explain why populations in Western countries appear to be developing allergic conditions at increasing rates [14]. The hygiene hypothesis suggests that reduced exposure to pathogens or their components early in life would no longer educate the maturing immune system and would result in an increased IgE response to allergens and a subsequent increase in allergic diseases. In the absence of infection, the default pathway of the immature immune system is thought to be toward a Th2-like immune response, which results in atopy. The hygiene hypothesis was mainly based on circumstantial evidence and little experimental support was available. More recently, an alternative interpretation of the hygiene hypothesis states that in the absence of sufficient microbial stimuli, normal regulatory immunological mechanisms may not develop resulting in a higher risk of diseases that have an immunological basis. In this respect, timely colonization of the intestine by the appropriate bacteria is a highly important stimulus to the developing immune system. Supporting this opinion, the composition of the gut microflora of allergic children was found to differ from that of healthy children [15]. Furthermore, antibiotic treatment which affects the composition of the microflora has been suggested to contribute to increased susceptibility to food allergy [16]. Similarly, oral tolerance induction is abrogated in germ-free mice [17]. The mechanism involved has been described as an imbalance of Th1/Th2 cells ([18, 19], paragraph 4), a reduced activation of Treg cells ([20], paragraph 5), or a failure in appropriate activation of innate effector cells and signalling molecules ([21], paragraph 6).

4. Th1/Th2 balance

Mosmann *et al* first described that $CD4^+$ T cell clones can be assigned in two different subsets [22]. Th1 cells are characterized by the secretion of IL-2 and IFN-γ and are associated with cell-mediated immunity. IL-12 produced by innate cells and IFN-γ produced by both NK cells and T cells polarize cells towards a Th1 cell differentiation through action of the signal transducer and activator of transcription 4 (Stat4), Stat1, and T box transcription factor T-bet. On the other hand, Th2 cells are characterized by IL-4, IL-5, and IL-13 secretion and are associated with humoral immunity. Differentiation of Th2 cells requires the action of GATA3 downstream of IL-4 and Stat6. Th1 and Th2 cells were described as being mutually inhibitory for the differentiation and effector functions of the reciprocal phenotype [23]. The commitment to the Th1 cell lineage is associated with the expression of the β2 chain

of the IL-12R complex and the IL-12Rβ2 induction is dependent on T-bet activation through IFN-γ and Stat1 signalling [24, 25]. The Th2 cell cytokine IL-4 appears to repress IL-12 signalling through inhibition of IL-12Rβ2 expression. Conversely, the Th1 cell cytokine IFN-γ represses Th2 differentiation by induction of T-bet. T-bet in turn induces Runx3 expression which together cooperates to further promote IFN-γ production by silencing the *Il4* gene and binding to the *Ifng* promoter and the *Il4* silencer [26, 27]. This cross-regulation may partly explain the strong biases towards a Th1 or Th2 response during many immune responses in mice and humans. However, it is now generally believed that $CD4^+$ T cell lineage differentiation is much more flexible and great plasticity has been observed in newly described subtypes, such as inducible Treg cells and Th17 cells, where the initial effector T cell responses are often followed by a shift to Treg cells needed to limit potential collateral tissue damage [28].

Food allergies are regarded as Th2-induced IgE-mediated immune reactions to otherwise harmless food components. Recent studies shown that this view is, however, oversimplified. A mixed Th1 and Th2 response in allergy has been recently observed in both human [29] and mice models [30], where sensitized cells are observed to induce not only the typical Th2 cytokines, such as IL-4, IL-5, IL-10, and IL-13, but also IFN-γ, as shown in figure 2. Manipulation of the Th1/Th2 balance shows to be either detrimental or beneficial for the development and treatment of allergic reactions. For instance, oral treatment of peanut-sensitized mice with liposome encapsulated rIL-12 caused an increase in IFN-γ but no difference in the Th2 cytokines produced, which shifted the Th2-balanced response to an enhanced Th1 response. This shift results in prevention and reversal of peanut hypersensitivity [31].

5. T regulatory cells

Oral tolerance, the specific suppression of cellular and/or humoral immune responses to an antigen by prior administration of the antigen by the oral route, is an important feature of the immune regulation in the gut [32]. Lack of oral tolerance due to the absence of optimal immunoregulation may be involved in causing food allergy. The mechanism by which oral tolerance is mediated includes deletion and anergy, as well as active suppression [33]. Active suppression, which involves the microenvironment and the presence of abundant IL-10 and TGF-β in the gut, favours the development of T cells with regulatory capacities. Preservation of oral tolerance is the result of a complex interplay of a number of cells, including a variety of subsets of Treg cells.

5.1 Naturally occurring and inducible CD4$^+$CD25$^+$ T regulatory cells

Murine naturally occurring Treg (nTreg) cells are characterized by an anergic phenotype, a constitutive high expression of the IL-2 receptor α chain (CD25), CD62, CD103, CTLA-4, GITR and the selective expression of the transcription factor forkhead box p3 (Foxp3). In contrast to murine nTreg cells, expression of FoxP3 on human nTreg cells does not seem to be specific for the regulatory phenotype. The development of CD4$^+$CD25$^+$ nTreg cells, occurs in the thymus but can also develop in the periphery and these cells can circulate back in the thymus, as reviewed by Rudensky [34]. Their immunosuppressive activity is most likely mediated by direct cell-cell contact in a process dependant on signalling by CTLA-4 [35] and cell-bound TGF-β [36]. However, other mechanisms, such as IL-10 and TGF-β secretion have been shown [37, 38].

The CD4$^+$CD25$^+$FoxP3$^+$ nTreg cells recognize mainly self-antigens and they have been shown to suppress the proliferation of other T cells *in vitro* and to protect from autoimmune and other inflammatory diseases *in vivo* in mice [39, 40]. It is unclear whether these cells can also recognize exogenous antigens. However, it has been demonstrated in mouse models that oral exposure to protein induces activation of functional CD4$^+$CD25$^+$ Treg cells in gut draining lymph nodes [41, 42]. These cells have been named inducible Treg (iTreg) cells and adoptive transfers of these cells have shown to regulate sensitization in ovalbumin TCR transgenic mice [43]. Van Wijk has shown that depletion of CD4$^+$CD25$^+$ Treg cells abrogates oral tolerance and increases sensitization to peanut [44]. Furthermore, inhibition of CTLA-4 which is present on both nTreg and iTreg cells can regulate the intensity of peanut allergic responses [45].

5.2 Adaptive T regulatory cells

In contrast to the CD4$^+$CD25$^+$FoxP3$^+$ nTreg and iTreg cells, adaptive T regulatory cells arise in the periphery from naïve cells. To date, there are no specific surface markers for adaptive Treg cells described, hindering studies on these cells. Adaptive Treg cells can be generated *in vitro* by repeated antigen stimulation and the presence of specific cytokines.

One subset of adaptive Treg cells are the Tr1 cells, which are characterized by low CD25 and no FoxP3 expression and its ability to produce high levels of IL-10 and significant amounts of TGF-β [46]. Tr1 cells have been identified in the intestinal mucosa of both mice and humans and can be induced *in vitro* in the presence of IL-10 [47-49]. *In vivo*, the generation of Tr1 is most likely controlled by subsets of immature or regulatory DCs, which express IL-10 and tolerogenic molecules [50] and their suppressive capacity can be reversed by neutralizing IL-10 and/or TGF-β [51]. Little is known about the antigen specificity of Tr1 cells, but studies have shown that

Tr1 cells specific for cecal bacterial antigens may be present in the intestine [52]. Furthermore, their role in the regulation of immune responses towards intestinal flora is indicated in mouse models of colitis [46].
A second subtype of adaptive Treg cells is the TGF-β-secreting Th3 cell. Weiner *et al* described these cells while studying oral tolerance. Feeding and sensitizing with an antigen leads to the activation of $CD4^+$ TGF-β-secreting cells which can suppress the activation of both Th1 and Th2 cells while inducing the production of IgA by B cells [53]. Although the mechanisms by which Th3 cells are induced are unknown, it is believed that the intestinal microenvironment contains high levels of TGF-β and IL-4 can stimulate Th3 differentiation [54]. Additionally, neutralization studies confirm that their suppressive property is mediated by TGF-β [55]. CTLA-4 signalling is implicated in their mode of action since anti-CTLA-4 antibodies can trigger TGF-β secretion by T cells [56]. However, since it has been reported that Th3 cells can express and are dependent on FoxP3, it is unclear if Th3 cells and inducible $CD4^+CD25^+FoxP3^+$ Treg cells are in fact the same cells [57].
Although Treg cells are activated in an antigen-specific manner, once activated, they can mediate bystander suppressive activity against other antigens [58]. All Treg cells described above have the ability to secrete TGF-β and IL-10 to different levels and both TGF-β and IL-10 can stimulate the induction of other Treg cells. Similarly, other non-$CD4^+$ mucosal regulatory cells have been described which may be affected by these regulatory cytokines, leading to their bystander regulation [59].

5.4 Other mucosal Treg cells

$CD8^+$ Treg cells were the first to be described in the 1980's and they were termed as "suppressor" T cells for their involvement in oral tolerance [60]. However, although different subsets have been described which regulate oral tolerance through soluble factors (IFN-γ and IL-6), cell-cell contact, and probably other undefined immunoregulatory molecules [61], $CD8^+$ Treg cells are not required for the induction and maintenance of oral tolerance [62].
Another intriguing mucosal regulatory T cell is the NK T cell. NK T cells from the liver of tolerized mice have been reported to transfer oral tolerance to naïve mice [63]. However, oral tolerance can still be induced in mice lacking NK T cells, indicating that they are not essential for the induction of oral tolerance. Furthermore, NK T cell activation was shown to abrogate oral tolerance [64]. Although the presence of NK T cells is necessary for the induction of Treg cells and oral tolerance to nickel [65], their function in the regulation of allergic sensitization remains controversial. NK T cells secrete both IFN-γ and IL-4, which may allow them to regulate both adaptive immune responses and the Th1/Th2 immune deviation [66, 67]. Additionally, NK T

cells share many surface markers with the innate NK cell, making it difficult to draw conclusions based on depletion and transfer experiments [68].
In addition, γδ T cells represent an important subset of mucosal T cells which have shown a homeostatic role in the maintenance of epithelium integrity through the production of cytokines and growth factors [71-74]. Furthermore, γδ T cells have been shown to secrete TGF-β in the intestine [75] and their presence is necessary for the induction of low-dose oral tolerance [76, 77].

6 Therapies

At present, there is no effective therapy available for the treatment of food allergy, and the only remedy is strict avoidance of the offending food. However, a number of novel forms of immunotherapy are being explored. A clinical trial study has shown that monthly injections of humanized recombinant anti-IgE antibodies significantly increased the threshold for allergic responses in peanut allergic-patients [78]. In theory, anti-IgE immunotherapy should protect against multiple allergens, but indefinite administration would be necessary to maintain its effect. Ideally, the most efficient treatment of the immune system would be a re-education of the immune system in order to (re-)establish oral tolerance, or at least cause a shift of the immune response to a less detrimental Th1 type immune response. As mentioned before, orally treating peanut-sensitized mice with liposome encapsulated rIL-12 which leads to an increase in IFN-γ results in prevention and reversal of peanut hypersensitivity [31]. Similarly, treatment with a food allergy herbal formula-2 (FAHF-2) has shown an induction of tolerance after an established peanut allergy which was mediated by IFN-γ producing T cells [79]. Treatments which involve the activation of the innate immune system have also demonstrated some beneficial effects, such as the use of CpG motifs which can also promote Th1 cytokine expression [80]. Stimulating tolerance induction by immunostimulation with tolerogenic epitopes, plasmid DNA immunotherapy or cytokine-modulated immunotherapy have also shown some promise in murine models of peanut anaphylaxis [81]. Similarly, although traditional immunotherapy has been too risky and unethical for the treatment of food allergies due to the high incidence of side effects, trials using powdered, aqueous or heat-denatured proteins have shown promising results [82-85].
Recently, there has been much focus on the role of Tregs in the prevention and therapy of mucosal inflammatory diseases, including food allergy. As previously mentioned, it was shown by van Wijk *et al* [44] that depletion of nTreg cells by anti-CD25 mAb treatment results in an increase of peanut specific allergic responses. It was also shown that depletion of nTreg cells abrogated peanut-specific oral tolerance.

Furthermore, children who outgrow their cow's milk allergy develop an increased population of $CD4^+CD25^+$ Treg cells in the peripheral blood. However, the specificity of the induced Treg cells is not known and a lack of knowledge on the specific signals which induced the differentiation of Treg cells hampers the development of treatment strategies. Furthermore, the role of other Treg cells in regulation of allergic responses and induction of oral tolerance should be further investigated.

Scope of this thesis

The use of animal models allows us to investigate the mechanisms involved in the onset of sensitization and breakdown of oral tolerance *in vivo* which is impossible to study in humans. In the present thesis, animal models for peanut oral sensitization and oral tolerance were used to explore different mechanisms of immunomodulation which may aid in the development of efficient therapies for food allergies in the future [30, 44]. The role of different immunoregulatory cells was studied (NK cells, chapter 2 and T regulatory cells, chapter 3). Additionally, microbial interventions were set under investigation, such as the effect of a reovirus infection during allergic sensitization (chapter 4). Lastly, a *Lactobacillus plantarum* strain of probiotics was considered for regulation of the peanut allergic response (chapter 5).

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CHAPTER 2

NK cells are Important Regulators of Allergic Sensitization and Oral Tolerance Induction to Peanut

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Abstract

Background Food allergies are important causes of life-threatening hypersensitivity reactions. Peanut sensitization in C3H/HeOuJ mice induces a strong peanut extract (PE)-specific serum IgE response, and this response is correlated in particular with the development of T-helper type 2 (Th2)-like T-cell responses which may be counteracted by type 1 cytokines, such as IFN-γ. Natural killer (NK) cells, being well-known IFN-γ-producing innate immune cells, may have an important role in shaping the immune response against peanut allergens.
Objective The aim of the current study was to determine the role of NK cells in sensitization and oral tolerance induction to peanut extract (PE)
Methods In C3H/HeOuJ mice models of allergic sensitization and oral tolerance to peanut, NK cells were depleted using an anti-Asialo GM1 (anti-ASGM1) antiserum prior to induction of low dose oral tolerance or during sensitization to PE. The induction of specific antibodies, mast cell degranulation, cytokines, and distribution of lymphocytes were analysed.
Results Four days following PE plus CT sensitization an increase in NK cells was measured in the MLN of sensitized mice compared to control mice. Anti-ASGM1 treatment showed 75-90% depletion of NK cells and subsequent PE sensitization resulted in higher levels of PE-specific antibodies, increased mast cell degranulation upon an oral challenge and increased production of Th2 cytokines following *in vitro* restimulation of splenocytes with PE. Additionally, oral tolerance to PE was abrogated in NK cell-depleted mice, which was apparent in particular at the level of IgE production and of cytokine production by T cells.
Conclusion Results indicate that NK cells are able to modulate oral sensitization and initiation of tolerance to peanut allergens. Hence, NK cells are interesting targets to design possible therapies for food allergies.

Introduction

Food allergy has emerged as a major health problem in westernized countries [1] and peanut allergy accounts for the majority of severe food-related allergic reactions, being most commonly associated with anaphylaxis and fatal outcome [2]. Peanut allergy usually results in a life-long allergy with high impact on daily life since no therapies are available and the only remedy is strict avoidance of the allergen [3]. Revealing the underlying mechanisms of sensitization to food proteins may contribute to the development of new treatment methods for peanut allergy.

During initiation of an adaptive immune response, many factors shape the commitment of naïve T cells, but the most important appears to be the cytokines present at the time of priming. A Th2-like activation is strongly promoted by IL-4 [4, 5]; while for a Th1-like activation both IFN-γ and IL-12 play a critical role [6, 7]. Allergic sensitization to peanut extract (PE) is classically characterized as a Th2-induced IgE-mediated phenomena counterbalanced by Th1 phenomena. Although recent studies have suggested that the Th2 hypothesis is oversimplified [8, 9], it is still generally believed that a Th1 shift in an allergic immune response may be beneficial in treating allergic disease [10].

Cells of the innate immune response may have an important role in shaping the initial T cell activation. Activated NK cells show rapid secretion of several cytokines, in particularly IFN-γ and they have been suggested to play an immunomodulatory role [11-14]. Trop *et al* (2003) has shown that NK cells are required for peripheral tolerance induction and that they can support anti-inflammatory responses [15]. More recent data has focused on human NK cells and their functions *in vitro*. Three functional subsets of NK cells may exist which are distinguished based on their cytokine secretion profiles. NK1 and NK2 cells have a Th1 and Th2-type cytokine profiles, respectively, while regulatory NK cells secrete mainly IL-10 [13, 16]. NK1 cells were able to inhibit the *in vitro* production of IgE by peripheral blood mononuclear cells (PBMC) and regulatory NK cells have been shown to suppress antigen-specific T cell responses *in vitro* in an IL-10-dependent manner. However, it is not clear if NK cells of mice also demonstrate regulatory characteristics [17] and the role played by NK cells in activation, induction, and regulation of immune responses to exogenous antigens such as peanut allergens *in vivo* remains controversial [18, 19].

The aim of the current study was to determine the role of NK cells in sensitization and oral tolerance induction to peanut extract (PE) in previously described mouse models [9]. Asialo-GM1 (ASGM1) is expressed on NK cells and to a lesser extent on activated macrophages, CD8 cells and NKT cells [19]. *In vivo* treatment with anti-ASGM1 antiserum showed depletion of NK cells, but not of NKT cells, in mesenteric

lymph nodes (MLN), spleen, and liver. Our results indicate that ASGM1$^+$ cells, presumably NK cells, are important regulators in the process of peanut-induced allergic sensitization as well as peanut-specific oral tolerance.

Materials and Methods

Mice

Female, specific pathogen-free C3H/HeOuJ Ico mice, 4-5 weeks of age, were purchased from Charles River (Lyon, France) and were left to acclimatize for 1 week. 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 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 Utrecht University.

Chemicals, reagents and monoclonal antibodies

Rabbit anti-ASGM1 was purchased by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cholera toxin (CT) was purchased from List Biological Laboratories, Inc (Campbell, California, USA). All other chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands) unless otherwise stated.

Preparation of peanut extract (PE)

Peanuts from Golden Peanut (Alpharetta, Georgia) were kindly donated by Imko Nut Products, the Nut Company (Doetinchem, the Netherlands). Protein extract was made by blending 100 g peanuts with 500 ml 20 mM Tris buffer (pH 7.2). After 2 h blending at intervals of 20 minutes for a minute at room temperature, the aqueous fraction was collected by centrifugation (3000 g, at 4°C for 30 min). The aqueous phase was subsequently centrifuged (10 000 g at 4°C for 30 min) to remove residual traces of fat and insoluble particles. Protein concentrations were determined using Bradford analysis with BSA as a standard. Extract (peanut extract, PE) contained typically 32 mg/ml protein and was stored at -20°C. Reducing SDS-PAGE from the extracts showed protein bands between 14 and approximately 100 kDa (not shown).

Anti-Asialo-GM1 antibody treatment on naïve mice

Naïve mice (n=3) were treated with 40 µl anti-Asialo-GM1 (anti-ASGM1), diluted to 400 µl in NaCl, 2 days before sacrifice. MLN, spleen, peyer's patches (PP), and liver lymphocytes were isolated for flow cytometry analysis.

Sensitization protocol

Mice (n=8) were orally exposed to PBS plus CT (control) or peanut extract (PE) plus CT (sensitization). Oral exposure was performed by intragastric dosing of 6 mg of PE plus 10 µg of CT on 3 consecutive days, followed by weekly dosing of PE plus CT (day 7, 14 and 21). During sensitization, mice were injected i.p. with 20 µl anti-ASGM1 (diluted to 200 µl in NaCl) on specified days (fig. 1). At day 28 all groups received an oral challenge with 16 mg PE. Mice were sacrificed on day 4 or day 29.

Oral Tolerance protocol

Mice (n=8) were exposed by intragastric dosing of 1 mg of PE in 200 µl PBS on 3 consecutive days. Control mice (n=8) were exposed to 200 µl PBS alone. Intraperitoneal challenges were given on day 21 and 28 with 100 µg PE in alum (25 mg/ml). Mice were injected i.p. with 20 µl anti-ASGM1 (diluted to 200µl in NaCl) on specified days (fig. 1). Mice were bled and sacrificed on day 36.

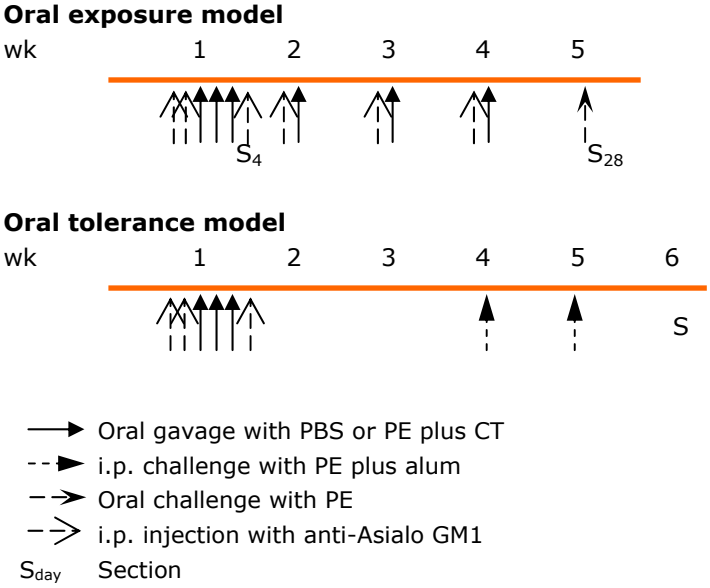

Figure 1
Schematic view of treatment protocols

Isolation of lymphocytes

To obtain single cells from PP, collected PP were shaken for 20 min at 37 °C in HBBS containing 15 mM Hepes, 100 U/ml penicillin, streptomycin 100 µg/ml (Invitrogen,

Paisley, Scotland), 5 mM EDTA, 1 mM DTT and 10% FCS (Greiner Bio-one, Germany) (HBBS/EDTA), washed once in RPMI/FCS, cut in small fragments, and digested in RPMI/FCS containing collagenase/DNAse. Cells were passed through a 70 µm filter to obtain single cells, and washed once in HBBS/ EDTA.
Lymphocytes from mesenteric lymph nodes (MLN) and spleen were obtained by carefully squeezing the organ through a 70 µm gauze, after which single cells were washed once in RPMI/FCS. To remove erythrocytes in spleen, cells were incubated with RBC lysis buffer (0.16M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA pH 7.4) for 1 min, and washed once in PBS. All cell suspensions were kept on ice.
Isolated liver was crushed through a stainless mesh and flushed with PBS. In order to remove hepatocytes, cells were centrifuged at 50 g for 1 minute and supernatant was collected and supernatant was centrifuged one more time. Thereafter, lymphocytes present in the supernatant were washed twice with PBS and passed through a 70 µm filter. Collected cells are further centrifuged at 250 g for 10 minutes. Cells are resuspended in 45% percoll at 3 mL per liver and 3 mL of 67.5% percoll is than carefully added. Cells are span for 20 minutes, 600 g at room temperature. Cells present in the interface was collected and washed once with PBS.

Flow cytometry
For flow cytometric analysis, 2 x 10^5 cells were pre-incubated with anti-CD16/CD32 (2.4G2) in staining buffer (PBS containing 0.25% BSA, 0.5 mM EDTA and 0.05% NaN_3) and thereafter cells were stained with fluorecein isothiocyanate (FITC)-, R-phycoerythrin (RPE)-, peridinin chlorphyll protein (PerCP)- or allophycocyanin (APC)-labeled antibodies. Cells were incubated for 30 min at 4 °C in the dark. All samples were stored in 0.4% formaldehyde until acquisition. Cell characterization was based on following antibodies (obtained from e-Bioscience San Diego, California, USA or BD-Pharmingen, San Diego, California, USA): CD49b (DX5); CD3 (145-2C11); CD8α (53.6-7); CD19 (1D3), CD11b (M1/70); CD11c (N418); CD103 (2E7); B220 (RA3-6B2). Data were acquired with a FACSCalibur (Becton Dickinson) and analysis was performed using Weasel (The Walter And Eliza Hall Institute of Medical Research, Melbourne, Australia).

Measurement of serum IgG1, IgG2a, and IgE antibodies
Blood samples were collected at various time-points and stored at -20°C until analysis. Levels of PE-specific IgE, IgG1 and IgG2a were determined by ELISA. Plates (highbond 3590; Costar, Cambridge, MA, USA) were coated overnight with 10 µg/mL PE (for IgG1 and IgG2a detection) or with 1.5 mg/mL purified rat anti-mouse IgE (BD Pharmingen) in PBS, followed by 1 h blocking with ELISA buffer (50 mM Tris, 136.9 mM NaCl, 2 mM EDTA, 0.5% Tween and 0.5% BSA). Each test serum was optimally

diluted and incubated for 2 hrs. For PE-specific Ab levels, a sera pool of mice sensitized with PE plus alum by i.p. injection once a week for 3 weeks was used as reference serum (start dilution 1 : 10) and a standard curve of the reference serum was included to determine antibody levels (arbitrary units). For detection of PE-specific IgG1 and IgG2a, alkaline phosphatase-conjugated IgG1, IgG2a (polyclonal goat anti-mouse), 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 Abs, a PE-digoxigenin (DIG) conjugate solution (diluted in High Performance ELISA Buffer, from Sanquin, San Diego, California, USA) 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.

Cell culture and cytokine measurement

Spleen and MLN single cell suspensions, 6.25 x 10^5 cells/mL in RPMI 1640 (Life Technologies, Paisley, Scotland) containing 10% fetal calf serum (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. The IL-10 (BD Pharmingen, San Diego, California, USA) and IFN-γ, IL-4, IL-5, IL-13 ELISA (eBiosciences, San Diego, California, USA) were performed in accordance with the manufacturer's instructions.

Measurement of serum 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 Scientific Ltd, Midlothian, Scotland). ELISA was performed according to the manufacturer's instructions.

Statistics

Data were analyzed using GraphPad Prism 4 software. The differences between group means were determined by using one-way ANOVA with Bonferroni as *post hoc* test. Statistical analyses were performed following logarithmic transformation (to achieve normal distribution).

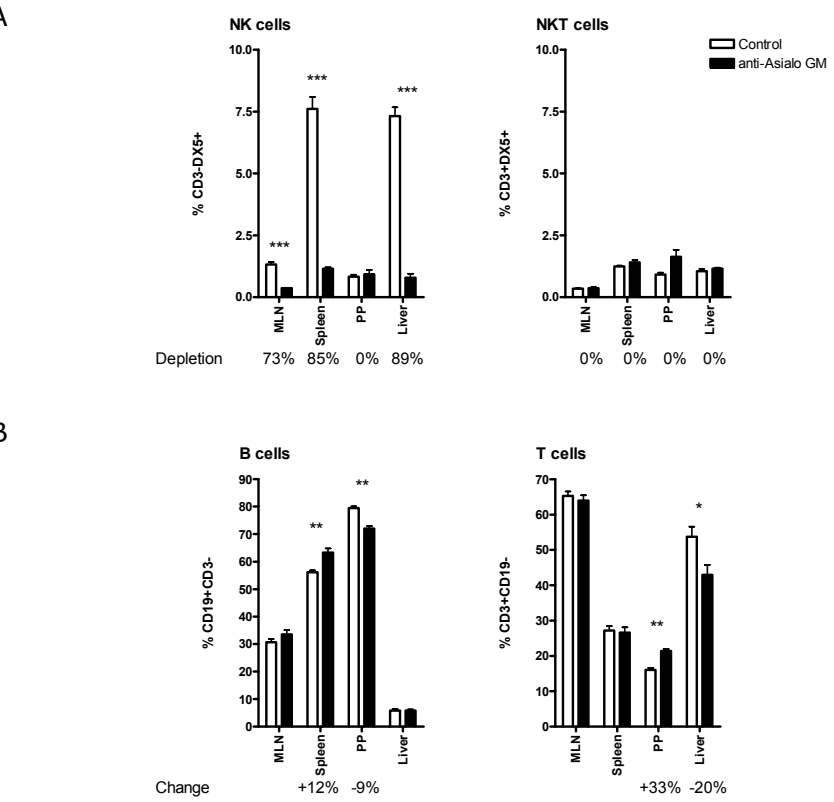

Figure 2
Effect of anti-ASGM1 treatment on different cell populations in the MLN, spleen, PP, and liver of naïve mice.
Naïve mice were treated with anti-ASGM1 or PBS. Two days after treatment, the MLN, spleen, PP, and liver lymphocytes were isolated. The expression of CD49b, CD3, and CD19 were determined by flow cytometry. Data are presented as percentage of total cells ± SEM of 3 mice per group. % of depletion (A) and % change (B) between anti-ASGM1 treated and PBS treated groups are stated. *, **, ***, Significantly different ($p<0.05$, $p<0.01$, $p<0.001$) from control and anti-ASGM1 treated group.

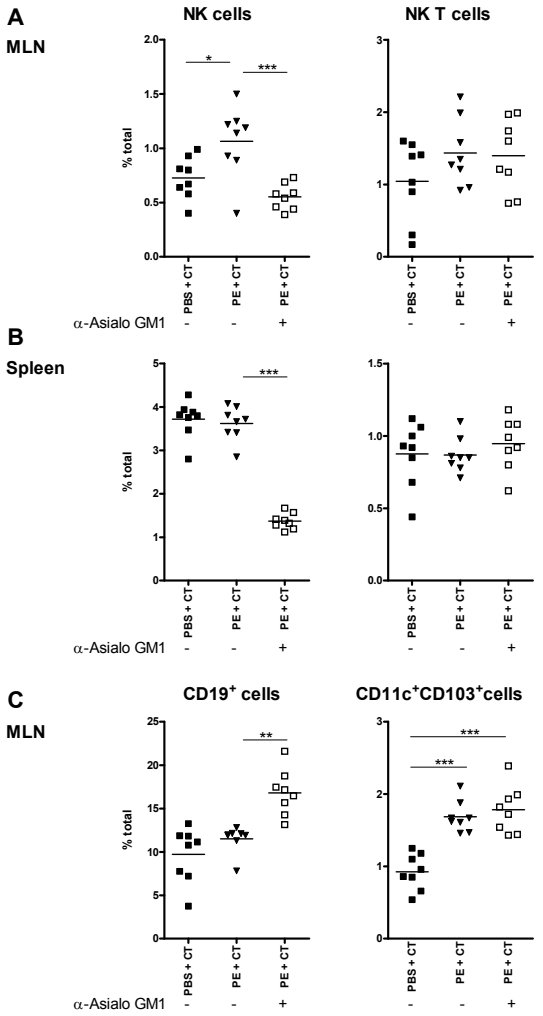

Figure 3
Effect of anti-ASGM1 treatment on the induction of allergic sensitization to PE.
Five weeks after oral exposure to PBS +CT or PE + CT, mice received an oral challenge with PE, after which they were sacrificed a day later. Indicated group was treated with anti-ASGM1 during the first 2 weeks of oral exposure. (A) PE-specific serum IgE antibody levels were determined in serum collected after 5 wks of oral exposure. (B) mMCP-1 level was measured in serum collected within 45 minutes after an oral challenge with PE. (C) Splenocytes were cultured for 96 hrs in the presence (black bars) or absence (white bars) of PE. In the supernatant IFN-γ, IL-10, IL-5, and IL-13 cytokine levels were determined by ELISA. Data are presented on a log scale as the mean arbitrary unit (A), ng/mL (B), or pg/mL (C) per group. *, **, ***, Significantly different ($p<0.05$, $p<0.01$, $p<0.001$) from PBS + CT control group. #, Significantly different ($p<0.001$) from PE + CT control group.

Results

Anti-ASGM1 antibody treatment in naïve mice leads to major depletion of NK cells but not of NKT cells in MLN, spleen, and liver.
Two days following treatment with anti-ASGM1, the number of NK cells ($DX5^+CD3^-$) in MLN, spleen and liver was decreased compared to controls by 73%, 85%, and 89%, respectively (Fig. 2a). No change in NK cell numbers was observed in the PP. $DX5^+CD3^+$ NKT cells were not affected by anti-asialo-GM1 treatment (Fig. 2a).
Since ASGM1 is not only expressed by NK cells, other cell populations were analyzed. For B ($CD19^+$) cells, a 9% decrease in the PP and a 12% increase in the spleen of treated mice were detected. Similarly, T ($CD3^+$) cells were less apparent in the liver by 20% and a 33% increase was measured in the PP of treated mice (Fig. 2b). No differences were observed for conventional ($CD11c^+$) dendritic cells (DCs) following anti-ASGM1 treatment in any of the organs analyzed (data not shown).

NK cells numbers are increased in the MLN of PE-sensitized mice.
To study the effect of anti-ASGM1 treatment on various immune cells during sensitization, mice were treated with anti-ASGM1 prior to PE sensitization with CT and sacrificed on day 4. Flow cytometric analysis showed that PE sensitization resulted in an increase in NK cell numbers compared to non-sensitized mice in the MLN, but not in spleen. Again, anti-ASGM1 treatment showed a drastic loss of NK cells in the MLN and spleen of PE sensitized mice (Fig. 3).
Further analysis of different cell populations revealed that PE sensitization in combination with anti-ASGM1 treatment increased B-cells in the MLN compared to both control and untreated PE-sensitized mice (Fig. 3). Among the various treatment groups no differences were observed within the different T cell subtypes ($CD3^+CD4^+$ and $CD3^+CD8^+$) measured in the MLN (data not shown). Furthermore, PE sensitization allowed for an increase in cDC numbers in the MLN, which is not affected by anti-ASGM1 treatment (Fig. 3).

Anti-ASGM1 antibody treatment increases allergic sensitization to PE.
To investigate the role of $ASGM1^+$ cells in controlling allergic sensitization to peanut, mice were orally sensitized to peanut for 4 weeks and anti-ASGM1 treatment was given weekly prior to each sensitization (Fig.1). Following PE sensitization, untreated mice developed PE-specific IgE, IgG1 and IgG2a responses (Fig. 4a), as previously observed [9]. Anti-ASGM1 treatment increased the levels of all PE-specific antibodies measured. Additionally, serum collected 30 minutes following PE challenge was analyzed for mMCP-1, a measure of mast cell degranulation.

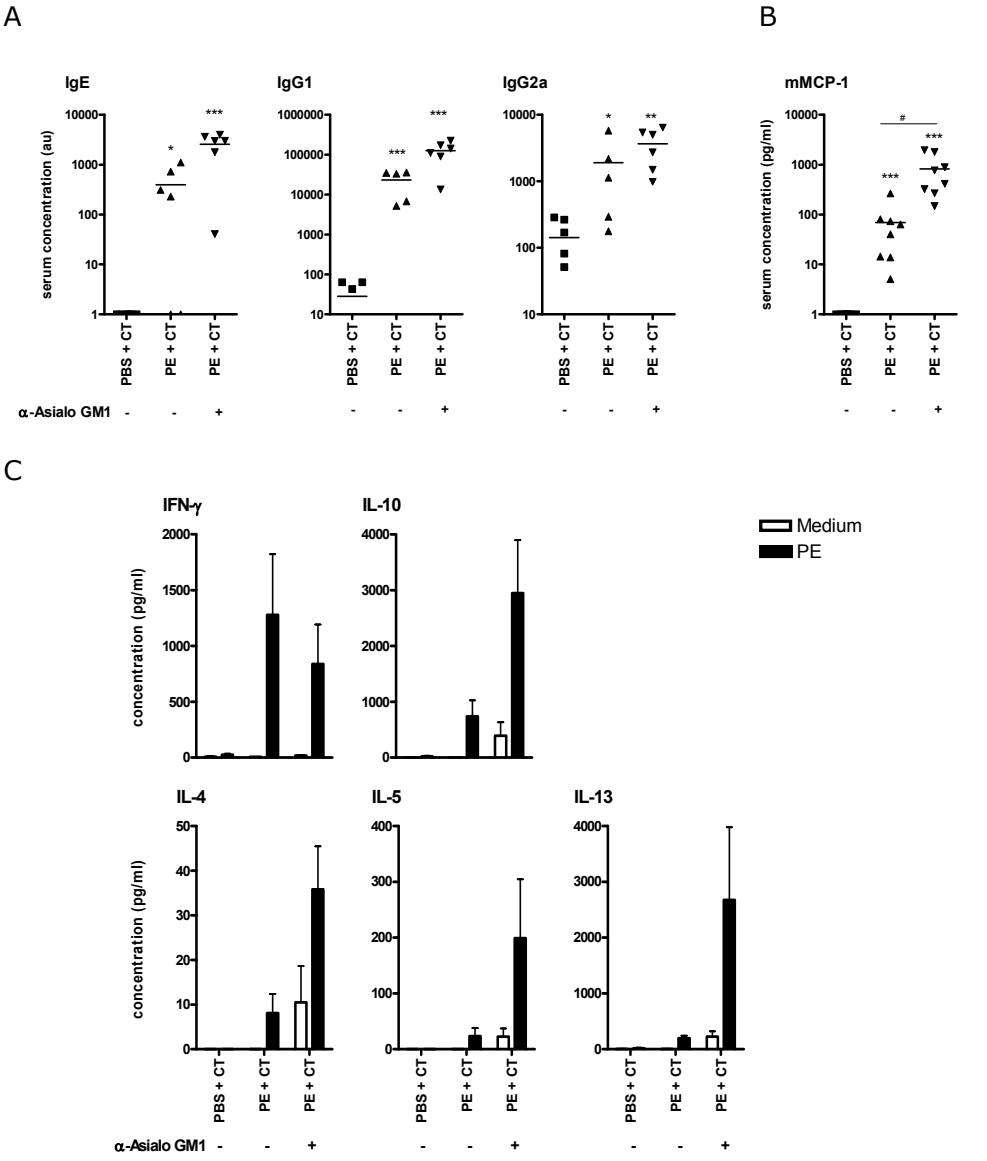

Figure 4
Effect of anti-ASGM1 treatment on different cell populations in the MLN and spleen of PE sensitized mice.
Mice were orally exposed to PBS + CT or PE + CT for 3 consecutive days. Indicated group was treated with anti-ASGM1 on day -2 and -1. Mice were sacrificed on day 4 and the MLN and spleen were isolated. The expression of CD49b, CD3, CD19, CD11c, B220, and CD103 were determined by flow cytometry. Individual mice are indicated and data are presented as percentage of total cells. *, **, *** Significantly different ($p<0.05$, $p<0.01$, $p<0.001$) from specified groups.

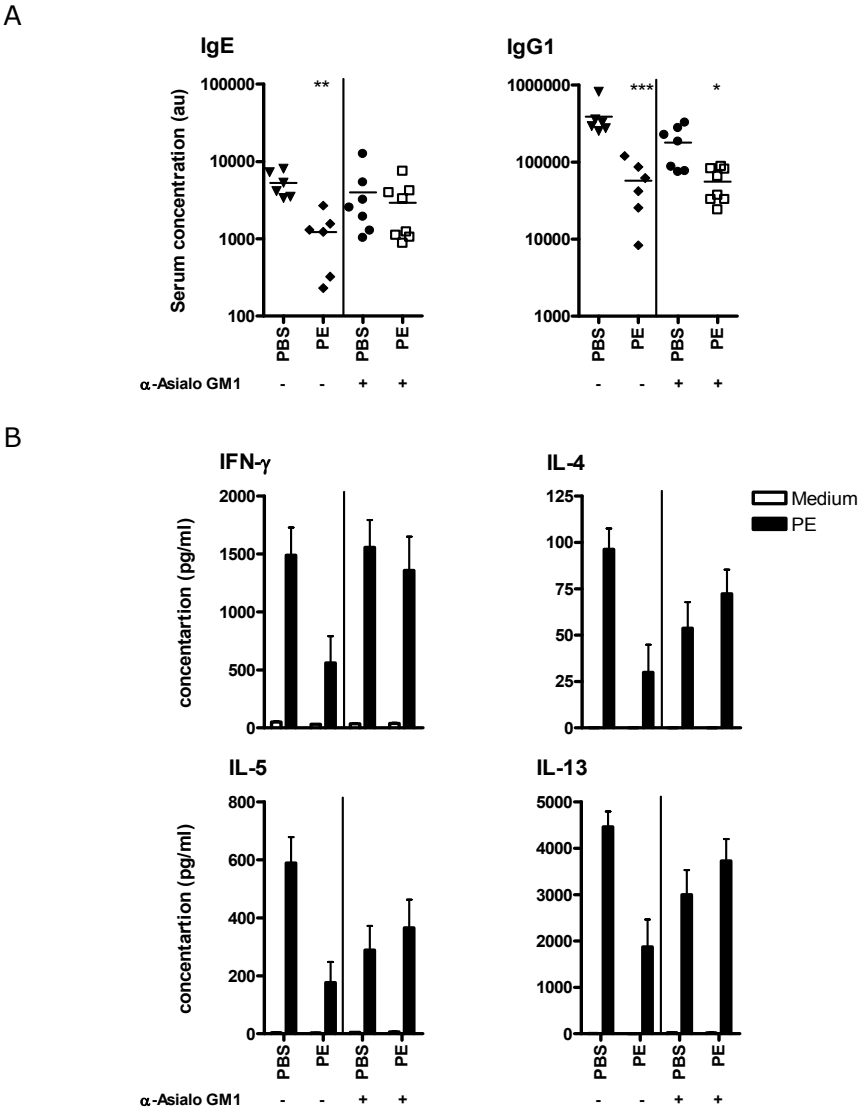

Figure 5
Effect of anti-ASGM1 treatment on the induction of oral tolerance to PE.
Before oral exposure to PE or PBS, mice were treated with anti-ASGM1 antibody or control antibody (Rabbit Ig). Twenty-one and twenty-eight days after the last oral exposure, the animals were immunized i.p. with PE plus alum, (A) PE-specific serum IgG1 and IgE antibody levels. Individual mice are indicated and presented in ^{10}log scale. (B) Cytokine levels in splenocyte culture supernatants. Cell suspensions were cultured in the presence (black bars) or absence (white bars) of PE. Data is presented with ±SEM of 6 (control groups) or 8(anti-ASGM1 treated groups). *, **, *** Significantly different ($p<0.05$, $p<0.01$, $p<0.001$) from the relevant PBS-exposed group.

Reflecting the increase in PE-specific IgE levels measured, anti-ASGM1 treatment also caused an increase of the mast cell release, as evidenced by enhanced serum levels of mMCP-1 upon PE challenge (Fig. 4b). To examine the effect of anti-ASGM1 treatment 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-5, IL-10, IL-13, and IFN-γ were measured in culture supernatants of PE-sensitized mice upon PE restimulation [9]. ASGM1$^+$ cell depletion increased the production of Th2 cytokines IL-4, IL-5, IL-13 and the regulatory cytokine IL-10. Moreover, the Th1 cytokine IFN-γ was decreased in the culture supernatants of anti-ASGM1-treated mice in comparison to untreated sensitized mice (Fig. 4c).

Anti-ASGM1 antibody treatment leads to partial abrogation of oral tolerance to PE.
As expected from earlier experience with the oral tolerance model (Fig. 1), oral pre-exposure to PE resulted in significantly lower splenic production of the cytokines IL-4, IL-5, IL-13 and IFN-γ. Additionally, lower serum levels of PE-specific IgG1 and IgE were measured in tolerized mice (Fig 5).
To determine if ASGM1$^+$ cells influences the establishment of oral tolerance, anti-ASGM1 was given before oral pre-exposure to PE or PBS. Anti-ASGM1 treatment appeared to prevent the decrease of PE-specific IgE production and of cytokine production by PE-restimulated splenocytes. A decrease of PE-specific IgG1 serum level due to PE pre-treatment was still measured (Fig. 5a).

Discussion

The ability of NK cells to secrete cytokines endows them with the ability to regulate responses of the adaptive immune system [11-14]. However, the role played by NK cells in activation and induction of immune responses to exogenous antigens *in vivo* remains controversial [18, 19]. Four days following PE plus CT sensitization an increase in NK cells was measured in the MLN of sensitized mice compared to control mice, indicating involvement of NK cells in the PE allergic response. Our results further indicate that ASGM1$^+$ cells, in particular NK cells, are important modulators of peanut allergic sensitization and that the depletion of these cells leads to partial abrogation of oral tolerance.
NK cells have no specific markers which can be used for explicit-target cell depletion. For this purpose, anti-ASGM1 antiserum has been frequently used [15, 18, 20]. However, ASGM1 is not only expressed in NK cells, but also to a lesser extent by activated macrophages, CD8+ T cells and NKT cells [19]. Anti-ASGM1 treatment was

here seen to deplete 75-90% of NK cells, showing no depletion of NKT cells, B and T cells, or cDC's. This indicates that anti-ASGM1 treatment used in our experiments was successful in depleting most exclusively NK cells. However, our results do not provide information as to whether changes in percentages of B and T cells are caused by a direct effect of anti-ASGM1 treatment or indirectly by depletion of NK cells. Further experiments are needed in order to clarify this uncertainty.

In the model used for PE allergic sensitization CT is a necessary mucosal adjuvant, as without it no hypersensitivity reaction is observed. It has been shown that CT entry into mammalian cells is mediated by binding of the pentameric B subunit of CT to gangliosides GM1 in the cell membrane, specifically to GD1(b) and ASGM1 [21]. Furthermore, it has been demonstrated that CT requires signaling through gangliosides GM1 in order to exert mucosal adjuvant properties [22]. Together these findings suggest that anti-ASGM1 treatment would interfere with CT adjuvanticity resulting in a decrease of PE allergic sensitization in mice treated with anti-ASGM1. Additionally, if anti-ASGM1 treatment would activate cells in a similar way as CT, one would expect allergic sensitization in mice exposed to PE in the absence of CT, but this was not the case (data not shown). However, our results indicate that the adjuvant effect of CT is not affected by anti-ASGM1 treatment since an increase in PE allergic sensitization following anti-ASGM1 treatment is observed. Note that in the PE oral tolerance model, the relation between CT and ganglioside GM1 is irrelevant as in this model CT is not used.

Overall, our data indicate that $ASGM1^+$ cells, presumably NK cells, are involved in modulating sensitization to PE. Four days following PE sensitization, changes in the cellular composition of MLN from PE-sensitized mice demonstrate an increase in B cells as a response to anti-ASGM1 treatment. Four weeks later, these effects appear to be reflected by higher PE-specific Ab levels and an increase in mast cell degranulation following a PE challenge. Additionally, PE-induced restimulation of splenocytes showed a decrease of IFN-γ production and an increase in Th2 cytokines in spleen cultures of anti-ASGM1-treated mice. These results indicate that depletion of NK cells may result in less IFN-γ production during sensitization to PE which may in turn lead to a stronger Th2 response to PE exposure. This finding closely links to observations in cancer and viral infection models in which NK cells have been proven to be important IFN-γ producers driving a Th1 phenomenon [23].

Th2 responses play a pivotal role in the development of allergic responses. It remains unclear, however, which mechanisms are responsible for the initiation of sensitization and a breakdown or failure to induce oral tolerance may be a proceeding process to allergic sensitization. Present results show that depletion of NK cells specifically prevents tolerance of IgE, but not of IgG1 responses, indicating a specific regulatory effect of NK cells on IgE responses. Previous *in vivo* studies in mice have shown that

NK cells only affect IgG2a isotype switching [24] but human *in vitro* studies have show that NK1 type cells can also inhibit IgE production [14]. Our results are in support of these human data.

In conclusion, our results clearly show that NK cells are important modulators of allergic sensitization and initiation of oral tolerance to peanut. Therefore, NK cells may become interesting targets in new strategies to treat food allergy.

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CHAPTER 3

$CD4^+CD25^+$ T Regulatory Cells do not Transfer Oral Tolerance to Peanut Allergens in a Mouse Model of Peanut Allergy

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Abstract

Background Recent studies have implicated $CD4^+CD25^+$ regulatory T cells (nTregs) in the maintenance of tolerance to oral antigens and in the regulation of the food allergic IgE response.
Objective The objective was to assess if nTregs can transfer allergen-specific oral tolerance to naïve, non-TCR transgenic mice and regulate peanut (PE)-specific hypersensitivity responses. Additionally, the role of the regulatory cytokines IL-10 and TGF-β in the modulation of peanut allergic sensitization was studied.
Methods CD25-enriched T cells from PE-tolerant mice were adoptively transferred to recipient mice, which were subsequently sensitized to PE. Depletion of $CD25^+$ cells and neutralization of IL-10 and TGF-β was compared in a CH3/HeOuJ mouse model of peanut allergic sensitization.
Results Transfer of $CD25^+$ Tregs-enriched cell populations did not affect the PE-specific cytokine production or PE-specific antibody levels compared to control mice however interestingly resulted in a decrease of mast cell responsiveness. On the contrary, transfer of $CD25^+$ Tregs-depleted cells caused an increase in non-specific cytokine production, in the absence of changes in PE-specific responses. TGF-β neutralization resulted even in a larger increase in spontaneous release of all cytokines measured (IL-4, IL-5, IL-10, IL-13 and IFN-γ), but surprisingly also to a higher PE-specific Th2-associated (IL-4, IL-5, IL13) cytokine production compared to depletion of CD25 cells or neutralization of IL-10. Similarly, depletion of CD25 cells and TGF-β neutralization but not of IL-10 neutralization lead to an increase in PE-specific antibody levels and elevated mast cell degranulation following a PE challenge.
Conclusions We conclude that $CD4^+CD25^+$ Tregs from non-transgenic tolerant mice cannot transfer specific oral tolerance of exogenous antigens to naïve mice and are more involved in general immune suppressive mechanisms , however we found evidence that TGF-β secreting Tregs (Th3) may play an important role.

Introduction

It is estimated that currently 3-4% of adult individuals are suffering from some kind of food allergy [1]. Peanut allergy accounts for the majority of severe food-related allergic reactions and is most commonly associated with anaphylaxis and fatal outcome [2]. In addition, peanut allergy does not usually resolve, resulting in a life-long allergy with a high impact on daily life. So far, no therapies are available and the only remedy is strict avoidance of the allergen [3]. Revealing the underlying mechanisms of sensitization to food proteins may contribute to the development of new treatment methods for peanut allergy.

Lack of oral tolerance due to absence of optimal immunoregulation is thought to be involved in causing food allergy. Preservation of oral tolerance is the result of a complex interplay of a number of cells, including a variety of subsets of regulatory T cells (Tregs). Within the $CD4^+$ T cell subpopulation, three classes of Tregs cells have been distinguished: Th3-type TGF-beta-secreting Tregs (Th3), IL-10-producing type 1 Tregs (Tr1), and naturally occurring $CD4^+CD25^+$ Tregs (nTregs) [4]. nTregs were first described by Sakaguchi *et al* [5] and are characterized by an anergic phenotype, a constitutive high expression of the IL-2 receptor α chain (CD25), and the expression of the transcription factor forkhead box p3 (Foxp3). Their immunosuppressive activity is most likely mediated by direct cell-cell contact involving for instance CTLA-4 [6] and cell-bound TGF-β [7]. In contrast to nTregs, Tr1 [8, 9] and Th3 [10, 11] cells do not express specific markers and they function through the immunosuppressive cytokines IL-10 and TGF-β, respectively. Additionally, although Tr1 and Th3 cells are activated in an antigen-specific manner, once activated, they can mediate bystander suppressive activity against other antigens, and this bystander suppression is mediated by the local release of IL-10 or TGF-β (Tr1 or Th3 cells, respectively) [12]. The three subsets of Tregs mentioned above have been implicated in the regulation of allergies [13-15].

It has been demonstrated in mouse models that oral exposure to ovalbumin induces activation of functional nTregs in gut draining lymph nodes [16] and that adoptive transfers of these activated cells regulate sensitization in ovalbumin TCR-transgenic mice [17]. Recently, van Wijk *et al* [13] have shown that depletion of nTregs by anti-CD25 mAb treatment results in increase of peanut specific responses, including IgE and mast cell responses. It was also been shown that depletion of nTregs abrogated peanut-specific oral tolerance. It remained unclear, however, if nTregs can transfer allergen-specific oral tolerance to naïve, non-TCR transgenic mice and indeed regulate peanut-specific hypersensitivity responses. To address this issue, CD25-enriched T cells from peanut-tolerant mice were adoptively transferred to recipient mice, which were consequently sensitized to peanut. Our results show that CD25-

enriched T cells from orally tolerized mice were unable to transfer tolerance to peanut. However, the presence of $CD25^+$ T cells was necessary to control the spontaneous responses transferred within the CD25-depleted population. These results prompted us to further study the role of regulatory cytokines in the modulation of peanut allergic sensitization. Comparing depletion of CD25 cells with neutralization of IL-10 and TGF-β indicates that both regulatory cytokines are important for general modulation of immune responses, but that TGF-β, hence Th3 T cells are particularly involved in modulating the Th2-biased peanut-specific response.

Materials and Methods

Mice

Female, specific pathogen-free C3H/HeOuJ Ico mice, 4-5 weeks of age, were purchased from Charles River (Lyon, France) and were left to acclimatize for 1 week. 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 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 Utrecht University.

Chemicals, reagents and monoclonal antibodies

Anti-CD25 (PC61), anti-IL10 (JES5.2A5), and anti-TGF-β (1D11) were produced by culturing hybridomas and purified using thiophilic agarose. Cholera toxin (CT) was purchased from List Biological Laboratories, Inc (Campbell, California, USA). All other chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands) unless otherwise stated.

Preparation of peanut extract

Peanuts from Golden Peanut (Alpharetta, Georgia) were kindly donated by Imko Nut Products, the Nut Company (Doetinchem, the Netherlands). Protein extract was made by blending 100 g peanuts with 500 ml 20 mM Tris buffer (pH 7.2). After 2 h blending at intervals of 20 minutes for a minute at room temperature, the aqueous fraction was collected by centrifugation (3000 g, at 4°C for 30 min). The aqueous phase was subsequently centrifuged (10 000 g at 4°C for 30 min) to remove residual traces of fat and insoluble particles. Protein concentrations were determined using Bradford analysis with BSA as a standard. Extract (peanut extract, PE) contained typically 32 mg/ml protein and was stored at -20°C. Reducing SDS-PAGE from the extracts showed protein bands between 14 and approximately 100 kDa (not shown).

Treatment protocols

Oral tolerance induction to PE. Mice (n=20) were exposed by intragastric dosing of 1 mg of PE in 200 µl PBS on 3 consecutive days. Control mice (n=8) were exposed to 200 µl PBS alone. To determine if tolerance induction was successful, animals were subsequently sensitized by intraperitoneal (i.p.) challenge on days 21 and 28 with 100 µg PE in alum (25 mg/ml). Mice were bled on day 35 and sacrificed on day 43 (Fig 1A)

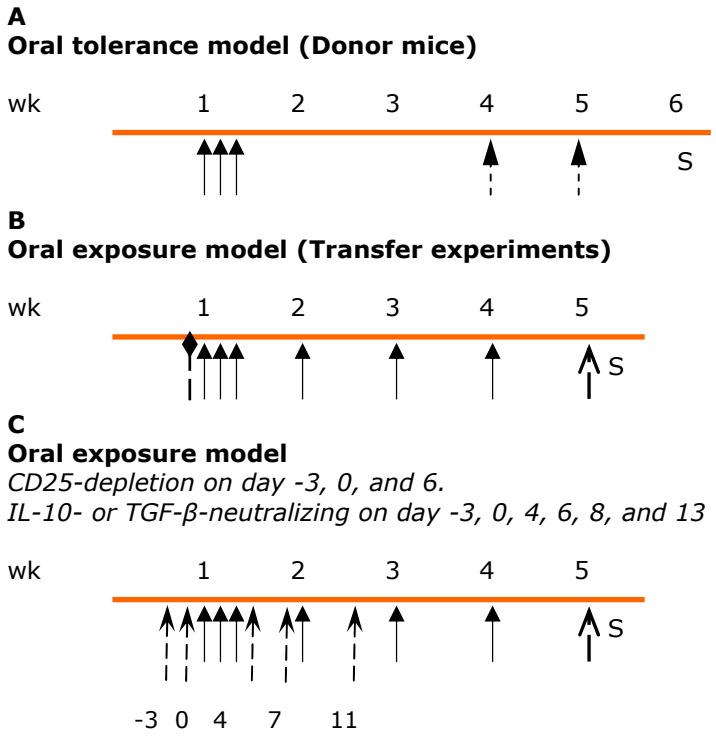


Figure 1
Schematic overview of treatment protocols.

Oral sensitization to PE. Mice (n=8) 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 of CT on 3 consecutive days, followed by weekly dosing of PE plus CT (days 7, 14 and 21). At day 28 all groups received an oral challenge with 16 mg PE and blood was collected 45 minutes later for measurement of mast cell degranulation. Mice were sacrificed on day 29. For adoptive transfer experiment, 2 x 10^6 cells were transferred one day before initial sensitization to PE (Fig 1B). For depletion and neutralizing studies, 200 µg of anti-CD25 mAb (PC61) was given on day -3, 0 and 7, or 500 µg of anti-IL-10 (JES5.2A5) or anti-TGF-β (1D11) mAbs were given i.p. on days -3, 0, 4, 7, and 11 (Fig 1C).

Enrichment of CD4+CD25+ cells from spleen and mesenteric lymph nodes of tolerized mice

Spleen and mesenteric lymph nodes (MLNs) were isolated from PE-tolerized mice. Single cell suspensions were acquired as previously described and pooled for $CD4^+CD25^+$ enrichment. $CD4^+CD25^+$ enrichment was carried out using a $CD4^+CD25^+$ Regulatory T Cell Isolation Kit from Miltenyi Biotec (Bergisch Gladbach, Germany) and was performed according to manufacturer's instructions.

Measurement of serum IgG1, IgG2a, and IgE antibodies

Serum were collected at various time-points and stored at -20°C until analysis. Levels of PE-specific IgE, IgG1, and IgG2a were determined by ELISA. Plates (highbond 3590; Costar, Cambridge, MA, USA) were coated overnight with 10 µg/mL PE (for IgG1 and IgG2a detection) or with 1.5 mg/mL purified rat anti-mouse IgE (BD Pharmingen) in PBS, followed by 1 h blocking with ELISA buffer (50 mM Tris, 136.9 mM NaCl, 2 mM EDTA, 0.5% Tween and 0.5% BSA). Each test serum was optimally diluted and incubated for 2 hrs. To determine antibody levels (arbitrary units) a standard curve of the positive reference serum was included. For detection of PE-specific IgG1 and IgG2a, alkaline phosphatase-conjugated IgG1, IgG2a (both polyclonal goat anti-mouse), 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 Abs, a PE-digoxigenin (DIG) conjugate solution (diluted in High Performance ELISA Buffer, from Sanquin, San Diego, California, USA) 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.

Cell culture and cytokine measurement

Spleen and MLN single cell suspensions, 6.25 x 10^5 cells/mL in RPMI 1640 (Life Technologies, Paisley, Scotland) containing 10% fetal calf serum (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. The IL-10 (BD Pharmingen, San Diego, California, USA) and IFN-γ, IL-4, IL-5, IL-13 ELISA (eBiosciences, San Diego, California, USA) were performed in accordance with the manufacturer's instructions.

Measurement of serum mouse mast cell protease-1

On day 28, blood was collected 45 min after oral challenge with PE and serum levels of mouse mast cell protease-1 (mMCP-1) were determined using an ELISA kit (Moredun Scientific Ltd, Midlothian, Scotland). ELISA was performed according to the manufacturer's instructions.

Statistics

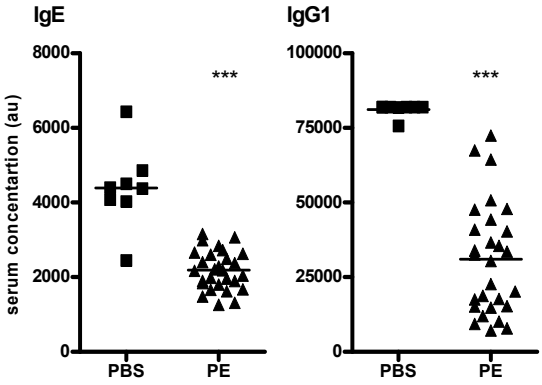
Data were analyzed using GraphPad Prism 4 software. The differences between group means were determined by using one-way ANOVA with Bonferroni as *post hoc* test. Statistical analyses were performed following logarithmic transformation (to achieve normal distribution).

Results

CD25 enrichment captured all FoxP3 expressing cells from spleen and MLN suspensions of tolerized mice

For the transfer studies, $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells were isolated from the spleen and MLNs of PE-tolerized donor mice. Oral tolerance was confirmed by lower PE-specific IgE and IgG1 levels in donor mice pretreated with PE than in control mice (Fig. 2A). In addition, splenocytes from donor mice tolerized to PE produced significantly lower amounts of cytokines IL-4, IL-5, IL-10, IL-13, and IFN-γ than those obtained from control mice (data not shown).

A



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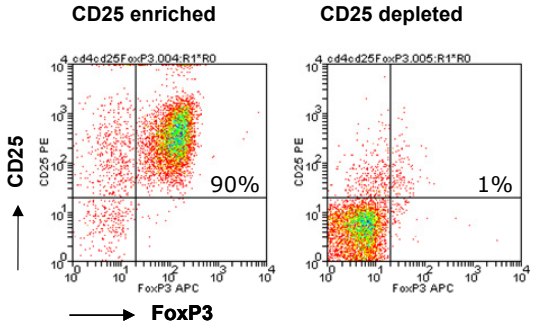

Figure 2
Characterization of donor spleen and MLN cells for adoptive transfers.
(A) PE-specific serum IgE and IgG1 antibodies following PE oral tolerance protocol. Data are represented as the mean serum concentration in arbitrary units of 8 or 28 mice per group (symbols indicate individual animals). ***, Significantly different (*p<0.001*) from control PBS pre-treated group. (B) Flowcytrometry analysis of stated cell isolations. Data is gated on $CD4^+$ live cells and $\%CD25^+FoxP3^+$ cells of total $CD4^+$ cells are indicated.

CD25 enrichment from the combined suspensions of spleens and MLN from tolerized mice resulted in complete capture of all $CD4^+CD25^+FoxP3^+$ Tregs (Fig. 2B). No $CD25^+$ or $FoxP3^+$ cells were present in the $CD25^+$ Tregs-depleted population.

$CD25^+$ Tregs cells do not affect PE specific cytokine production but are rather general immune suppressive.

$CD25^+$ Tregs-enriched and -depleted cell populations from PE tolerized donor mice were transferred to recipient mice one day before the standard oral sensitization protocol (with PE+CT) was started (Fig. 1B). After four weeks, spleen cells were isolated and cultured in the presence or absence of PE. In agreement with previous studies, significant levels of IL-5, IL-13, and IFN-γ were found upon restimulation with PE in culture supernatants of PE-sensitized mice (Fig. 3).

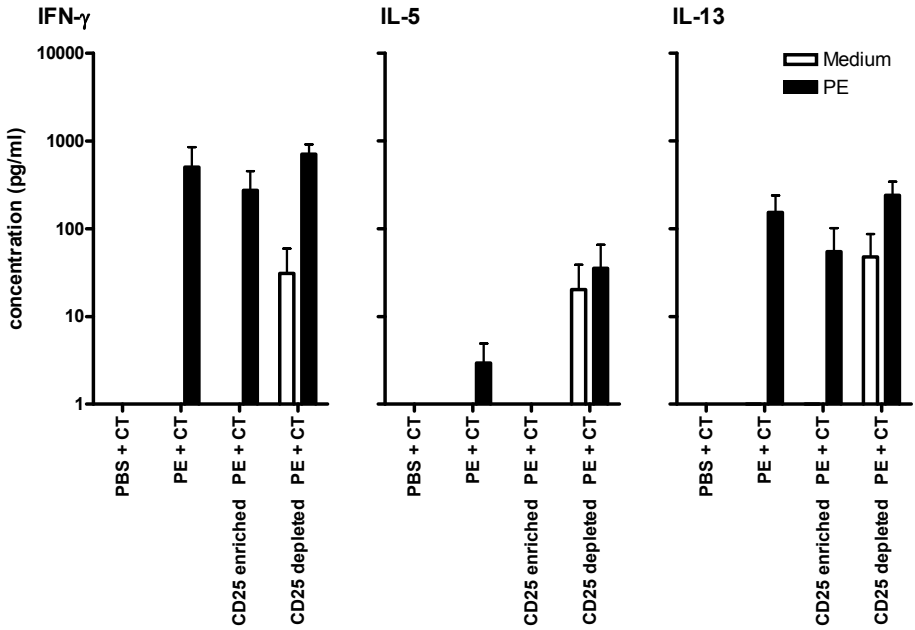

Figure 3
Effect of CD25-enriched and -depleted cells adoptive transfers on cytokine levels of restimulated splenocytes.
Mice were orally sensitized to PE by exposure to PE + CT or left unsensitized (PBS + CT treatment). Indicated groups received CD25 depleted or enriched spleen and MLN cells one day before initial sensitization. Following a 4 week sensitization protocol, splenocytes were cultured for 96 hrs in the presence (black bars) or absence (white bars) of PE. In the supernatant IL-5, IL-13, and IFN-γ cytokine levels were determined by ELISA. Data are represented as the group mean of 4 (unsensitized group) or 8 (sensitized groups) mice per group. Groups are significantly different ($p<0.01$) from unsensitized group.

However, these PE-specific T cell responses were not affected by transfer of $CD25^+$ Tregs-enriched or -depleted cells from PE-tolerized donor mice. On the other hand, spontaneous release of all cytokines was increased in splenocyte cultures of mice which had received $CD25^+$ Tregs-depleted cells compared to non-treated sensitized mice and mice which had received $CD25^+$ Tregs-enriched cells.
These results show that $CD4^+CD25^+$ $FoxP3^+$ Tregs of PE tolerized mice cannot transfer PE tolerance, but that they are essential for the general suppression of immune responses.

Transfer of $CD25^+$ Tregs-enriched populations do not affect PE-specific antibody levels but results in a decrease of mast cell responsiveness.
After four weeks of exposure to PE plus CT, mice developed PE-specific IgG1, IgG2a, and IgE antibody responses. Adoptive transfers of $CD25^+$ Tregs-enriched or -depleted cells had no effect on serum levels of Th1-associated (IgG2a) or Th2-associated (IgG1 and IgE) PE-specific antibody levels compared to non-treated sensitized mice (Fig. 4A).

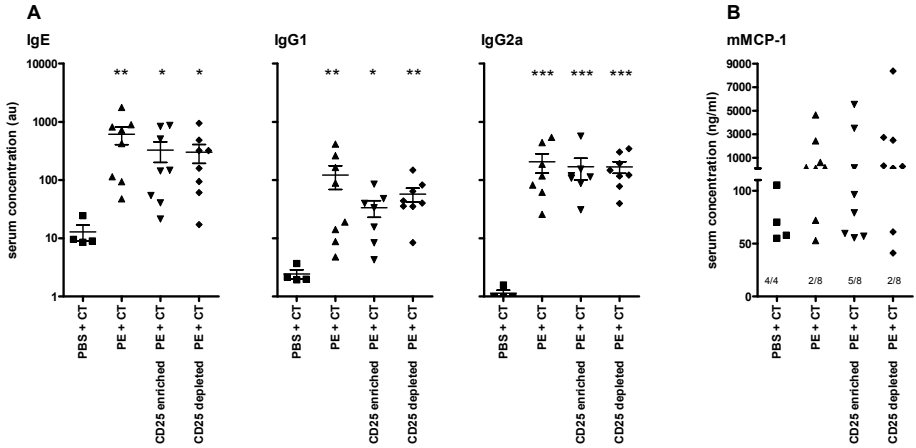

Figure 4
Effect of CD25-enriched and -depleted spleen and MLN cells adoptive transfers on PE-specific antibody production and mast cell degranulation.
Mice were orally sensitized to PE by exposure for 4 weeks to PE + CT. One day prior to the initial sensitization, mice received adoptive transfers of indicated cells. (A) PE-specific serum antibody levels in serum collected after 4 weeks of oral exposure. (B) mMCP-1 levels were determined in serum collected within 45 minutes after an oral challenge with PE. Symbols indicate individual mice and data are presented on a log scale as the mean arbitrary unit per group (A) or in ng/mL (B). *, **, ***, Significantly different ($p<0.05$, $p<0.01$, $p<0.001$, respectively) from control unsensitized group. Ratio of responsive mice (mMCP-1 level in serum above highest level in unsensitized group) is stated per group.

After the 4 week oral regime, all mice received an oral challenge with PE and the serum mMCP-1 concentrations at 45 min after oral challenge was used as a measure for the mast cell response. Clear mast cell degranulation was observed in 6 out of 8 mice that were sensitized and not treated with cell transfers (Fig. 4B). Although the mean mMCP-1 serum concentration per treated group showed no significant differences to non-treated sensitized mice, the presence of $CD25^+$ Tregs-enriched cells in recipient mice resulted in fewer responsive mice (3 out of 8 are positive) as compared to control groups (6 out of 8 are positive).

Depletion of CD25 cells or neutralization of TGF-β or IL-10 during PE sensitization results in a profound increase of PE-specific cytokine production.

Next to the role of $CD4^+CD25^+FoxP3^+$ Tregs, we aimed at investigating the role of regulatory cytokines TGF-β or IL-10, which may also originate from other, e.g. Th3 and/or Tr1, regulatory cells.

Depletion of CD25 Tregs or neutralization of regulatory cytokines TGF-β or IL-10 was carried out during the first 2 weeks of sensitization (Fig. 1C). All antibody treatments resulted in a drastic increase of PE-induced as well as spontaneous production of all cytokines measured (Fig 5). Remarkably, in comparison to anti-CD25 and anti-IL-10 antibody treatment TGF-β neutralization resulted in a larger increase of spontaneous release of all cytokines measured but interestingly also to a higher PE-specific Th2-associated (IL-4, IL-5, IL13) cytokine production. Additionally, IL-10 production was higher in anti-TGF-β treated mice compared to other treatment groups. These data indicate that the regulatory cytokine TGF-β is not only important for general homeostasis, but particularly involved in modulating the Th2-biased PE-specific response.

Depletion of CD25 cells and TGF-β neutralization but not of IL-10 neutralization results in an increase of PE-specific antibody levels and elevated mMCP-1 release.

Since anti-CD25, anti-IL-10 and anti-TGF-β all resulted in increased PE-induced cytokine responses, it raised the question whether the humoral response, PE-specific antibody levels and mMCP-1 levels, were also altered.

Treatment with anti-CD25 antibodies significantly increased PE-specific antibody (IgE, IgG1, and IgG2a) production following PE sensitization (Fig 6A), which confirms previous studies [13]. Treatment with anti-TGF-β showed a slight increase in PE-specific antibodies IgG1 and IgG2a, however, this increase was not observed for PE-specific IgE levels. On the other hand, IL-10 neutralization did not lead to increased PE-specific antibody responses.

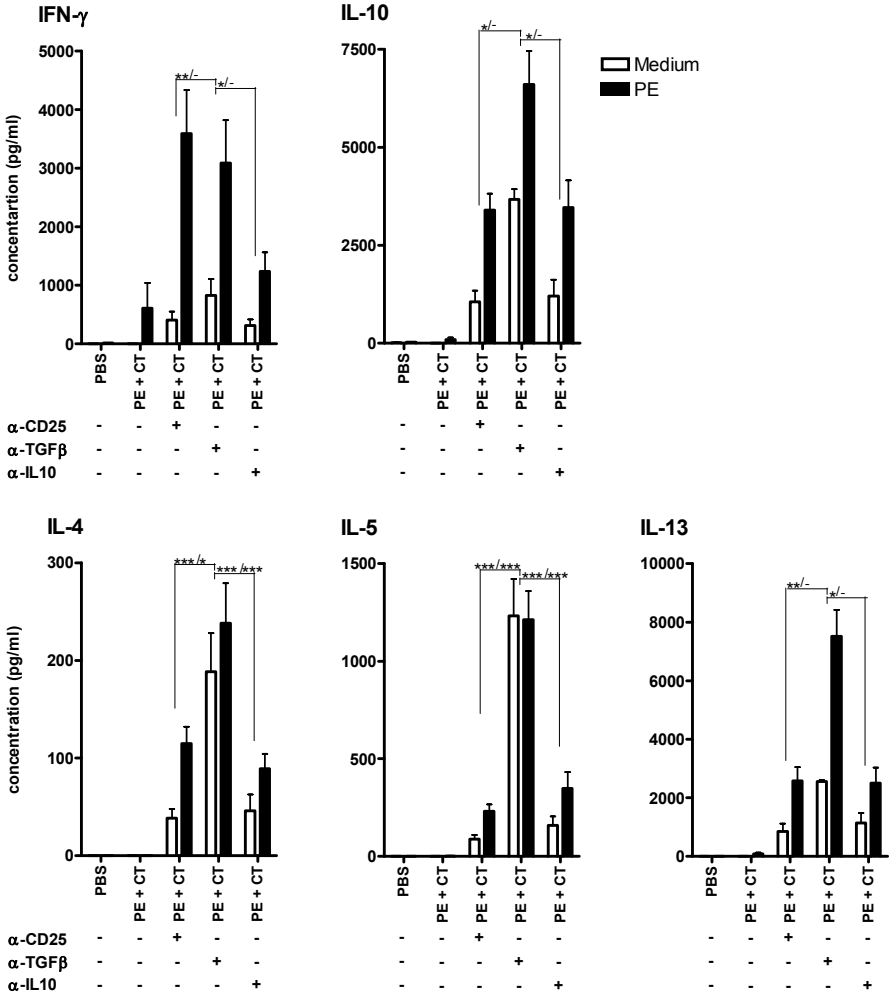

Figure 5
Effect of CD25 cell depletion or neutralization of TGF-β and IL-10 cytokines on cytokine levels of restimulated splenocytes from PE sensitized mice.
Mice were orally sensitized to PE by exposure to PE + CT or left unsensitized (PBS + CT treatment). Indicated groups received depleting anti-CD25 or neutralizing anti-TGF-β or anti-IL-10 mAbs during the first 2 weeks of PE sensitization. Following a 4 week sensitization protocol, splenocytes were cultured for 96 hrs in the presence (black bars) or absence (white bars) of PE. In the supernatant IL-4, IL-5, IL-10, IL-13, and IFN-γ cytokine levels were determined by ELISA. Data are represented as the group mean of 5 (unsensitized group), 6 (sensitized control group), and 8 (sensitized treated groups) mice per group. *, **, ***, Significantly different (*p<0.05, p<0.01, p<0.001*, respectively) from indicated groups and restimulation condition (medium / PE). All treated groups are significantly different from sensitized mice (*p<0.05*).

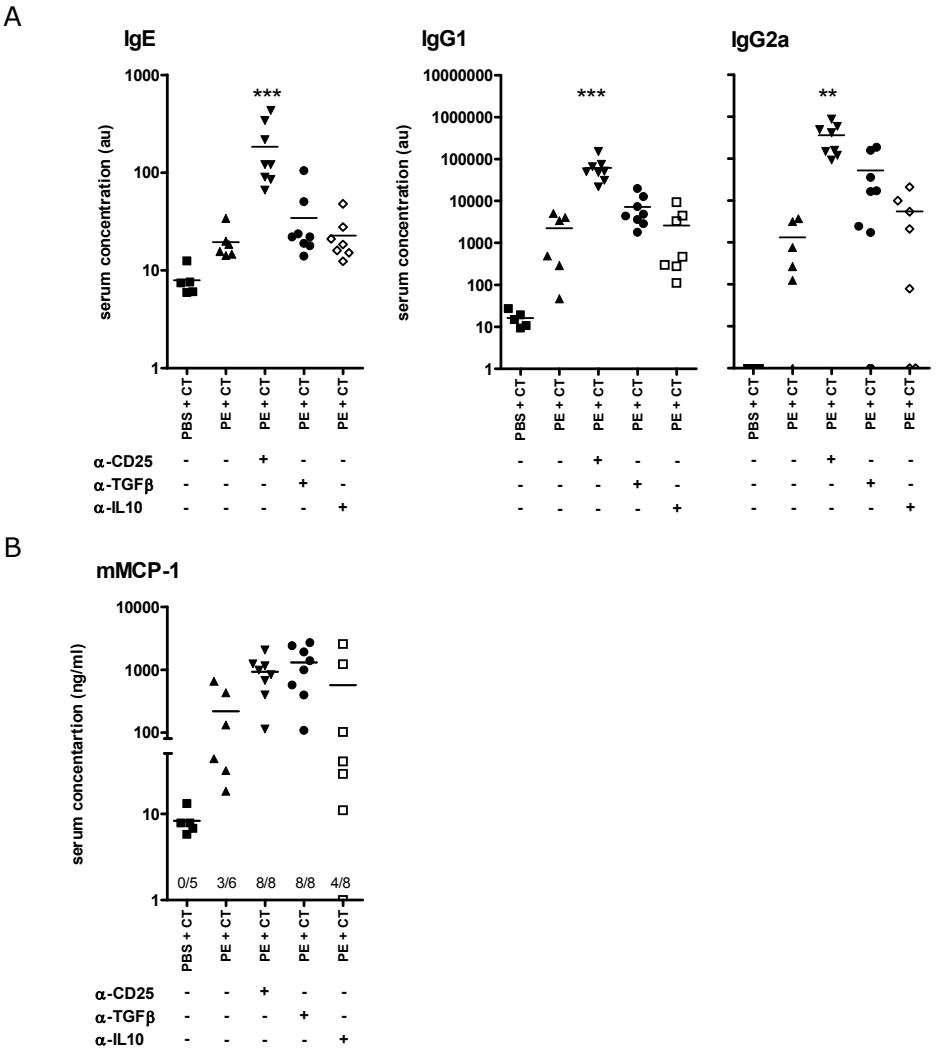

Figure 6
Effect of CD25 cell depletion or neutralization of TGF-β and IL-10 cytokines on PE-specific antibody production and mast cell degranulation of PE-sensitized mice.
Mice were orally sensitized to PE by exposure to PE + CT or left unsensitized (PBS + CT treatment). Indicated groups received depleting anti-CD25 or neutralizing anti-TGF-β or anti-IL-10 mAbs during the first 2 weeks of PE sensitization. (A) PE-specific antibody levels in serum collected after 4 weeks of oral exposure. (B) mMCP-1 levels were determined in serum collected within 45 minutes after an oral challenge with PE. Symbols indicate individual mice. Data was logarithmically transformed and are presented linear as the mean arbitrary unit per group (A) or in ng/mL (B). *, **, ***, Significantly different (*p<0.05, p<0.01, p<0.001*, respectively) from sensitized group. Ratio of responsive mice (mMCP-1 level in serum above highest level in unsensitized group) is stated per group.

In accordance with the increase of PE-specific IgE antibody levels, anti-CD25 treatment also resulted in an increase in mMCP-1 serum level following a PE oral challenge compared to non-treated sensitized mice (Fig 6B). This increase in mast cell degranulation was also observed following TGF-β neutralization, but not following IL-10 neutralization.
These results show that in contrast to IL-10 neutralization, CD25 depletion and TGF-β neutralization are able to influence both the PE-specific antibody and also mast cell responses.

Discussion

The role of $CD4^+CD25^+$ Tregs in specific modulation of exogenous antigen sensitization in non-transgenic mice is still unclear. Since depletion studies have shown that $CD4^+CD25^+$ Tregs can modulate sensitization of orally administered PE [13], we investigated whether transfer of isolated $CD4^+CD25^+$ Tregs from tolerized mice could prevent oral sensitization to PE. Our results show that $CD4^+CD25^+$ Tregs are unable to transfer PE specific oral tolerance to naïve mice but that they are essential for homeostasis, since transfer of $CD25^+$ Tregs-depleted cells showed an increase in non-specific cytokine production. This is in agreement with the present and previous depletion studies, showing an increase in both non-specific and PE-specific cytokine responses when $CD25^+$ cells are depleted [13]. However, depletion of $CD25^+$ cells also resulted in increases of serum levels of PE-specific antibodies and mMCP-1, while this effect was not observed when CD25-depleted cells were transferred. We have used the current approach to investigate whether transfer of regulatory T cells could down-regulate orally induced food allergy responses. However, PE+CT-treatment itself also induces these (and other) regulatory cells [13] and they may interfere with the efficacy of transferred cells. Nonetheless, with the transfer of CD25-depleted cells we observed a clear change in spontaneous cytokine production. It is important to note in this respect that adoptive transfer of Tregs is a much more gentle manipulation of the system than depletion of cell populations or neutralization of cytokines. But to the best of our knowledge, no data has yet been published which otherwise states that $CD4^+CD25^+$ Tregs from non-transgenic mice can transfer exogenous antigen-specific oral tolerance to naïve mice.
Since Sakaguchi et al first described $CD4^+CD25^+$ Tregs [5], a number of studies have meticulously studied their function and suppressive properties, both in vitro and in vivo. They have shown the importance of $CD4^+CD25^+$ Tregs in preventing development of autoimmune diseases [5, 18-20] and in effective tumor immunity [21-23]. Adoptive transfer studies have shown a role of $CD4^+CD25^+$ Tregs in

modulation of auto-immune diseases [5, 18, 20]. In respect to non auto-antigens, it has been shown that oral administration of ovalbumin (OVA) to OVA TCR-transgenic (DO11.10) mice can induce $CD4^+CD25^+$ Tregs that can transfer specific-tolerance to wild type mice [17]. However, TCR-transgenic mice of the DO11.10 strain, harbouring a majority of T cells that recognizes only a single antigen, do not reflect a normal situation. One could speculate that nTregs are necessary for the general homeostasis and auto-tolerance in the periphery, while other suppressive immune cells or factors may be necessary to establish and maintain oral tolerance in the gut. The immunosuppressive environment of the gut has been reviewed by many in the last years [24, 25], and the regulatory cytokines IL-10 and TGF-β have frequently been suggested as important regulatory cytokines. $CD4^+CD25^+$ Tregs are believed to function through cell-cell contact but they may still modulate sensitization to exogenous antigens by bystander cytokine-mediated suppression [12]. Similarly, both Tr1 and Th3 Tregs may be responsible in bystander suppression. In all cases, the regulatory cytokines IL-10 and TGF-β may be very important.

Previous studies show that TGF-β-secreting Th3 cells mainly inhibit Th1 responses, while regulation of Th2 responses, including IgE responses to oral allergens has been particularly attributed to IL-10-producing Tr1 cells [26, 27]. Nevertheless, peanut allergy in the present mouse model implicates both Th1 and Th2 immune responses [28], permitting a possible role for both Tr1 and Th3 cells for the modulation of allergic sensitization to PE. However, Tr1 cells have been described to express CD25 in response to antigen activation [29], which means their presence within the $CD25^+$ Tregs-enriched population cannot be excluded. Tr1 cells have furthermore been frequently implicated in tolerance in the periphery, while Th3 cells have been extensively described in the gut [10, 11]. In the present study, results of neutralization of IL-10 suggest that IL-10 does not have a strong regulatory effect. In another study (chapter 5) we show that by using more frequent dosing of neutralizing antibodies during all 4 weeks of PE sensitization, IL-10 is very well capable of regulating Th1/Th2 cytokine production and mast cell responses. However, data of the present and previous study suggest that IL-10 is less efficient in modulating food allergy responses than for instance IFN-γ (chapter 5) and TGF-β (present chapter).

Th3 cells constitute a unique T-cell subset which primarily secretes TGF-β. Our neutralization studies show that TGF-β serves as an important regulatory cytokine during oral PE sensitization since TGF-β neutralization has lead to significantly more cytokine production by restimulated splenocytes than neutralization of IL-10 or depletion of $CD25^+$ cells, and to an increase in PE-specific antibodies and mast cell degranulation. This is in line with other studies which have shown that immune responses to soluble antigens in the gut involve the generation of cells that secrete

TGF-β (Th3 cells) [30]. Furthermore, TGF-β is a pleiotropic cytokine that contributes to maintenance of the intestinal epithelial barrier and to reduction of proinflammatory stimuli [31].

We conclude that $CD4^+CD25^+$ Tregs from non-transgenic tolerant mice do not transfer specific oral tolerance of exogenous antigens to naïve mice under the used conditions. Even so, much more research is needed into the role of regulatory T cells, in particular of the Th3 cells, in the modulation of allergic sensitization and maintenance of oral tolerance.

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CHAPTER 4

Enteric Reovirus Infection Stimulates Peanut-Specific IgG2a Responses in a Mouse Food Allergy Model

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Abstract

Background Food allergies mediated by immunoglobulin (Ig)-E antibodies are an important cause of life-threatening hypersensitivity reactions. Orally administered peanut antigens mixed with the mucosal adjuvant cholera toxin (CT) induce a strong peanut extract (PE)-specific serum IgE response, and this response is correlated with the development of T-helper type 1 (Th1) and T-helper type 2 (Th2)-like T-cell responses.
Objective The purpose of this study was to determine if respiratory enteric orphan virus (reovirus), a non-pathogenic virus that induces robust Th1-mediated mucosal and systemic responses, could modulate the induction of *PE*-specific allergic responses when co-administered with *PE*.
Methods Young C3H/HeJ mice were orally exposed to PE mixed with CT, reovirus, or both CT and reovirus. The induction of specific antibodies, cellular immunity, cytokines, and distribution of Peyer's patch (PP) lymphocytes were assessed.
Results Following oral administration, reovirus demonstrated systemic and mucosal immunity characterized by prototypic Th1-dominant responses in the presences of CT and PE. As expected, CT promoted PE-specific serum IgE, IgG1, and IgG2a and intestinal IgA production, as well as splenic Th1- and Th2-associated cytokine recall responses. Reovirus did not alter the PE-specific serum IgE and IgG1 levels, but it did substantially increase the PE-specific IgG2a response when co-administered with PE with or without CT, demonstrating a Th1 shift in the PE-specific Th1/Th2 balance. Additionally, reovirus significantly decreased the percentage of PP $CD8^+$ T-cells and $Foxp3^+CD4^+$ T-regulatory cells when co-administered with PE suggesting mobilization of these cell populations out of the PP.
Conclusion The results from this study demonstrate that an acute mucosal reovirus infection and subsequent Th1 immune response is capable of modulating the PE-specific Th1/Th2 balance. The reovirus-mediated shift in the PE-specific Th1/Th2 balance may have therapeutic implications as increased levels of non-allergenic *PE*-specific IgG2a could block *PE* antigens from binding to IgE-sensitized mast cells.

Introduction

Food allergies pose significant risk of morbidity and mortality, particularly in young children. Among foods that cause allergies, peanuts are a major problem since accidental ingestion is common, allergy to peanuts is severe and sometimes fatal, and peanut allergies resolve in only about 20% of cases [1;2]. It has been estimated that approximately 1-2% of children develop peanut allergies [3] and over the past several decades, the rate of children that developed peanut allergies had increased in developed countries [4;5].

The reason why food allergies were increasing in developed countries is not known, but for almost 20 years the hypothesis described by Strachan [6] and now popularly referred to as the 'hygiene hypothesis' has been invoked to explain why populations in developed countries appear to be developing pathologies associated with allergic conditions at increasing rates. Broadly, the hygiene hypothesis suggests that reduced exposure to pathogens or their components early in life results in an increased IgE response to allergens and a subsequent increase in allergic diseases. The mechanism for this increased predilection for mounting IgE responses has been described as an imbalance of Th1/Th2 cells [7;8], reduced activation of T-regulatory cells (Tregs) [9], or failures in appropriate activation of innate effector cells and signalling molecules [10].

Despite not knowing the precise immune mechanisms that account for increased frequencies of atopic patients, one approach to testing the hygiene hypothesis has been to identify inverse relationships between rates of allergic hypersensitivities and immunity to pathogens in hypersensitive patients. These studies have included analyses of responses to bacterial [11;12], parasitic [13;14], and viral [15;16] pathogens. In some studies seropositivity to Hepatitis A [17], and Epstein Barr virus [18;19] has been correlated with reduced incidence of atopy. In addition, immunity to herpes virus [20] and cytomegalovirus [21] [22] have been inversely correlated with atopy. These mucosally transmitted viruses have high frequencies of occurrence in human populations and are frequently transmitted under conditions of hygiene that are less than ideal, suggesting that viruses could be an important role in regulation of hypersensitivity. However, to dissect cellular and molecular mechanisms that are potentially involved in virus-mediated regulation of allergy, an animal model of both allergy and mucosal virus infection is needed.

Previous studies have documented that mice of the C3H strain produce substantial PE-specific serum IgE antibody following oral immunization with PE and the mucosal adjuvant CT [23;24]. This immune response is characterized by the appearance of PE-specific Th1 and Th2 responses [25] and activation of innate effectors including dendritic cells [26]. The present study examines the capacity of a Th1 promoting

mucosal virus infection to modulate the allergic response to PE and CT in an animal model of allergy and infection. Reovirus was used as the mucosal viral pathogen because the virus is well characterized, naturally infects humans and mice, and is found ubiquitously in nature. In fact, its presence in water sources is commonly used as a sign of fecal contamination [27;28]. Following intestinal infection in C3H mice, reovirus induces stereotypic Th1-driven responses characterized by the development of high titers of virus-specific serum IgG2a antibody [29], and the induction of IFN-γ-producing T-cells [30;31]. Reovirus activates intestinal dendritic cells *in vivo* [32-34] and under some circumstances reovirus [35] or its hemagglutinin [36] can induce oral tolerance, indicating that the immune response to reovirus in mice has the potential to regulate allergic responses by a variety of mechanisms. Here we found that the robust Th1 responses to reovirus modulated the PE-specific immune responses in mice. Although the PE-specific IgE response developed normally in sensitized mice that received reovirus, these mice demonstrated an enhanced PE-specific IgG2a antibody response, suggesting an increased PE-specific Th1 response. These experiments provide evidence for a role of enteric viruses in regulating induction of PE-specific immune responses.

Methods

Virus

Third passage stocks of reovirus serotype 1, strain Lang (T1/L) were prepared in L929 cells and purified by 1,1,2-trichloro-1,2,2-trifluoroethane (freon) extraction and CsCl gradient centrifugation [37]. The concentration of virions in purified preparations was determined by spectrophotometry where 1 optical density U at 260 nm = 2.1×10^{12} particles/mL [38] and by plaque assays [39].

Preparation of peanut extract

Peanuts from Golden Peanut (Alpharetta, Georgia) were kindly donated by Imko Nut Products, the Nut Company (Doetinchem, the Netherlands). Protein extract was made by blending 100 g peanuts with 500 ml 20 mM Tris buffer (pH 7.2). After 2 h blending at intervals of 20 minutes for a minute at room temperature, the aqueous fraction was collected by centrifugation (3000 g, at 4°C for 30 min). The aqueous phase was subsequently centrifuged (10 000 g at 4°C for 30 min) to remove residual traces of fat and insoluble particles. Protein concentrations were determined using Bradford analysis with BSA as a standard. Extract (peanut extract, PE) contained typically 32 mg/ml protein and was stored at -20°C. Reducing SDS-PAGE from the extracts showed protein bands between 14 and approximately 100 kDa (not shown).

Mouse Treatment Protocol

All experiments were performed under a protocol approved by the WVU Institutional Animal Care and Use Committee. The oral sensitization was performed as previously described [40;41] with some modifications. Four week old C3H/HeJ female mice (Jackson Mice, Bar Harbor, ME), were orally gavaged with 0.25 mL of either phosphate buffered saline (PBS), PE (6 mg), PE plus CT (1mg/mL, List Biologicals, Campbell, CA), PE plus T1/L (10^7 plaque forming units), PE plus CT and T1/L, or T1/L plus CT. The mice were treated on days 1, 2, 3, 8, 15, and 21. All mice were orally dosed with 12 mg of PE alone on day 30 and sacrificed on day 31.

Cytokine analysis by splenocytes

Spleen cultures were performed as previously described [42]. Splenocytes were cultured in 150 µL of tissue culture medium (TCM) (RPMI 1640 (Cellgro, Herndon, VA), 10% FBS, 10mM N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) hemisodium salt, 2 mM L-glutamine, 100 U penicillin/mL, 0.1 µg streptomycin/mL (Biowhittaker), 50 µM β-mercaptoethanol) in 96 well sterile U-bottom plates at a concentration of 2.5×10^6 cells/mL in the presence or absence of 200 µg/mL of PE. The cells were cultured for 96 hours at 37°C in 5% CO_2 atmosphere. Following the 96 hour incubation, the plates were centrifuged for 10 min at 150 × *g* and supernatants were collected and stored frozen at -70°C for further analysis. Levels of cytokines in the supernatants were determined by sandwich enzyme linked immunosorbent assay (ELISA) as described [43]. 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, UK) for 4 h at room temperature. Samples and cytokine standards were added in serial 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, 3,3',5,5'-tetramethylbenzidine (TMB) substrate (0.1 mg/mL) was added and the colour reaction was stopped with 2M H_2SO_4. Absorbance was measured at 450nm using an ELISA reader ELX800. IL-10 ELISA (BD Pharmingen, San Diego, CA) was performed in accordance with the manufacturer's instructions.

Splenic $CD8^+$ effector function

Single cell suspensions of spleen cells from mice that were infected with T1/L were prepared as previously described [44]. Cells were resuspended to a final concentration of 1×10^7 cells/mL and incubated with T1/L at a multiplicity of infection (MOI) of 1 for 45 minutes at room temperature. Following incubation, cells were washed with TCM, resuspended to 4×10^6 cells/mL, and incubated at 37°C in

an atmosphere of 5% CO_2 for 7-10 days. The effector cells were then restimulated with syngeneic thioglycolate-elicited peritoneal exudate cells (PECs) pulsed with T1/L for 45 min at 25°C (MOI=1) at a ratio of five effectors to one PEC, cultured at a final concentration of 2.5×10^6 effectors/mL in TCM supplemented with 10% conditioned medium from concanavalin A (Sigma, St. Louis, MO) stimulated rat splenocytes and 25 mM α-methylmannoside. Cultures were incubated for an additional 5 days at 37°C in an atmosphere of 5% CO_2. Ficoll-Hypaque gradient enriched $CD8^+$ effector cells were treated with GolgiStop™ (BD Pharmingen) and cultured with target L929 cells infected with T1/L at an effector to target ratio of 25:1 in 96 well V-bottom plates. As a positive control, some effector cells were polyclonally activated by incubating with plate bound anti-CD3/anti-CD28 antibodies. Cultures were incubated for 4-6 hours at 37°C at a 5% CO_2 atmosphere. Following incubation, cells were stained for flow cytometry analysis.

Lamina Propria fragment cultures

Lamina propria (LP) fragment cultures were established as previously described [45]. Initially, PPs were removed and the small intestines were longitudinally split and cut into approximately 1-cm segments. Luminal contents were removed by washing the intestinal segments in PBS followed by incubation on a rotator at 37°C for 30 minutes in PBS supplemented with 10 mM EDTA and 1 mM dithiothreitol (DTT) to remove the epithelium. The PBS with EDTA and DTT was replaced and the fragment cultures incubated for an additional 30 min on the rotator at 37°C. The LP fragments devoid of epithelium were incubated in TCM for 5 days at 37°C in 5% CO_2. After the 5 day incubation, the supernatants were collected and the total, reovirus-specific, and *PE*-specific antibody concentrations were measured by ELISA.

Serum and LP supernatant ELISA

ELISA was performed as previously described [46]. EIA/RIA flat bottom plates were coated overnight at 4°C with purified T1/L reovirus (2.5×10^{10} particles/mL), 20 µg/ml PE, or 1 µg/ml goat anti-mouse IgA in 50 µl of 0.1M $NaHCO_3$. Plates were twice washed with PBS supplemented with 0.05% Tween (PBST) and blocked with 3% bovine serum albumin (BSA) in PBS for 1 hour at room temperature (RT). Plates were additionally washed two times with PBST. Mice sera and LP supernatants were serially diluted in PBS with 10% fetal bovine serum (FBS), added to the coated wells, and incubated overnight at 4°C. Immune reference serum of a known titer was added to additional reovirus coated wells and known concentrations of purified mouse IgA (Southern Biotechnology, Birmingham, AL) were added to anti-IgA coated wells to generate standard curves for determination of antibody titers or quantitation of antibody concentrations as indicated. Following four washes, biotinylated goat anti-

mouse antibodies specific to IgG (H + L) or IgG2a, (Southern Biotechnology) in 10% serum were added to wells with serum and biotinylated goat anti-mouse antibody specific for IgA (Southern Biotechnology) were added to wells with LP supernatant for one hour at RT. Plates were washed six times and strepavidin peroxidase was added for one hour at RT at a concentration of 2.5 µg/mL in 10% FBS. Following eight washes, reovirus-specific (serum total IgG, IgG1, and IgG2a and LP IgA) antibodies as well as total and PE-specific LP IgA antibodies were detected through the subsequent colour development using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) plus 0.03% H_2O_2 and analyzed at 405 nm with an ELISA plate reader. PE-specific serum IgE and PE-specific IgG1 and IgG2a antibodies were determined, respectively, by capture and indirect ELISA as described [47].

Peyer's Patch Lymphocyte Preparation

Excised PP were mechanically disrupted with 18-gauge syringe needles and enzymatically dissociated with Collagenase Blendzyme Liberase I at 0.28 Wünsch units/mL (Roche, Indianapolis, IN) and DNase 1 grade 1 at 220 U/mL (Roche) for 30 min at 37°C. The disrupted PP were then treated with 0.1M EDTA for 5 min at RT and expressed through a 70 µm cell strainer yielding a single cell suspension that was subsequently stained for flow cytometry analysis.

Flow cytometric analysis

Splenic effector and PP cells were stained for expression of surface markers. CD8+ effector cells were stained with anti-mouse CD8α-allophycocyanin (APC) (1:100) (BD Pharmingen) and PP cells were stained with anti-mouse CD8α-fluorescein isothiocyanate (1:100) (BD Pharmingen), CD45R/B220-phycoerythrin (1:300) (BD Pharmingen), or CD4-phycoerythrin (1:200) (BD Pharmingen) for 30 min at 4°C in the dark. Cells were twice washed with FACS buffer (1X Dulbecco's PBS supplemented with 0.5% BSA and 2mM EDTA), and fixed and permeabilized. Splenic effector cells were fixed with Cytofix/Cytoperm™ solution (BD Biosciences) according to the manufacturers' instructions, intracellularly stained with anti-mouse IFN-γ-phycoerythrin (1:100) (BD Biosciences), and analyzed by flow cytometry. PP cells were fixed for 2 hours at 4°C in the dark with eBioscience Foxp3 fixation/permeabilization solution (eBioscience, San Diego, CA), twice washed with permeabilization buffer (eBioscience), and intracellularly stained with anti-mouse/rat Foxp3-APC (1 µg/10^6 cells) (eBioscience) in permeabilization buffer for 30 min at 4°C. Cells were washed, resuspended in FACS buffer, and analyzed by flow cytometry. All stained cells were analyzed with a FACSCaliber flow cytometer (BD Biosciences). Data were analyzed with CellQuest Pro, ver. 3.2 (Becton Dickinson).

Statistical analysis

Data were analyzed using one way analysis of variance (ANOVA) followed by a Tukey's or Bonferonni post hoc test. Bonferonni post hoc was used for the PE-specific serum antibody and splenic cytokine analyses. Tukey's post hoc was used for all other analyses. A *P* value of <0.05 was considered significant.

Results

Reovirus Induces Robust Th1 and IgA responses in the presence of CT and PE

C3H mice orally infected with reovirus mount a robust anti-reovirus Th1-response. To ensure that mice infected with reovirus in the presence of PE and CT mounted a prototypic systemic and mucosal immune response to reovirus, serum samples and LP-fragment culture supernatants were analyzed for reovirus-specific IgG and IgA, respectively. The anti-reovirus serum IgG titers were very high in serum from infected mice, but mice that received CT + PE + reovirus had a statistically significant decrease compared to mice that received reovirus + CT (Fig. 1a). Virus-specific IgG was non-detectable in mice that did not receive reovirus.

LP-fragment cultures from reovirus-infected mice made substantial reovirus-specific IgA responses, irrespective of whether they received CT or PE (Fig. 1b). Mice that did not receive reovirus made no detectable anti-reovirus IgA. Whereas CT + reovirus did not enhance the anti-reovirus response, mice that received CT + PE + reovirus did have a statistically higher anti-reovirus IgA response than mice receiving PE + reovirus.

All mice that received reovirus whether mixed with PE, CT, or both, mounted robust virus-specific IgG2a responses, however, a significantly lower titer was observed in mice that received PE + CT + reovirus compared to mice that received CT + reovirus, reflecting the levels found when total virus-specific IgG was assessed (Fig. 2a). Together these data suggest that T-cell help for humoral antibody responses were dominated by IFN-γ producing Th1 cells [48]. That Th1-dominated anti-reovirus responses were induced in virus-infected mice was further supported by the observation that *in vitro* IFN-γ production was readily detected following *in vitro* restimulation of $CD8^+$ splenic T-cells from mice infected with reovirus with or without PE and CT (Fig. 2b).

Together these data indicate that reovirus is a potent immunogen in these mice, and that neither CT nor PE negatively affected the immunogenicity of reovirus.

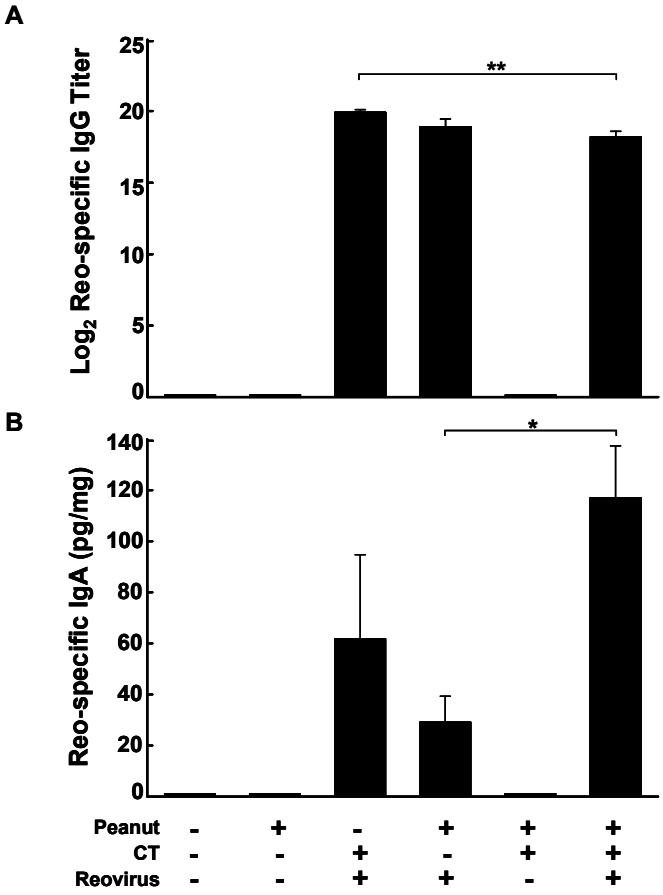

Figure 1
Induction of reovirus-specific systemic and intestinal humoral immune responses.
Total reovirus-specific serum IgG antibody titers from treated mice were determined as described in materials and methods (A). Reovirus-specific IgA concentrations were determined as described in materials and methods and are presented as picograms of specific antibody per milligram of total IgA (B). Results shown are combined from two independent experiments. Error bars indicate standard error among 7-9 mice per group. Significance indicated with (*) $P<0.05$ (**) $P<0.001$.

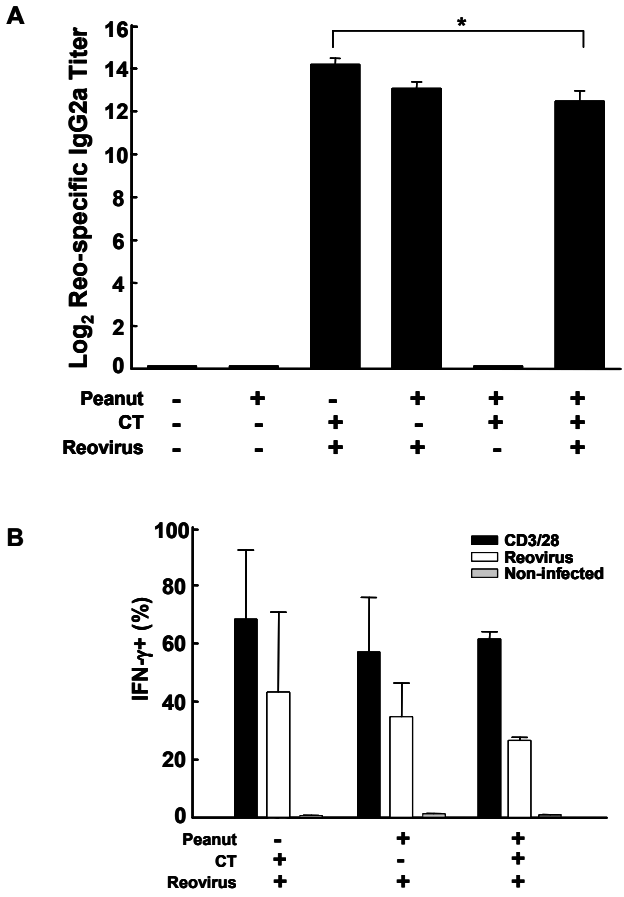

Figure 2
Induction of Th-1 dominant reovirus-specific humoral and cell-mediated immune responses. Reovirus-specific serum IgG2a isotype antibody titers from orally treated mice were determined as described in materials and methods. Results shown are combined from two independent experiments. Significance indicated with (*) $P<0.05$. Error bars indicate standard error among 7-9 mice per group (A). Splenic $CD8^+$ T-cells were harvested from reovirus infected mice and restimulated *in vitro* with reovirus. Activation of $CD8^+$ effector T-cells was assayed by the production of intracellular IFN-γ when challenged with non-infected (gray bars) or reovirus-infected (open bars) L929 mouse fibroblast cells, or polyclonally activated with anti-CD3α/CD28 antibodies (black bars). Bars represent the percentage of CD8/IFN-γ double positive T-cells following *in vitro* stimulation. Error bars indicate standard deviation of replicates (B).

CT promotes mixed PE-specific serum antibody responses and reovirus modulates PE-specific serum IgG2a

As expected, oral administration of PE + CT promoted production of robust PE-specific IgE, IgG1, and IgG2a serum antibody responses (Fig. 3). There was little or no IgE detected in mice receiving PE alone or PE + reovirus. IgG1 and IgG2a were detectable in the PE alone group but were significantly increased when CT was co-administered. Reovirus did not alter the CT-mediated increase serum levels of PE specific IgE and IgG1. However, co-administration of reovirus with PE caused a significant 30-fold increase in the PE-specific IgG2a response when compared to treatment with PE alone. Co-administration of reovirus with PE and CT exhibited a trend to an 8-fold increase in PE-specific IgG2a compared to PE+CT treatment. These increases demonstrate the Th1-skewing potential of reovirus on PE-specific IgG2a responses.

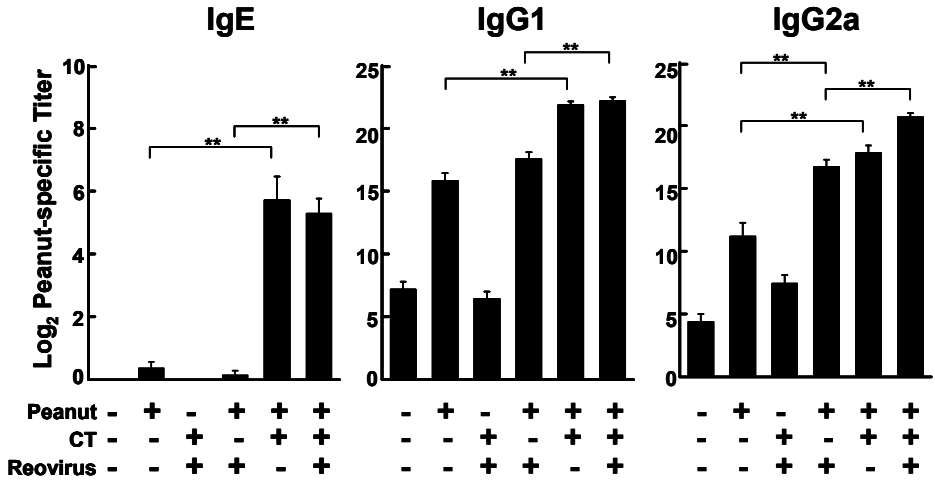

Figure 3
Effects of reovirus on PE-specific serum antibody production.
Mice were orally treated as described in the materials and methods. At the end of the treatment protocol, serum from treated mice was collected and the PE-specific serum IgE, IgG1, and IgG2a antibody titers were analyzed by ELISA. Data were analyzed for statistically significant differences by ANOVA followed by Bonferonni post hoc test. Significance indicated with (**) $P<0.001$. Results shown are combined from two independent experiments. Error bars indicate standard error among 7-9 mice per group.

Reovirus does not affect the CT-mediated PE-specific mucosal IgA response

In addition to promoting systemic antibody responses to PE, CT promotes production of mucosal anti-PE IgA [22]. To determine whether reovirus affects the mucosal anti-

PE response, LP-fragment cultures of intestinal tissue were established from mice treated with PE mixed with CT, reovirus, or both CT and reovirus (Fig. 4). Anti-PE IgA was readily detected in intestinal cultures from mice receiving PE + CT, irrespective of whether reovirus was also administered. Interestingly, reovirus had no detectable adjuvant properties on the mucosal anti-PE IgA response.

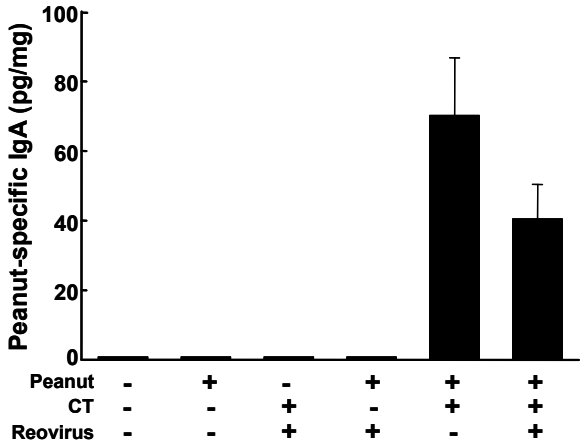

Figure 4
Effects of reovirus on PE-specific IgA production.
Lamina propria fragment cultures from treated mice were established *in vitro* as described in the materials and methods. After 5 days, the culture supernatants were collected and the presence of PE-specific IgA was determined by ELISA. Data were analyzed for statistically significant differences by ANOVA followed by Tukey's post hoc test. Results shown are combined from two independent experiments. Error bars indicate standard error among 7-9 mice per group. PE-specific IgA concentration is shown as picograms per milligram of total IgA.

Reovirus does not affect the CT-enhanced PE-specific splenic cytokine responses

Previous studies have indicated that spleen cells from PE-sensitized mice demonstrate a PE-induced Th1/Th2 mixed cytokine response [49]. Therefore the effects of CT and reovirus on the PE-specific recall cytokine response was examined (Fig. 5). Spleen cells from treated mice were stimulated *in vitro* with or without PE, and the Th1-associated (IFN-γ and Th2-associated (IL-4, IL-5, and IL-10) cytokine levels were measured in culture supernatants. Spleen cells from mice receiving PE + CT demonstrated significantly increased levels of IL-4, IL-5, and IL-10 compared to the PE alone group. There was an increase in IFN-γ for the PE + CT group albeit not significant. Reovirus did not affect the CT-enhanced cytokine response to PE

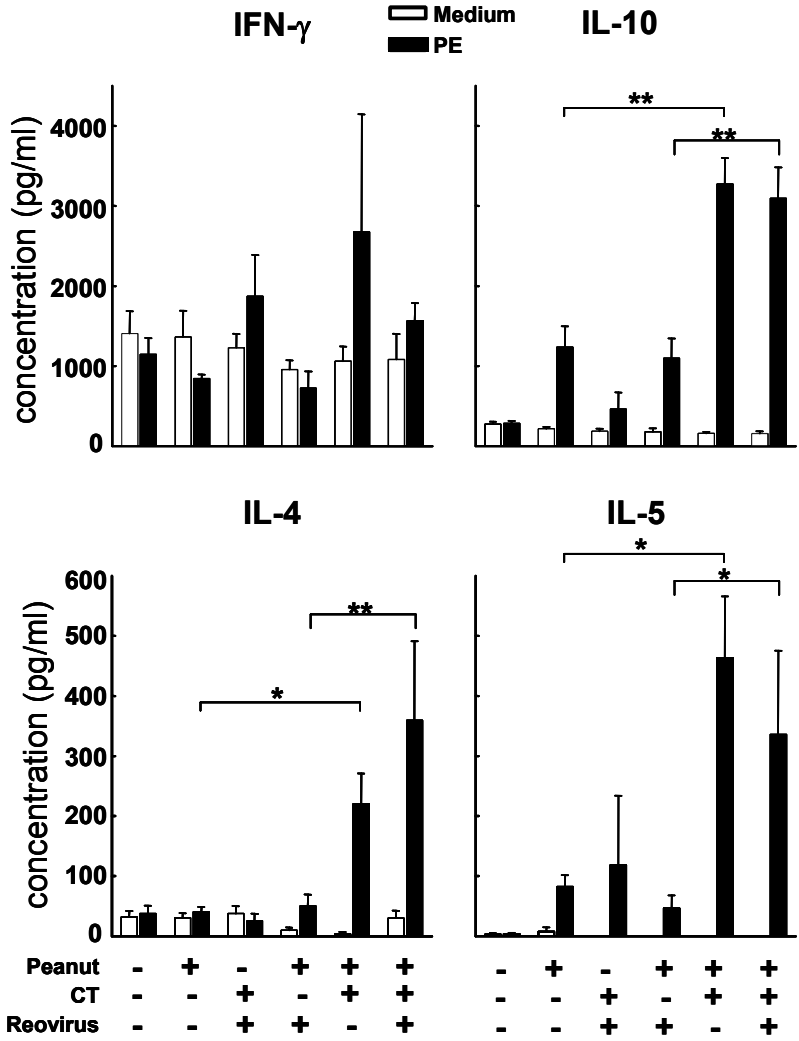

Figure 5
Effects of reovirus on cytokine levels in splenocyte cell culture supernatants.
Splenocytes from treated mice were cultured *in vitro* in the presence (filled bars) or absence (open bars) of 200µg/ml of PE for 96 hours. Following incubation, the cell culture supernatants were harvested and the IFN-γ, IL-10, IL-4, and IL-5 cytokine levels induced by restimulation were analyzed by ELISA. Cytokine concentration is shown as picograms per milliliter. Data were analyzed for statistically significant differences by ANOVA followed by Bonferonni post hoc test. Significance indicated with (*) $P<0.05$, (**) $P<0.001$. Results shown are combined from two independent experiments. Error bars indicate standard error among 7-9 mice per group.

Reovirus modestly affects the PP lymphocyte distribution when co-administered with PE

Despite the development of robust antigen-specific responses to PE and reovirus, most of the treatments did not affect the percentages of B-cells, T-helper cells, T-cytotoxic cells, and Tregs in the PP (Table 1). Only in the PP of the PE + reovirus group a slight but statistically significant decrease was detected in the $CD8^+$ T-cells and $CD4^+CD25^+Foxp3^+$ Tregs. The percentage of $CD4^+$ T-cells also appeared to be trending downward in the PE + reovirus group, but did not reach statistical significance.

Table I. Lymphocyte distribution in Peyer's patches

	Percent (S.D.)			
Groups	**CD4**	**CD8**	**B220**	**CD4/Foxp3**
PBS	21.7 (1.8)	2.7 (0.3)	72.8 (3.0)	7.5 (0.3)
Peanut	21.8 (3.2)	3.6 (0.8)	70.8 (2.5)	11.6 (0.8)
Reovirus + CT	23.0 (2.3)	3.6 (1.1)	67.0 (3.9)	10.4 (1.1)
Peanut + Reovirus	19.1 (1.5)	1.8 (0.2)*	72.0 (2.0)	4.8 (0.6)*
Peanut + CT	21.7 (2.2)	3.9 (1.0)	69.2 (4.9)	11.1 (0.2)
Peanut + Reovirus + CT	24.8 (3.9)	3.7 (0.7)	66.4 (4.8)	12.3 (0.5)

[a] PP cells were stained for expression of CD4, CD8α, CD45R/B220, and Foxp3 and analyzed with flow cytometry.
[b] Analyzed using ANOVA followed by a Tukey post hoc test. Significance is indicated with an asterisk (*) ($P<0.05$)

Discussion

Epidemiological and experimental data support the hygiene hypothesis as an explanation for the observed increase in the prevalence of atopic disorders in developed countries, despite the lack of a well-defined mechanism [50]. One variant of the hygiene hypothesis suggests that lack of microbial stimulation results in a Th1/Th2 imbalance with a resulting Th2-bias that predisposes individuals to allergic responses. In this study, it was hypothesized that induction of a robust anti-viral Th1-type immune response induced by reovirus would inhibit the prototypic Th2-type PE-specific food allergic response. Several experimental efforts biasing the Th1/Th2 balance toward a Th1-type immune response have successfully demonstrated suppression of Th2-type food allergic responses. For example, IL-12, which drives production of IFN-γ that results in cell mediated immunity and class switching to IgG2a, has been shown to inhibit *PE*-induced anaphylactic reactions, decrease *PE*-

specific IgE levels, and reverse the *PE*-specific IgG1/IgG2a ratio when orally administered to mice in a model of peanut allergy [51]. Additionally, in an indirect manner, the toll-like receptor (TLR)-ligands CpG (TLR9) [52] and LPS (TLR4) [53], and the herbal extract food allergy herbal formula-2 (FAHF-2) [54] induced a Th1-type shift in the immune response resulting in abrogation of *PE*-specific allergic responses.

The systemic and mucosal responses to reovirus were highly specific and characteristic of 'conventional' Th1-dominant responses (serum IgG2a and IFN-γ-producing cells), even in the presence of CT. Thus it was concluded that virus infection did establish a milieu that promoted formation of antigen-specific Th1-cells following oral exposure. Having established that reovirus induced an immunologic environment that promoted Th1 responses, the next question was what effect this inflammatory setting had on the *PE*-specific antibody, and particularly the IgE response in the context of CT. Although IgE antibody responses to antigen are typically thought of as driven by Th2 cells, the roles of Th2 and Th1 cells in this model are complex because CT promotes the activation of both PE-specific Th2 and Th1 cells [55]. In addition CT modifies the activity of regulatory cells [56] and antigen presenting cells [57] following exposure to PE.

As expected, CT enhanced production of PE-specific IgE, IgG1, and IgG2a and also of PE-induced splenic production of IL-4, IL-5, and IL-10. Reovirus did not alter the PE-specific serum IgE or IgG1 responses; however, virus substantially increased the levels of PE-specific IgG2a. This finding would suggest an increase in PE-specific Th1 responses without a concomitant increase in Th2 activity, and is consistent with the hypothesis that reovirus has Th1-promoting activity. However, an increase in PE-specific memory Th1 cells was not observed in the spleens of infected mice as measured by increased IFN-γ production, which argues against the idea that reovirus activates PE-specific Th1-cells.

The mechanism of how reovirus promotes a response to an unrelated and physically unlinked antigen has not yet been determined, but the virus has been previously shown to enhance humoral immunity to unlinked antigens [58]. One hypothesis that could account for the observations is that reovirus infection acutely increases local IL-12 production that promotes PE-specific Th1 cells and IgG2a production. This hypothesis is consistent with previous findings from our group, showing that following oral infection with reovirus, increased levels of IL-12 and IFN-γ mRNA were detected in mouse PP and mesenteric lymph node in the first 96 hours after infection [59]. The cellular source of this and other proinflammatory cytokines has not been firmly established. Although reovirus did not modify the PE-specific cytokine recall response from spleen cells restimulated with PE *in vitro*, it is plausible that *in vivo*, early IL-12 production by innate immune cells and IFN-γ produced by virus-activated

T-cells, including $CD8^+$ T-cells, enhanced the PE-specific IgG2a response. An analysis of the kinetics of IL-12 and IFN-γ during the sensitization protocol would help to determine their possible role in the PE-specific IgG2a enhancement.

Earlier studies demonstrated that reovirus acted as a polyclonal activator of the intestinal IgA responses in mice if given prior to weaning. This polyclonal response was paralleled by the initiation of germinal centres in the Peyer's patches in pre-weaned mice [60]. The mice used in the present study were young but weaned. Nevertheless, it was thought that perhaps reovirus infection in these young but weaned mice would increase the IgA response to PE antigen, which could prevent the uptake of PE and potentially serve as a barrier to initiation of IgE production and PE-specific allergic responses. Although CT increased the IgA response to PE and reovirus, reovirus by itself did not enhance the IgA response to PE. It is possible that the mixture of PE and reovirus would be more effective at inducing PE-specific IgA in mice prior to weaning. Alternatively, whether PE-conjugated reovirus could induce strong PE-specific IgA responses in the absence of CT remain to be determined.

Reovirus modestly altered the distribution of the $CD4^+CD25^+Foxp3^+$ Tregs in the PP of mice treated with PE and reovirus. Although further analysis is needed, we can only speculate that an increase in another undefined cell type in the PP or the migration of the Tregs from the PP to another tissue accounts for this decrease. $CD4^+CD25^+$ Tregs have been shown to play an important role in the induction of oral tolerance [61]. The change in the relative percentage of these cells in the PP of mice receiving PE and reovirus suggests that reovirus may have an effect on the induction of the oral tolerance response.

To the best of our knowledge, this is the first study that examines the effects of an enteric virus on the development of food allergies following oral sensitization. A recent epidemiological study by Reimerink et al. [62] investigating the relation between early-life intestinal viral infections and the development of atopic disorders, pointed to an association between infants suffering from recurrent wheeze at age 1 and 2 and increased rotavirus seropositivity in their first year of life. However, the authors also reported an inverse association between norovirus seropositivity and allergic sensitization in the first year of life, highlighting the importance for studying enteric virus infections and their roles in the development of atopic disorders. Overall, our data demonstrates that an acute infection with an enteric virus and the subsequent mucosal and systemic development of a virus-induced Th1 immune response is capable of enhancing the PE-specific IgG2a antibody response. Moreover, the reovirus-mediated increase in PE-specific IgG2a may have therapeutic implications. Strait et al reported that allergen-specific IgG can block IgE-mediated anaphylaxis *in vivo* through antigen interception and FcγRIIb-mediated inhibition [63]. Future studies examining the potential of reovirus-induced increased PE-specific

IgG2a levels to block or inhibit IgE-mediated allergic responses in a mouse model of peanut allergy are needed. Given that peanut allergies can be life-threatening, are rarely outgrown, and avoidance being the only absolute effective treatment, further studies testing mechanisms that have therapeutic prospects need deeper exploration.

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CHAPTER 5

Translational approach to predict the efficacy of probiotics to influence peanut allergy

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Abstract

Background Probiotics may be considered for treatment of allergic individuals on the basis of their immune modulatory properties. Here, we have used a translational strategy to assess the allergy-modulating capacity of probiotics.
Methods The efficacy of different strains of probiotics (*Lactobacillus plantarum 256* (LP256), *Lactobacillus plantarum* NCIMB 8826 (LP8826), and *Lactobacillus reuteri* strain DSM20016) to modulate cytokine production was tested using human peripheral blood mononuclear cells (PBMC). Next, all strains were screened for their modulatory potential in a mouse peanut allergy model. In depth studies investigated the effect of LP256 on intestinal TGF-β production and the influence of neutralization of IFN-γ (using anti- IFN-γ; clone XMG1.2) or IL-10 (using anti-IL-10; clone JES5.2A5) on the effects of LP256, thus allowing further characterization of the modulatory capacity of LP256.
Results All probiotic strains increased the production of IFN-γ, IL-12, and IL-10 by human PBMC. *In vitro* added LP256 stimulated IL-10 as well as IFN-γ production by splenocytes from peanut sensitized mice. In the peanut allergy model LP256 caused a small increase in serum levels of peanut-specific IgE, mast cell responses, and *ex-vivo* IFN-γ production. In addition, LP256 appeared to induce intestinal activation of TGF-β. Irrespective of treatment with LP256, neutralization of IFN-γ and especially of IL-10 induced high ex-vivo production levels of Th2 cytokines (IL-5, IL-13 and IL-10). Neutralization of IL-10, and of IFN-γ only in combination with LP256, caused an increase of mMCP-1 responses, without affecting IgE levels.
Conclusion Selected probiotics appeared to have a moderate effect on peanut sensitization, despite the fact that they appeared to profoundly stimulate cytokine production *in vitro.* Combined neutralization studies, however, point to the capacity of LP256 to modulate mast cell responses providing that the strong regulatory influence, of in particular IFN-γ, was absent. If more detailed knowledge of the mechanisms underlying the immunological effects of food allergy becomes available, it may be possible to improve therapy by using cocktails of different probiotics. Our study however shows that careful strain selection is difficult. The translational approach presented here, using human PBMC, mouse splenocytes as well as animal models, and including means to neutralize existing regulatory mechanisms, may be helpful in selecting and characterizing immunomodulatory probiotics.

Introduction

It is estimated that currently 3-4% of adult individuals are suffering from some kind of food allergy [1]. Peanut allergy, among other food allergies, deserves particular attention. It accounts for the majority of severe food-related allergic reactions and is most commonly associated with anaphylaxis and fatal outcome [2]. In addition, peanut allergy does not usually resolve, resulting in a life-long allergy with a high impact on daily life [3]. So far, no therapies are available and the only remedy is strict avoidance of the allergen [4]. Accidental ingestion of peanut allergens, however, is common due to trace amounts of peanut in many food products and cross-contamination during processing [5]. Therefore, the development of new treatment methods for peanut allergy is necessary, for which more mechanistic studies are required.

An apparent decline in microbial exposure during early life is proposed as a possible cause for the increase incidence of allergic diseases over the past few decades [6-9]. In the absence of sufficient microbial stimuli, normal regulatory immunological mechanisms may not develop resulting in a higher risk of diseases that have an immunological basis. In this respect, timely colonization of the intestine by the appropriate bacteria is a highly important stimulus to the developing immune system. Supporting this opinion, the composition of the gut microflora of allergic children has been found to differ from that of healthy children [10]. This provides a strong rationale for using probiotics to shape the immune response of the host, especially in infancy. Probiotics are live bacteria exhibiting health-promoting activities. Studies have demonstrated that probiotics can prevent pathogen colonization of the gut and reduce the incidence or relieve the symptoms of various diseases caused by a dysregulated immune response, such as diabetes [11], arthritis [12, 13] and colitis [14-16]. Most studies have focused on the role of certain strains of lactobacilli and bifidobacteria as probiotics and they have been observed to influence the immune system in a number of ways. There are, however, very few studies in which several alleged probiotic strains have been compared *in vivo* and it is, for this reason, unknown to what extent a finding using a certain bacteria strain is relevant for other strains, or even for the same species. To date, there are only a few strains that have been reasonably well documented in clinical studies and they mostly show a positive but temporary effect on atopic dermatitis and eczema, in regard to allergic diseases [17-25].

Several mechanisms by which probiotics can exert a beneficial effect on health have been proposed. Originally, it was thought to stem from improvements in the intestinal microbial balance. However, there is now substantial evidence that probiotics can also provide benefits by modulating immune functions, since they may

affect enterocytes, antigen presenting cells, regulatory T cells (Tregs), and effector B and T cells. For beneficial effects on allergy, it is thought that probiotics can skew the Th2 balance immune response towards Th1 immunity [26-28] and/or stimulate Tregs [29-31], thereby alleviating the allergic response. Probiotics have been shown to decrease IL-6, which is known to deactivate regulatory cells [36] and to increase IL-10 and IL-12 production by PBMC [32-35]. IL-10 is known for its immunoregulatory role [37] and IL-12 is known to promote the differentiation of Th1 cells as well as natural killer (NK) cell activity [38]. Under these conditions (low levels of IL-6, and high levels of IL-10 and Il-12), cellular immunity and modulation by all kinds of regulatory T cells is favored. Regulation may result from IFN-γ-mediated skewing of the immune response from Th2- to Th1-driven. Exposure to probiotics in the gut can also stimulate the intestinal epithelial cells (IEC) to secrete anti-inflammatory cytokines, such as TGF-β [39], which can promote the differentiation of immature dendritic cells (DC) into regulatory DC [40-42]. Probiotics may in addition directly trigger the differentiation of regulatory DC and together lead to a further induction of Tregs [31]. Finally, clinical trials have observed that probiotics induce a low-grade activation of the immune response, characterized by elevated IgE, IgA and IL-10. Such a low-grade inflammation may also induce regulatory cells and thereby contribute to establishment of oral tolerance [43, 44].
To study the role of probiotics in the modulation of peanut allergy, mice were orally treated with different strains of probiotics prior and during the induction of sensitization. From this initial screening, in combination with probiotic-induced cytokine production profiles by human PBMC, *Lactobacillus plantarum* 256 (LP256) strain was selected to be further investigated. LP256 treatment appeared to have a moderate influence on peanut sensitization and this influence became more apparent in case IFN-γ was neutralized.

Materials and Methods

Mice
Female, specific pathogen-free C3H/HeOuJ Ico mice, 4-5 weeks of age were purchased from Charles River (Lyon, France) and 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 a 12-h light/dark cycle. Drinking water and standard laboratory food pellets were provided *ad libitum*. The experiments described in this manuscript were all approved by the animal experiments committee of the Faculty of Veterinary Medicine, Utrecht University.

Chemicals, reagents and monoclonal antibodies
Anti-IL-10 (JES5.2A5) and anti-IFN-γ (XMG 1.2) were produced by culturing the hybridoma's and purified using thiophilic agarose. Cholera toxin (CT) was purchased from List Biological Laboratories, Inc (Campbell, California, USA). All other chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands) unless otherwise stated.

Preparation of peanut extract
Peanut was kindly donated by Imco Nut Products, the Nut Company (Doetinchem, the Netherlands) and was supplied by Golden Peanut, plant at Alpharetta (Georgia). Protein extract was made by blending 100 g peanut with 500 mL 20 mM Tris buffer (pH 7.2). After 2 h blending at intervals of 20 min for 1 min at room temperature, the aqueous fraction was collected by centrifugation (3000 g, at 4°C for 30 min). The aqueous phase was subsequently centrifuged (10 000 g at 4°C for 30 min) to remove residual traces of fat and insoluble particles. Protein concentrations were determined using Bradford analysis with BSA as a standard. Extract (peanut extract, PE) contained typically 32 mg/mL protein and was stored at -20°C. Reducing SDS-PAGE from the extracts showed protein bands between 14 and approximately 100 kDa (not shown).

Probiotics
Lactobacillus plantarum 256 (LP256), *Lactobacillus plantarum* NCIMB 8826 (LP8826), and *Lactobacillus reuteri* strain DSM20016 were streaked on MRS, solidified with 1.4% agar and incubated at 37 ºC (all probiotics were kindly provided by TNO Quality of Life, Zeist, The Netherlands). Next, single colonies obtained were cultured at 37 ºC in MRS. The number of bacteria was estimated by measuring the optical density at 600 nm (OD_{600}) and resuspended to an estimated concentration of 5×10^9 colony forming units per mL (cfu/mL). The bacterial suspensions were aliquoted and stored at – 80 ºC. Aliquots of these stock suspensions were used to determine the actual number of cfu's per mL.

In vitro cultures with human PBMC's
PBMC's were isolated in a flow cabinet at room temperature. The blood (Sanquin, Nijmegen) was diluted 1:1 with Iscove's Modified Dulbecco's Medium (IMDM) containing GlutaMAX (Invitrogen, Breda, The Netherlands). The PBMC's were isolated by density gradient centrifugation (Ficoll-Plaque PLUS, Amersham, Roosendaal, The Netherlands). PBMC's were cultivated in IMDM containing 1% heat-inactivated human AB serum, 1% penicillin, streptomycin (v/v) (Invitrogen, Breda, The Netherlands). The cells were left unstimulated or were stimulated with LPS (1 µg/ mL) or with

bacteria (LP256, LP8826, L. reuteri 1:1 ratio (based on CFU counts)). After 24 hr incubation, the concentrations of IL-10, IL-12 and IFN-γ present in the co-culture supernatants were determined using the BD cytometric bead array and flow cytometry (FACSCantoII).

Sensitization protocol
Mice (n=8) 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 of CT on 3 consecutive days, followed by weekly dosing of PE plus CT (day 7, 14 and 21). From day -14 until day 27, mice were treated by gavage 3 times a week with 1 x 10^9 cfu of different lactobacilli strains (LP256, LP8826 or *Lactobacillus reuteri*), diluted in 0.2 M $NaHCO_3$. For cytokine-neutralization experiments, mice were treated i.p. on day -1 with 1 mg of anti-IL-10, anti-IFN-γ, or 200 µl NaCl followed by twice a week dosing of 0.5 mg antibodies or NaCl until day 27.

LP256 and TGF-β treatment protocol
Mice were orally treated for 3 consecutive days with PBS (negative control), 10^9 cfu of LP256 or with 5µg of TGF-β on day 3 only (positive control). Three hours after the last treatment, mouse ileum tissues were isolated.

Immunohistochemical staining
Acetone fixed cryostat sections of the small intestine were incubated for 1h with anti-phospho-SMAD2/3 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:5 in PBS, 1% BSA. The antibodies were then visualized by anti-goat FITC (DAKO, Denmark) 1:20 in PBS. Sections were mounted with DAKO Fluorescent Mounting Medium. Images were acquired using an Olympus BX60 microscope with colorview III digital camera and Cell Imaging software.

Measurement of serum IgG1, IgG2a, and IgE antibodies
Blood samples were collected at various time-points and stored at -20°C until analysis. Levels of PE-specific IgE, IgG1, and IgG2a were determined by ELISA. Plates (highbond 3590; Costar, Cambridge, MA, USA) were coated overnight with 10 µg/mL PE (for IgG1 and IgG2a detection) or with 1.5 mg/mL purified rat anti-mouse IgE (BD Pharmingen) in PBS, followed by 1 h blocking with ELISA buffer (50 mM Tris, 136.9 mM NaCl, 2 mM EDTA, 0.5% Tween and 0.5% BSA). Each test serum was optimally diluted and incubated for 2 hrs. For PE-specific Ab levels, a sera pool of mice sensitized with PE plus alum by i.p. injection once a week for 3 weeks was used as reference serum (start dilution 1 : 10) and a standard curve of the reference serum was included to determine antibody levels (arbitrary units). For detection of

PE-specific IgG1 and IgG2a, alkaline phosphatase-conjugated IgG1, IgG2a (both polyclonal goat anti-mouse), 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 Abs, a PE-digoxigenin (DIG) conjugate solution (diluted in High Performance ELISA Buffer, from Sanquin, San Diego, California, USA) 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.

Cell culture and cytokine measurement

Spleen and MLN single cell suspensions, 6.25 x 10^5 cells/mL in RPMI 1640 (Life Technologies, Paisley, Scotland) containing 10% fetal calf serum (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. The IL-10 (BD Pharmingen, San Diego, California, USA) and IFN-γ, IL-4, IL-5, IL-13 ELISA (eBiosciences, San Diego, California, USA) were performed in accordance with the manufacturer's instructions.

Measurement of serum 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 Scientific Ltd, Midlothian, Scotland). ELISA was performed according to the manufacturer's instructions.

Statistics

Data were analyzed using GraphPad Prism 4 software. The differences between group means were determined by using one-way ANOVA with Bonferroni as *post hoc* test. Statistical analyses were performed following logarithmic transformation (to achieve normal distribution).

Results

Restimulation of human PBMC by probiotics leads to production of IL-10, IL-12, and IFN-γ

PBMC from four different healthy donors were cultured in the presence of LP256, LP8826, L. reuteri, LPS, or medium alone. Cytokine levels in the supernatant shows that LPS and all 3 probiotics stimulated the production of the regulatory cytokine IL-10 and the Th1 skewing cytokines IL-12 and IFN-γ (Fig. 1). In particular, LP256 stimulated the production of all three cytokines relatively the best, in comparison to L. reuteri, which clearly induced less of the Th1 cytokines IL-12 and IFN-γ, and LP8826 that seems to be less potent than LP256 for IL-10 and IL-12 production.

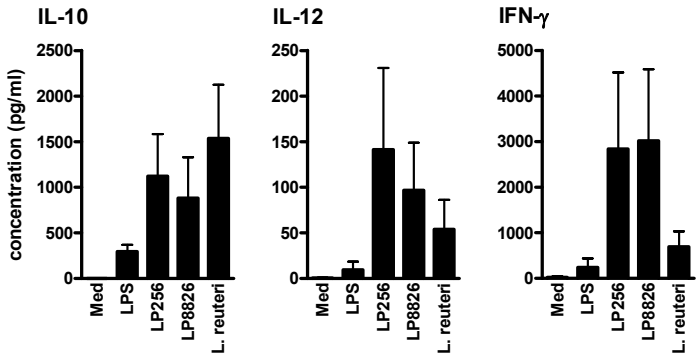

Figure 1
Effect of probiotic treatment on *in vitro* co-culture with human PBMC's
Human PBMC's (10^6 cells per well) from 4 donors were co-cultured with LPS (1µg/mL) or with indicated probiotic strain (1:1 ratio) for 24 hrs. In the supernatant IL-10, IL-12, and IFN-γ cytokine levels were determined using the BD cytometric bead array and flow cytometry (FACSCantoII). The mean cytokine levels of 4 individual donors are presented.

In vivo treatment of PE sensitized mice with different strains of probiotics results in a slight enhancement of PE-specific immune responses

In order to investigate if treatment with probiotics modulates the PE-specific immune responses, mice were treated 3 times a week with $1x10^9$ cfu of LP256, LP8826 or L. reuteri starting from 2 weeks before being sensitized with PE and CT. After 4 weeks of exposure to PE and CT, mice developed PE-specific IgG1, IgG2a, and IgE antibody responses. None of the probiotics caused a significant change in levels of different isotypes (Fig. 2a). But despite this absence of significant changes in particular the

LP256 strain caused some increase in IgE levels, whereas LP8826 appeared to slightly stimulate IgG2a production (Fig. 2a).

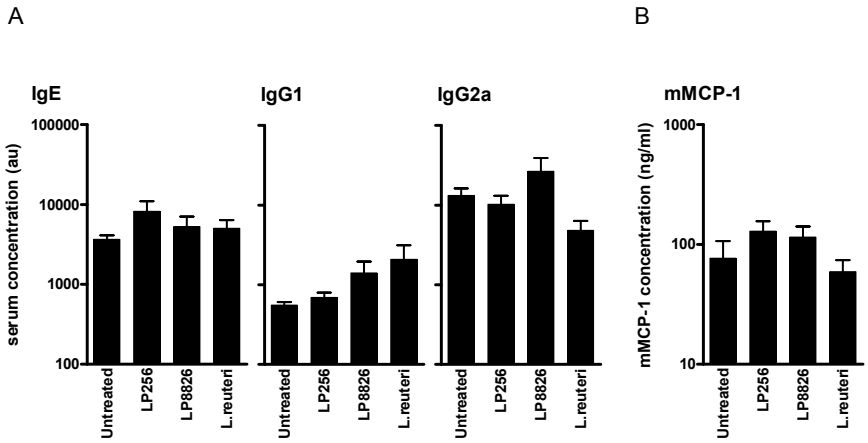

Figure 2
Effect of probiotic treatment *in vivo* on PE-specific antibody response and mast cell degranulation in a mouse model to peanut allergy
Mice were orally sensitized to PE by exposure for 4 weeks to PE + CT. Two weeks prior until 4 weeks after the start of the sensitization protocol, mice were orally treated 3 times a week with 1 x 10^{9} cfu probiotics, or left untreated. (A) PE-specific serum antibody levels in serum collected after 4 weeks of oral exposure. (B) mMCP-1 levels were determined in serum collected within 45 minutes after an oral challenge with PE. Data are presented on a log scale as the mean arbitrary unit per group (A) or in ng/mL (B) (n=8).

After a 6 week probiotic treatment and a 4 week oral exposure regime to PBS or PE with CT, all mice received and oral challenge with PE. Concentration of mMCP-1 levels in serum taken within 45 minutes of challenge was used as a measure of mast cell degranulation. In accordance with the PE-specific IgE levels, mast cell degranulation was observed in the PE and CT-exposed mice and this was slightly increased in LP256- and LP8826-treated mice (Fig. 2b).
On the other hand, *L. reuteri* showed a decrease in the Th1 associated (IgG2a) and an increase in Th2 associated (IgG1 and IgE) PE-specific antibody levels compared to sensitized but untreated mice (Fig. 2a), confirming the human PBMC results. However, this difference has resulted in unchanged mast cell degranulation measured by the mMCP-1 level in serum following PE challenge compared to control mice.
Together, these data show that all studied probiotic strains had no or only a minor effect on PE-specific immune responses, although their potent *in vitro* modulatory capacity. Since oral treatment with LP256 induced the highest changes in PE-specific

clinical responses (mMCP-1) and IgE, we addressed the question as to whether LP256 is able to modify *ex vivo* PE-specific responses of splenocytes from sensitized mice.

Ex vivo co-cultures of LP256 and splenocytes from PE-sensitized mice stimulate cytokine production

Splenocytes from sensitized mice were isolated, restimulated with PE, and co-cultured with increasing amounts of LP256. Exposure to probiotics during PE-specific restimulation led to a dose dependent increase in IL-10 production. In contrast, IFN-γ secretion by splenocytes was increased by LP256 but peaked at a concentration of $2x10^5$ cfu per mL of LP256 (Fig 3). Additionally, Th2 cytokines (IL-4, IL-5, and IL-13) were also increased at a concentration of $2x10^5$ cfu per mL of LP256 (data not shown).

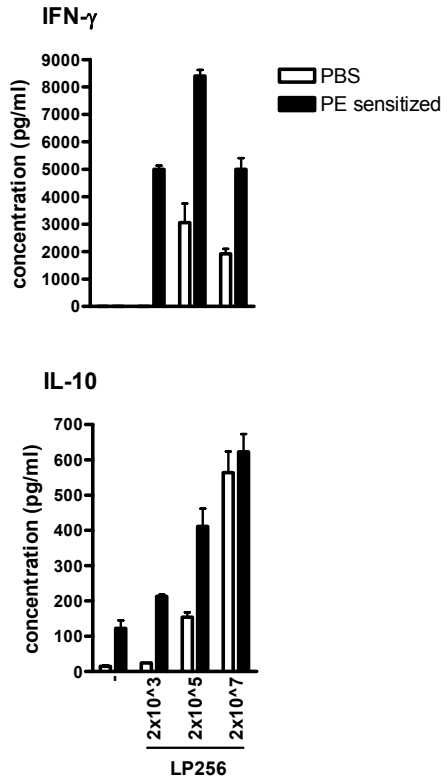

Figure 3
Effect of co-culturing of LP256 on PE-specific restimulation of splenocytes from control and PE sensitized mice
Mice were orally exposed to PBS + CT (control, white bars) or PE + CT (sensitized, black bars) for 4 weeks. Splenocytes were cultured for 96 hrs in the presence of PE and different concentrations of LP256 (as indicated). In the supernatant IFN-γ and IL-10 cytokine levels were determined by ELISA.

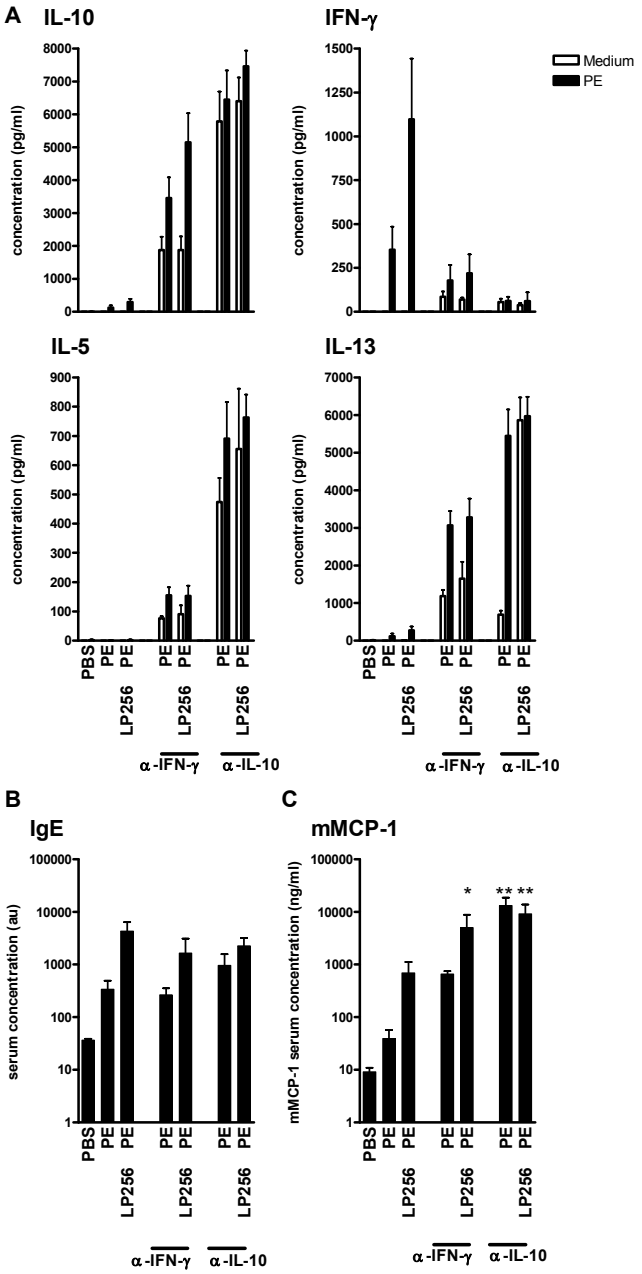

Figure 4
Effect of IFN-γ and IL-10 neutralization on the modulation of PE sensitization during LP256 treatment
Mice were orally sensitized to PE by exposure for 4 weeks to PE + CT or left unsensitized (PBS + CT treatment). Indicated groups received 1 x 10^9 cfu of LP256 3 times a week from day -14 and throughout the PE oral exposure protocol. Indicated groups received neutralizing anti-IFN-γ mAb or neutralizing anti-IL-10 mAb during LP256 treatment. (A) Splenocytes were cultured for 96 hrs in the presence (black bars) or absence (white bars) of PE. In the supernatant IFN-γ, IL-10, IL-5, and IL-13 cytokine levels were determined by ELISA. (B) PE-specific serum IgE antibody levels were determined in serum collected after 4 weeks of oral exposure. (C) mMCP-1 level in serum was measured in serum collected within 45 minutes after an oral challenge with PE. Data are presented on a log scale as the mean arbitrary unit (B), ng/mL (C), or pg/mL (D) per group. *, significantly different ($p<0.001$) from the PBS, PE and ($p<0.05$) from the PE + LP256 exposed group. **, significantly different ($p<0.001$) from the PBS, PE and PE + LP256 exposed group.

These data substantiate the *in vivo* data described above, indicating that LP256 treatment may be able to regulate Th2 immune responses increasing production of IL-10 as well as of IFN-γ, although the ratio of IL-10/IFN-γ is higher at higher doses.

In vivo neutralization of IFN-γ or IL-10 does not influence the LP256 effect

The effect of LP256 may be masked by regulatory mechanisms induced by this probiotic, for instance IL-10 or IFN-γ cytokine production. To examine this possibility, PE-sensitized and control mice were treated with LP256 in combination with neutralizing anti-IFN-γ and anti-IL-10 mAbs during the 4-week long sensitization protocol.

In comparison to sensitized but untreated mice, treatment with LP256 along side exposure to PE caused a particular increase in production of IFN-γ (Fig. 4a). The serum levels of PE-specific IgE and of mMCP-1 were also increased following LP256 treatment.

Neutralization of IFN-γ or IL-10 during sensitization both caused a general dysregulation of the T cell response characterized by an increase in all Th2 cytokines measured (IL-4, IL-5, IL-13, and IL-10) and a decrease of the Th1-like cytokine IFN-γ (Fig 4a). In both cases, higher levels of all cytokines were detected in supernatant of splenocytes that were cultured without PE. Interestingly, dysregulation by neutralizing IL-10 appeared to have a more profound effect on all cytokine levels than neutralization of IFN-γ (Fig. 4a).

Neutralization of IL-10 or IFN-γ did not significantly affect the levels of IgE, although in the case of capturing IL-10 the levels of IgE seemed somewhat increased in the absence of LP256. In contrast, mMCP-1 levels in serum were increased in mice neutralized with anti-IFN-γ and these were even increased when combined with LP256 treatment. IL-10 neutralization had a much larger impact on mast cell degranulation, but additional LP256 treatment did not further increase mMCP-1 levels.

Oral administration LP256 induces phosphorylation of Smad2 in the mouse intestine

Since the immune-stimulating activity of LP256 was not solely based on IFN-γ and/or IL-10, the question was raised whether biologically active TGF-β could play a role in the LP256-induced immunomodulation. In order to analyze the activity of TGF-β locally in the intestinal mucosa, the induction of cytoplasmic Smad2 phosphorylation was assessed. Phosphorylation of Smad2 is a crucial step for initiation of TGF-β signal transduction [45-47].

To this end, ileum tissues were isolated from mice treated with 10^9 cfu of LP256 for 3 consecutive days. Control mice were either treated with PBS (negative control) or on day 3 with 5 μg of TGF-β (positive control). Immunohistochemical stainings showed

increased expression of phosphorylated Smad2 in the ileum of mice that received either LP256 or TGF-β, and not in sham-treated mice. Staining was detected mainly in the cytoplasma of lamina propria lymphocytes and weakly in some epithelium cells. These data show that orally administered LP256 induces intestinal activation of TGF-β.

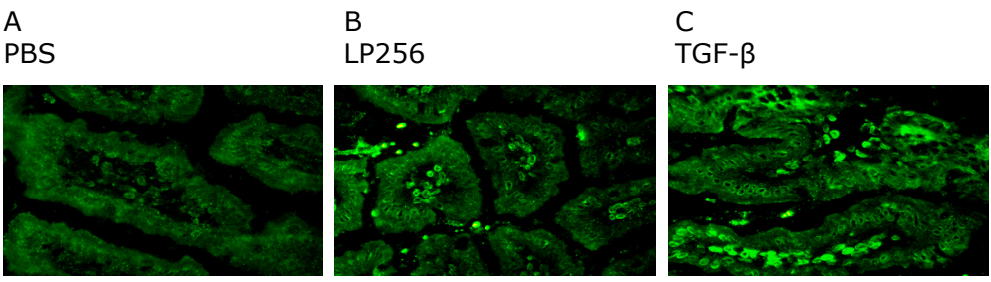

Figure 5
Oral administration LP256 induces phosphorylation of Smad2 in the mouse intestine.
Mice were orally treated for 3 consecutive days with PBS (A, negative control), 10^9 cfu of LP256 (B) or with 5µg of TGF-β on day 3 only (C, positive control). Three hours after the last treatment, mouse ileum tissues were isolated and tissue frozen sections were stained with anti-phosphorylated Smad2 antibody. Representative photographs are shown. Positive stainings are indicated by arrows.

Discussion

Over the past decades, there has been increasing interest in the potential health effects of probiotics. In order to study the possible therapeutic role of probiotics in food allergy, the modulatory capacity of three different probiotics strains (*Lactobacillus plantarum 256* (LP256), *Lactobacillus plantarum* NCIMB 8826 (LP8826), and *Lactobacillus reuteri* strain DSM20016) were studied *in vitro* by using human PBMC or mouse splenocyte cultures of PE-sensitized mice and *in vivo* using a mouse peanut allergy model.
In vitro, all three probiotic strains showed the potential to stimulate the production of both Th1 cytokines IFN-γ and IL-12 and the regulatory cytokine IL-10. *L. reuteri*, being an extensively studied probiotic strain [17, 30, 48], was a very poor stimulator of the Th1-linked cytokines IL-12 and IFN-γ, compared to both *Lactobacillus* strains. The LP256 strain appeared to stimulate IFN-γ and IL-10 production by splenocytes from PE-sensitized mice and *in vivo* studies showed that LP256 caused a moderate stimulation of serum levels of PE-specific IgE, mMCP-1 and of IFN-γ production by PE-restimulated splenocytes. Recent human studies have also indicated that probiotic treatment can lead to a chronic low-grade inflammation, characterized by elevated levels of IgE, IgA and IL-10, which in turn appeared to modulate allergic symptoms

and protect from eczema [43]. Apparently, stimulation of immune responses by probiotics is not unique for LP256 but a more general effect of probiotics, as our *in vitro* studies with other probiotic strains also suggest. Others that have used this same model have interpreted increases in PE-specific IgE levels and mast cell degranulation as a worsening of food allergy [49, 50]. In contrast, helminth infections, accompanied by increases in IgE and IL-10, also protect against allergies. In this case, the stimulation of the Th2 response is accompanied by induction of local regulatory mechanisms, such as TGF-β production in the intestine [51, 52]. TGF-β, in turn, can lead to activation of Tregs [53]. Here, we show LP256 is able to stimulate Smad2 phosphorylation in the ileum. From this, and from the *in vitro* data showing upregulation of IL-10 and IFN-γ, we speculate that LP256 treatment may eventually induce a state of immunoregulation. No changes were found in the percentage of $CD4^+CD25^+$ T cells in LP256-treated sensitized mice in comparison to untreated sensitized mice (data not shown), but naturally occurring $CD4^+CD25^+$ T regulatory cells have been shown to control PE-specific allergic responses [54]. However, other regulatory cells , such as Tr1 (IL-10 secreting) or Th3 (TGF-β secreting) cells, and in case of food allergy also Th1 cells, may play a more imperative role in probiotic modulation [55] .

Neutralization of IL-10 and IFN-γ, both cytokines known to have immunoregulatory potential, had a strong modulating effect on cytokine responses to PE and on mast cell responses, but not on IgE serum levels. Additional LP256 treatment did only affect mast cell responses, and not the production of cytokines or IgE. Remarkably, the mMCP-1 response was increased by neutralization of IFN-γ and this effect was further enhanced by LP256. These *in vivo* neutralization data may be combined with *in vitro* cytokine production results to explain the effects of LP256. LP256 induced a concentration-dependent increase of IL-10 and IFN-γ production by mouse splenocytes, being IFN-γ stimulated at a lower concentration of probiotics than IL-10. Thus at a certain concentration, LP256 may stimulate IFN-γ producing Th1 cells that regulate Th2 cell activation and mast cell activity. When IFN-γ is neutralized Th2 responses and mast cell responses become enhanced. Remarkably, it appears that only when regulation by IFN-γ is absent, LP256 can further stimulate mast cell responses. At higher concentration of the probiotic, IL10-producing cells may be more prominent for regulation. IL-10 seems more important in suppressing mast cell responses than IFN-γ, since when IL-10 neutralization lead to an increase in mast cell responses (even without presence of LP256).

Together, all studies point towards a low grade stimulatory effect of LP256 which is difficult to link to a straightforward beneficial effect in food allergy. Several *in vitro* experiments have evaluated the effects of *Lactobacillus* strains in the modulation of the immune responses. In these cases, they were found to induce maturation of DC

[56], stimulate the production of IFN-γ, IL-12, IL-10, and IL-18 [27], activate human monocytes [57], inhibit the production of Th2 classified cytokines, and induce IL-10-producing Tregs [28]. Conflicting data on IFN-γ production have been published by Kruisselbrink et al, who report that LP256 treatment inhibits IFN-γ production in restimulated splenocytes from OVA-allergic mice [58]. Furthermore, Smits et al. have reported that *L. casei* and *L. reuteri*, but not *L. plantarum*, are able to prime DC to drive the development of IL-10-producing Tregs [31]. This shows that, despite our own findings, no generally conclusive evidence can be delivered regarding the modulation of cytokine production upon *L. plantarum* treatment.

We have shown that LP256 has the potential to increase cytokine production, but also that these changes do not translate into profound effects on PE sensitization in the mouse model here used. But similarly for the human situation, it has been reviewed recently that supplementation of probiotics during pregnancy and early infancy does not reduce the incidence of allergic rhinitis or asthma during childhood [60]. It was also suggested that any beneficial effect of probiotics may not only depend on the composition, concentration and timing of dosing of the probiotics but also on multiple other factors including genetic background of allergic patients, general immunological condition, the composition of their natural microbial flora, and the consistency of the probiotic treatment.

Our study shows only moderate effects of probiotics in the mouse peanut allergy model. We can however not exclude that other probiotics or mixtures of probiotics may have strong beneficial effects on peanut or other food allergies. But as also shown in this study, multiple mechanisms might play simultaneous regulatory roles, making it difficult to select suitable probiotics. A translational approach as presented here, using human PBMC, *in vitro* animal studies as well as *in vivo* animal models, including means to neutralize existing regulatory mechanisms, may be very helpful to select and characterize the probiotic(s) that may be most relevant to pursue.

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CHAPTER 6

Summarizing Discussion

The usual response to harmless gut antigens is the induction oral tolerance. Lack of oral tolerance due to absence of optimal immunoregulation may be involved in causing food allergy. However, the underlying mechanisms that control oral tolerance versus oral sensitization remain to be elucidated (reviewed in chapter 1). Revealing the mechanisms of sensitization to food proteins may contribute to the development of new treatment methods for food allergies. In the present thesis, animal models were used to explore different immunoregulatory mechanisms. Additionally, the possible therapeutic potential of microbial interventions was set under investigation.

1. Summary of this thesis

The use of animal models allows us to investigate the mechanisms involved in the onset of sensitization and breakdown of oral tolerance *in vivo* which are impossible to study in humans. In the present thesis, animal models for peanut oral sensitization [1] and peanut oral tolerance [2, 3] were used to explore different mechanisms of immunomodulation. This may aid in the development of efficient therapies for food allergies in the future.

In **chapter 2**, the role of the innate immune system on the initiation of oral sensitization and tolerance was studied by depletion of NK cells. It was observed that NK cells are important regulators of the allergic response and are involved in the induction of oral tolerance. The results indicate that NK cells, probably by IFN-γ production, are able to control an initiation of oral sensitization by favoring a Th1 response.

Chapter 3 focuses on the role of T regulatory (Treg) cells in the regulation of peanut allergic responses. Van Wijk *et al* [3] showed that depletion of naturally occurring $CD4^+CD25^+$ Treg (nTreg) cells by anti-CD25 mAb treatment resulted in an increase of peanut extract (PE)-specific IgE and mast cell responses. It was also shown that depletion of nTreg cells abrogated PE-specific oral tolerance. We show that although nTreg cells have an unequivocal role in homeostasis, they are unable to transfer oral tolerance to PE following PE plus CT sensitization. Comparing depletion of CD25 cells with neutralization of IL-10 and TGF-β indicates that both regulatory cytokines are important for general modulation of immune responses, but that TGF-β is particularly involved in modulating the Th2-biased PE-specific response. These data indicates that the Th3-type TGF-β-secreting Treg cells (Th3) are especially important in the regulation of PE oral hypersensitization.

Together, these results and previous studies describe the mechanisms for an increased allergic response as an imbalance of Th1/Th2 cells, a failure in appropriate activation of innate effector cells and signalling molecules ([4] and chapter 2), or a reduced activation of Treg cells ([5, 6] and chapter 3). In real-life, virus and bacterial infections may be important factors regulating the above mentioned features leading to allergic responses. **Chapter 4** examines the capacity of a Th1-promoting mucosal virus infection (reovirus) to regulate the allergic response to PE in an animal model of allergy and infection. We found that the robust Th1 responses to reovirus modulate PE-specific immune responses in mice demonstrated by an enhanced PE-specific IgG2a antibody response, suggestive of an increased PE-specific Th1 response. These experiments provide evidence for a role of enteric viruses in regulating allergic immune responses.

Similarly, an apparent decline in microbial exposure during early life is proposed as a possible cause for the increase incidence of allergic diseases over the past few

decades. Probiotics, live bacteria exhibiting health-promoting activities, may be considered for treatment of allergic individuals on the basis of their immune modulatory properties. In **chapter 5**, we have used a translational strategy to assess the allergy-modulating capacity of probiotics. Selected probiotics appeared to have a moderate effect on peanut sensitization, despite the fact that they appeared to profoundly stimulate cytokine production *in vitro.* However, combined neutralization studies indicated that the probiotic strain LP256 does not have the capacity to modulate peanut allergic responses. Our study shows that careful strain selection is difficult. The translational approach presented here, using human PBMC, mouse splenocytes as well as animal models, and including means of exploring existing regulatory mechanisms, may be helpful in selecting and characterizing immunomodulatory probiotics.

2. Mouse models of oral sensitization and tolerance

Animal models of food allergy have been used to study the mechanisms involved in the development of hypersensitization reactions to food proteins as well as the immunologic mechanisms of the adverse reactions to allergen re-exposure [7, 8]. One major goal of food allergy researchers has been to develop an adjuvant-free animal model of allergic sensitization which is responsive to an oral re-exposure of the allergen resulting in immediate hypersensitivity symptoms representative of human disease [9]. However, the normal immune response of allergens encountered via the gastrointestinal tract is that of oral tolerance, resulting in the induction of allergen specific T cells with regulatory activity. The use of mucosal adjuvants and manipulation of epithelial barrier function by different means have frequently been used in described animal models [9]. Cholera toxin (CT) is the most common adjuvant used for oral sensitization in rodent models of food allergy [8, 9].

2.1. Oral sensitization

Snider *et al* [10] showed that oral administration of an antigen in combination with CT resulted in antigen-specific IgE production and anaphylaxis upon a re-exposure with the antigen. Li and others [8, 11] later modified this protocol to generate models of milk- and PE-specific anaphylaxis. It was shown that multiple feeds of antigen plus CT results in allergen-specific IgE production and following oral challenge, mice develop anaphylaxis characterized by histamine release and a drop in body temperature. However, the magnitude of the sensitization and particularly the challenge response appeared to be strain-dependent (Smit, unpublished data). Li *et al* selected the TLR4 mutant C3H/HeJ mouse strain for their model of allergic sensitization which has been demonstrated to be more susceptible to the induction of

food allergy and anaphylaxis than its wildtype strain C3H/HeOuJ [12]. Interestingly, C3H/HeJ mice contain a point mutation in the coding region for the *tlr4* gene resulting in LPS-hyporesponsiveness, demonstrating a role for the innate immune system in the regulation of allergic reactions. Furthermore, Matharu *et al* showed that in the absence of TLR4-mediated signals, secretion of proinflammatory and immunoregulatory cytokines is dysregulated [13].
In the sensitization model used in this thesis, C3H/HeOuJ mice are sensitized with PE in combination with CT for three consecutive days, following weekly sensitizations for a further three weeks with an oral challenge of PE alone on the fifth week. In contrast to systemic or subcutaneous challenges, an oral challenge in C3H/HeOuJ mice results in measureable mast cell degranulation but not in clinical signs of anaphylaxis (unpublished data). But also European derived C3H/HeJ mice do not develop the expected anaphylactic responses following PE challenge (van Wijk, unpublished data), revoking its advantage over its wild type counterpart. Possibly, C3H/HeJ breeding stocks in the USA and in Europe respond differently, although it remains uncertain if the lack of anaphylactic responses is due to differences between the mice strains or to detailed differences in treatment protocols (e.g. dosage, housing conditions). However, the hypersensitivity model used has been proven very suitable to study the mechanisms of oral sensitization, including challenge responses (as mast cell activity).
Although the mechanisms involved in the adjuvant effect of CT were not under investigation in this thesis, studying the *in vivo* effects of CT may provide important lessons about the mechanisms by which a Th2 response to oral proteins is induced (summarized in figure 1). It may very well be that in a real-life situation, similarly adjuvant effects are taking place during allergen-encounter. One example is the CT dependent decrease of γδ T cells from the intestine of PE plus CT treated mice as well from the intestine of mice treated with CT alone (Bol-Schoenmakers, unpublished results). Additionally, we have shown that depletion of γδ T cells can decrease induction of peanut-specific allergic responses (Bol-Schoenmakers, unpublished results). One of the challenging topics of future research is defining the factors that may act as initiators of food allergic sensitization in susceptible individuals.

2.2. Oral tolerance

Oral tolerance is the specific suppression of cellular and/or humoral immune responses to an antigen. It can be induced by oral pre-administration of the particular antigen, and has probably evolved to prevent hypersensitivity reactions to food proteins and products from commensal mucosal flora. Oral tolerance in C3H/HeOuJ mice can be induced by oral exposure to low doses of PE. PE plus aluminum hydroxide (alum) systemic sensitization is used in order to test for the

efficacy of PE tolerization. Mice which have been pre-exposed to PE produce a much lower PE-specific response to PE plus alum sensitization than control mice. However, it is important to note that oral tolerant mice in this model still show PE-specific allergic responses. Evidently, tolerance as induced here is not complete but relative.

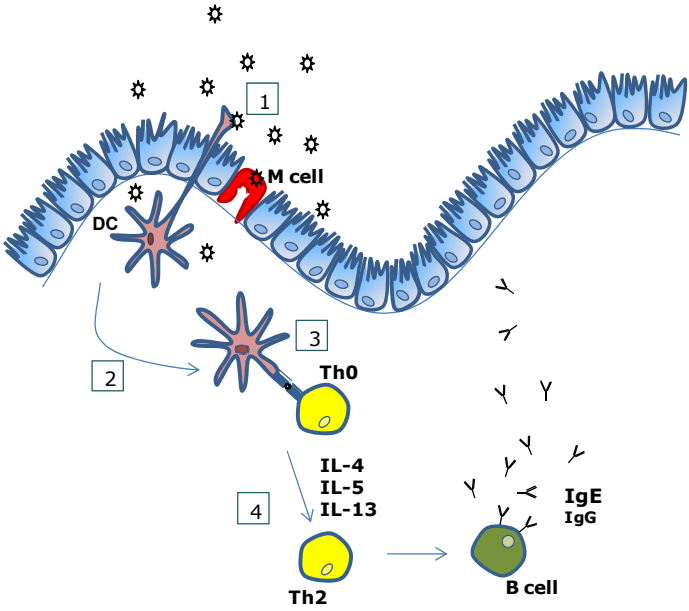

Figure 1. Adjuvant effects of Cholera Toxin (CT)
The mucosal adjuvant CT can affect antigen availability by an increase in intestinal permeability, enhancement of antigen uptake by DCs and enhancement of antigen transport over the epithelial layer (1). It can effect DC recruitment and migration (2), as well as DC maturation by increasing stimulatory molecules (3). CT itself can also promote Th2 polarization (4).*Reviewed by Sanchez et al [14].*

This indicates that oral tolerance is not an all-or-nothing response and that active tolerance induced by oral antigen pre-exposure can regulate future sensitizing events, this concept has been in the past termed as a multi-step process in tolerance induction [15]. This rationale was assumed during the adoptive transfer experiments in chapter 3 of this thesis. Unexpectedly, transfer of $CD4^{+}CD25^{+}$ Treg cells from PE orally tolerized mice did not regulate PE plus CT sensitization. The question remains whether CT is able to break local oral tolerance, at which the PE-specific transferred tolerance may not have any effect following PE plus CT treatment. Due to the strength of the CT adjuvant effect, Treg cells alone may not be potent enough to control the allergic response. It has been proposed that food allergy is a dynamic

process, influenced not only by Treg cells, but also by the regulation of specific T-cell cytokines and other cells of the immune system ([16] and this thesis).

3. T cell differentiation and regulation

It is believed that dendritic cells (DCs) play a key role in regulating the balance between tolerance and immunity by controlling differentiation of naïve T cells. T cell activation depends on interaction with co-stimulatory molecules, pro-inflammatory cytokines produced by DCs and, to some extent, by the strength of the interaction of the T cell receptor with the antigen [17].

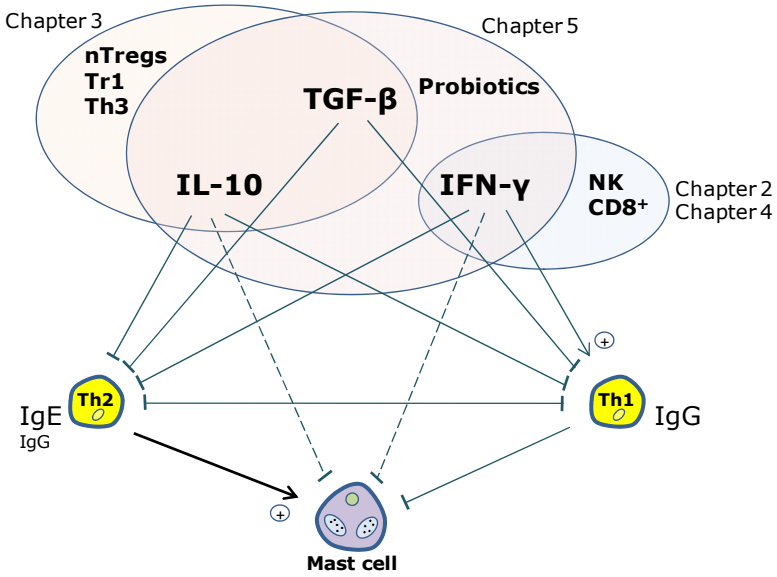

Figure 2. Regulation of the T cell allergic response
IgE-mediated food allergic reactions are characterized by antigen-specific IgE dependent release of mast cells. In this thesis, we observe that three different cytokines, IFN-γ, IL-10, and TGF-β, are most important for the regulatory environment during food allergic sensitization. IFN-γ is an important Th1 skewing cytokine, which was shown to be beneficial in food allergies. TGF-β and IL-10 are both regulatory cytokines able to decrease both Th1 and Th2 specific responses during an allergic reaction. Furthermore, IL-10 and IFN-γ are both able to inhibit mast cells. Different cell types are able to produce these cytokines, and thus regulate an allergic response. Additionally, microbes during infection or probiotic treatment may stimulate the production of the above mentioned cytokines.

Th1 cells are characterized by their production of IFN-γ and are required for cellular immunity. IL-12 produced by innate cells and IFN-γ produced by both NK cells and T cells polarize cells towards a Th1 cell differentiation through action of the signal transducer and activator of transcription 4 (Stat4), Stat1, and T box transcription factor T-bet. Th2 cells produce IL-4, IL-5, and IL-13 and are involved in humoral immunity. Differentiation of Th2 cells requires the action of GATA3 downstream of IL-4 and Stat6. Treg cells are able to regulate both Th1 and Th2 cell immune responses. The presence of abundant IL-10 and TGF-β in the gut, favours the development of T cells with regulatory capacities. Different subtypes of Treg have been described which produce mainly TGF-β and IL-10. However, their immunosuppressive activity may also involve direct cell-cell contact mechanisms (summarized in chapter 1).
In the case of allergic sensitization, three different cytokines, IFN-γ, IL-10, and TGF-β, seem to be most important for the regulatory milieu [18] (Fig. 2).

3.1. Th1/Th2 balance

Food allergies are regarded as Th2-induced IgE-mediated immune reactions to otherwise harmless food components. Recent studies have shown that this view is, however, oversimplified [19]. A mixed Th1 and Th2 response in allergy has been recently observed in both humans [16, 20] and mice models [1, 21], where sensitized cells are observed to induce not only the typical Th2 cytokines, such as IL-4, IL-5 and IL-13, but also IFN-γ and IL-10. However, previous studies have additionally shown that this dynamic process of allergic sensitization can be modulated by a Th1-type shift in the immune response [22-25].
In this thesis, we have observed that NK cells, which are strong IFN-γ producers, are able to regulate oral tolerance and an allergic response in which their depletion further increases the specific Th2 response induced by PE plus CT sensitization. Human *in vitro* studies have also shown that a small fraction of NK cells secrete IL-10 and display regulatory functions [26]. However, our model shows no indication of a role for IL-10 following NK cell depletion. *Ex vivo* restimulation of splenocytes from PE-sensitized and NK cell depleted mice showed a decrease in IFN-γ, but an increase in IL-10 and other Th2-type cytokine production.
In Chapter 4, we show that an acute Th1 virus infection increased the PE-specific Th1 antibody response. Reovirus oral infection shows increased levels of IL-12 and IFN-γ mRNA in mouse PP and mesenteric lymph nodes in the first 96 hours after infection [27]. Additionally, it was shown that reovirus-activated DC up-regulates IFN-gamma production, increases NK cytolytic activity and enhances T cell cytokine secretion (IL-2 and IFN-gamma) [28].
Furthermore, chapter 5 shows that neutralization of IFN-γ leads to an increase in the PE-specific Th2 cytokine production by restimulated splenocytes and an increase in

mast cell degranulation. Supporting our data, Lee *et al* [22] have orally treated peanut sensitized mice with liposome encapsulated rIL-12 which led to an increase in IFN-γ with no difference in the Th2 cytokines produced. This shift from a Th2-balanced response to an enhanced Th1 response resulted in prevention and reversal of peanut hypersensitivity. Furthermore, specific targeting of neutralizing anti-IL-12 antibodies in the allergy resistant BALB/c mouse strain showed an increase in susceptibility to food allergy [29].
The molecular basis of Th2 cell differentiation by IFN-γ has been extensively studied. IFN-γ induces T-bet, which in turn induces Runx3. Runx3 then cooperates with T-bet to further promote IFN-γ production while silencing the *Il4* gene by binding to the *Ifng* promoter and the *Il4* silencer, respectively [30, 31]. Thus, IFN-γ exerts a direct inhibitory effect on Th2 cytokines while promoting Th1 differentiation. Furthermore, IFN-γ induces IL-12 production by antigen presenting cells (APC), such as DCs and macrophages [32, 33]. APCs provide the first contact of naive $CD4^+$ T cells with antigen. Therefore, this IL-12 production is of great importance on the differentiation pathway towards a Th1 phenotype. In fact, IFN-γ is a pleiotropic cytokine known to induce and regulate an array of mechanisms which may affect allergic responses (summarized by Teixeira *et al*, [34]).
When taking all this data into account, it is clear that an allergic immune response is a complex and dynamic process in which a Th2-biased response can be considered detrimental and a Th1-shift in response by IFN-γ production beneficial. This Th1/Th2 balance and the role of other regulatory cytokines in the regulation of an allergic reaction are depicted in figure 2.

3.2. $CD4^+$ Regulatory T cells

Various types of mucosal T cells have been shown to play a central role in maintenance of homeostasis and in the establishment of controlled immune responses, such as oral tolerance. Within the $CD4^+$ T cell subpopulation, three classes of Treg cells have been distinguished: Th3-type TGF-β-secreting Treg (Th3), IL-10-producing type 1 Treg (Tr1), and naturally occurring $CD4^+CD25^+$ Treg (nTreg) (summarized in chapter 1). However recently, the existence of some of these regulatory T cells as separate population has been challenged [35]. In any case, the effects of nTreg cells and various regulatory cytokines have been examined in chapter 3 of this thesis through specific depletion of $CD25^+$ cells and neutralization of the regulatory cytokine IL-10 and TGF-β for their role in allergic sensitization.
Previous studies have shown the importance of nTreg cells in the regulation of oral sensitization and oral tolerance induction[3]. However, the role $CD4^+CD25^+$ Treg cells in oral tolerance specific transfer to naïve mice was unclear. Chapter 3 shows that $CD4^+CD25^+$ Treg cells cannot regulate PE-specific oral tolerance in mice which are

later sensitized with PE plus CT. As stated in paragraph 2.2, it may be that Treg cells alone are not potent enough to regulate the strong immune stimulating effects of CT. For further clarification, an additional experiment is required in which PE-specific tolerance is induced in naïve mice prior to PE plus CT sensitization. If hyporesponsiveness is measured following PE pre-exposure in this oral allergy model it would mean tolerance to oral re-exposure of an immunogenic PE treatment can indeed be established. It would follow that modulation of the PE-allergic response by Treg cells transfers would be more complex than here studied.

Much more likely, a combination of various Treg cells together with an array of other regulatory cells such as innate T cells, DC, and other leukocytes forming the regulatory milieu may be involved in specific oral tolerance (Fig. 2). For instance, we observed that neutralization of TGF-β during an early stage in sensitization allowed for drastic increases in splenic PE-specific cytokine responses and an increase in mast cell degranulation. IL-10 neutralization early during sensitization allowed for lower increases in cytokine responses than TGF-β, but cytokine levels were still drastically higher than from control mice. For both IL-10 and TGF-β neutralization early during sensitization, no difference in PE-specific IgE response was measured, but anti-CD25 treatment significantly increased PE-specific IgE levels.

Timing of IL-10 administration or neutralization may be of importance while studying its role in allergic sensitization. IL-10 has been observed to reduce the release of pro-inflammatory cytokines by mast cells [36]. In chapter 5, an additional drastic increase in mast cell degranulation was observed following IL-10 neutralization. However, in this study anti-IL-10 mAb was administered twice a week for 6 weeks, up to 3 days before PE oral challenge. The increase in mast cell degranulation measured may be a direct effect of IL-10 neutralization on mast cells [36]. The indication in chapter 5 that IL-10 may be a more important cytokine for probiotic regulation of peanut allergy stems from the probiotic concentration-dependent increase of splenic IL-10 production, and not by the increase in mast cell degranulation. However, probiotic treatment also induced TGF-β signaling in the lamina propria of treated mice which, again, indicates a role for both Tr1 and Th3 cells for probiotic induced regulation of food allergy (Fig. 2).

Our findings are illustrative that *in vivo* IL-10 and TGF-β (but also IFN-γ) are probably just a few of the plethora of factors necessary for proper regulation of the PE-specific allergic response. In addition, these regulatory factors may be influenced by external players such as infections and probiotics.

4. Infections: Regulators or risk factors

"Nothing in Biology makes sense except in the light of evolution"
Theodosius Dobzhansky, 1973

The hygiene hypothesis described in chapter 1 suggests that reduced exposure to pathogens or their components early in life results in an increased IgE response to allergens and an increase in allergic diseases. The mechanism for this increased predilection for mounting IgE responses has been described as an imbalance of Th1/Th2 cells, reduced activation of Treg cells, or failures in appropriate activation of innate effector cells and signalling molecules [37]. In Chapter 4, we have shown that a reovirus infection can indeed affect a peanut-specific response. Likewise, repeated mild virus infections in early life may have a protective role in the development of asthma or atopy by driving the immune system towards Th1 responses. Moreover, skin sensitization to peanut was less frequent among Hepatitis-A seropositive subjects, further suggesting that lower hygiene standards may prevent atopic sensitization to food allergens [38].

However, evidence on this hypothesis is not always consistent as far as respiratory viruses and asthma are concerned where infections may provide the required adjuvant effect to initiate or exacerbate an allergic response [39]. Several factors, including the presence of an atopic environment, timing of exposure and severity of the infection contribute to the allergy-infection relationship. Contrasting evidence, with respect to the effects of particular microorganisms on the regulation of allergic responses, has led to the proposal that it is the rate rather than the kind of infection affecting the development of allergic diseases [40]. Nonetheless, timing of the infection may also play a role. We have shown that effector cells of the innate immune system can influence the allergic response possible by early IFN-γ production. Indeed some infections, particularly virus infections, may cause NK cell activation, thus contributing as well to a regulatory milieu in the intestine.

Contradicting the Th1/Th2 balance theory is the fact that individuals with helminths infections have an enhanced Th2-type response but are less likely to develop allergic sensitization. Likewise, treating the infection leads to an increase in allergic response. It is believed that helminth infections are able to induce a highly tolerant environment where regulatory T and DC cells induce bystander and specific tolerance to exogenous antigens [41, 42].

The idea that microorganisms can modulate a specific immune response to bystander antigens and the fact that the composition of the gut microflora of allergic children has been found to differ from that of healthy children [43] has led to the rationale of using probiotics to shape the immune response of the host, especially in infancy. Our present data shows that the particular probiotic LP256 has at best only marginal

effects on a peanut allergy response. However, results suggest that other (combinations of) probiotics or prebiotics may have beneficial effects on gut health and food allergy.

5. Therapies and future research

Peanut allergy accounts for the majority of severe food-related allergic reactions and is most commonly associated with anaphylaxis and fatal outcome. In addition, peanut allergy does not usually resolve, resulting in a life-long allergy with a high impact on daily life. So far, no therapies are available and the only remedy is strict avoidance of the allergen. Revealing the underlying mechanisms of sensitization to food proteins may contribute to the development of new treatment methods for peanut allergy. Specific immunotherapies with modified antigens/epitopes, anti-IgE antibodies, regulatory cytokines, immunomodulatory microorganisms and alternative medicine have been described [44, 45].

In this thesis, regulation of peanut allergy was observed by counterbalancing the established Th2 allergic response and by the induction of Treg cells. Although probiotics are capable of stimulating production of Th1 cytokines and inducing Treg cells, their regulating capacity in food allergy has shown to be minimal for both human and animal studies. In chapter 5, we have shown that a broad approach is necessary in order to select and characterize probiotics which may be beneficial for food allergy therapy. However, considering the dynamic processes involved in allergic sensitization and oral tolerance induction, it is more likely that an efficient therapy will arise from careful selection of immunoregulatory entities which can both counter-balance a Th2 response and induce modulatory cells. For instance, a trial with prebiotic oligosaccharides reported a reduction in eczema in high risk formula fed infants [46], although more information is needed to determine whether this finding persists over a longer period of time, applies to other manifestations of allergic disease, or is associated with reductions in allergen sensitisation. In a mouse model for cow's milk allergy, a particular prebiotic mixture provided during sensitization, reduced allergic effector responses [47]. This model showed the potential for dietary intervention with prebiotics in reducing the allergic response to food allergens. Similarly, exposure to pathogen-associated molecular patterns (PAMPs), that signal through different Toll-like receptor (TLR)s, and can influence both T cell differentiation and effector T cell activation has shown some promising results in pre-clinical studies for allergic diseases [48]. Furthermore, studies on helminths infection and their role in the regulation of allergic immune responses may prove beneficial in discovering new immunomodulating factors for use in future treatments of allergic diseases [49].

Concluding Remarks

Allergic reactions to food components can cause serious illness and sometimes life-threatening anaphylactic reactions. Elucidating the immunological mechanisms during allergic sensitization and oral tolerance is essential for the development of effective therapies for food allergy. This thesis has shed some light on the dynamic process of allergic sensitization and oral tolerance, involving cells of the innate as well as the adaptive immune system. Furthermore, it has discussed many factors influencing the development of allergic sensitization, including genetic predisposition, immunological state, and composition of the microbial flora. Further knowledge will likely give rise to a safer form of personalized immunotherapy which will utilize a combination of tolerogenic and/or Th1 skewing entities in combination with specific immunotherapy.

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Samenvatting

Het onderzoek beschreven in dit proefschrift is gericht op het vinden van mogelijkheden om voedselallergie te genezen. Op dit moment wordt geschat dat 8% van de kinderen en 3 tot 4% van de volwassenen last hebben van voedselallergie. Pinda-allergie is de oorzaak van het merendeel, en ook van de meest ernstige allergische reacties tegen voedsel. In ontwikkelde landen is het aantal kinderen met voedselallergie de laatste decennia gestegen al lijkt nu het aantal gestabiliseerd.

Om mogelijkheden te onderzoeken die een bijdrage kunnen leveren aan het genezen van voedselallergie is het van belang te weten hoe voedselallergie ontstaat: welke factoren zijn er bij betrokken en welke biologische processen spelen een rol?

Het ontstaan van voedselallergie (*Allergie* is afgeleid van het Latijn *All Erg* voor 'vreemd reageren') is deels genetisch bepaald, en de genetische gevoeligheid wordt ook wel aangeduid met atopie. Daarnaast zijn er tal van andere niet genetische factoren die kunnen bijdragen aan het ontwikkelen van een allergie. De leeftijd van de persoon waarop het contact met het allergeen het eerst plaatsvindt, kan bijvoorbeeld een rol spelen. Maar ook de samenstelling van het dieet zou kunnen bijdragen. De soort voeding (bijv. borst- of flesvoeding) en ook infectieziekten kunnen onder andere invloed hebben op de samenstelling van de darmflora, die onder normale omstandigheden bestaat uit "goede" bacteriën.

Na inname van voedsel worden voedselbestanddelen, na vertering, opgenomen door de darm. Eenmaal opgenomen in het darmweefsel komen de verteerde voedselbestanddelen in contact met het aanwezige immuunsysteem. Het is goed te weten dat het immuunsysteem van de darm, in het Engels Gut-Associated Lymphoid Tissue (GALT) genoemd, het grootste en meest complexe onderdeel van het gehele afweersysteem is. Dit is niet zo verwonderlijk als men bedenkt dat de darm voortdurend wordt blootgesteld aan voedsel dat tal van chemicaliën bevat en vrijwel nooit steriel is, dat wil zeggen altijd wel vreemde, ongewenste micro-organismen bevat. Het immuunsysteem van de darm heeft als bijna onmogelijke taak te zorgen dat die ongewenste micro-organismen geen kwaad doen, terwijl tegelijkertijd de behoefte aan voeding gestild moet worden. Met andere woorden, het afweersysteem mag niet reageren tegen gewenste voedselbestanddelen, maar moet wel reageren op ongewenste voedselbestanddelen. Dit tegen de achtergrond dat voedselbestanddelen lichaamsvreemd zijn. De GALT moet dus een zeer moeilijke functie vervullen, gelijk aan de douane van een land dat voortdurend door ongewenste indringers bedreigd wordt, maar gewenste bezoekers welkom wil heten.

De GALT omvat een veelheid aan afweercellen om de moeilijke douanefunctie te vervullen. Cellen met uitlopers (dendritische cellen, of DC) zullen als eerste tal van voedselcomponenten opnemen en zodanig verwerken dat ze herkenbaar worden voor zogenaamde helper T cellen (Th cellen). Deze Th cellen zijn de dirigenten van het afweersysteem. DC en Th cellen maken fysiek contact in de darm of in lymfklieren die in, en rond de darm aanwezig zijn. De omstandigheden waaronder het contact tussen DC en Th cellen plaatsvindt, bepalen wat de Th cel vervolgens gaat doen. Als er een kwaadwillende bacterie wordt 'gepresenteerd' aan de Th cel, zal deze andere cellen gaan aanzetten tot een reactie die moet leiden tot verwijderen en doden van die bacterie. Als er een nuttig eiwit wordt gepresenteerd zal de Th cel zorgen voor onderdrukking van de agressieve werking van het immuunsysteem. Daarbij spelen ook T cellen een rol die "regulatoire" T cellen of Tregs worden genoemd. Een van de gedachten is dat in voedselallergische patiënten deze Tregs niet of niet optimaal functioneren. Het aanzetten van andere cellen om hetzij agressief te reageren of om immuunreacties te onderdrukken gebeurt deels door middel van hormoonachtige stoffen die afgegeven worden door immuuncellen en ook wel cytokines of interleukines genoemd worden.

Mogelijk als gevolg van het niet optimaal functioneren van Tregs ontwikkelt zich een typische allergische reactie. Er ontstaan zogenaamde Th2 cellen die gekenmerkt worden door de mogelijkheid om een specifiek palet van interleukines (IL4, IL5, IL13) te produceren. Hiermee wordt de productie van speciale anti-lichamen gestimuleerd, namelijk IgE anti-stoffen. Deze IgE anti-stoffen zijn weer verantwoordelijk voor het activeren van zogenaamde mestcellen in geval van opname van een bepaald voedselallergeen. Mestcellen zijn cellen van het immuunsysteem die, vanwege de mogelijkheid om onder andere histamine af te geven, van groot belang zijn bij het aanzetten tot ontstekingsreacties. In geval van allergie kan de afgifte van mestcelproducten echter leiden tot kortademigheid, bloeddrukdaling en in het ergste geval tot een dodelijke anafylactische shock. Normaal worden de Th2 cellen, die in sommige gevallen heel nuttig werk verrichten, in bedwang gehouden door Tregs, maar ook door andere Th cellen, zoals Th1 en Th3 cellen, of cellen zoals 'Natural Killer' (NK) cellen.

Van zowel Th1 als NK cellen is bekend dat ze van belang zijn in de afweer tegen virussen en een van de ideeën is dat virusinfecties zouden beschermen tegen het ontstaan van een allergie doordat de interleukines (interferon-gamma, of IFN-γ) geproduceerd door Th1 of NK cellen het ontstaan van Th2 cellen remmen. Als dat inderdaad zo is zouden virusinfecties mogelijk beschermen tegen het ontstaan van voedselallergie. Dit idee komt tot uitdrukking in de zogenaamde *Hygiëne Hypothese*.

Deze hypothese zou de toename van allergie gedurende de laatste 20-30 jaar kunnen verklaren en stelt dat dit komt door een afname van infectieziekten (mede als gevolg van hygiëne, maar ook door toename in vaccinaties). Minder infectieziekten zouden het immuunsysteem, en met name de Th1 en NK cellen lui maken, waardoor de "regulatoire" balans binnen het immuunsysteem verstoord wordt. Het gevolg is dat Th2 cellen minder in bedwang gehouden worden en allergieën zich makkelijker kunnen ontwikkelen

Het zal al duidelijk zijn dat de regulatie in de GALT inderdaad heel complex is, wat waarschijnlijk een gevolg is van een evolutionair proces waarbij de GALT zich voortdurend heeft aangepast aan veranderende omstandigheden. Onduidelijk is hoe de "regulatoire" cellen precies met elkaar in verband staan en welke van deze cellen van cruciaal belang zouden kunnen zijn in het voorkomen of genezen van voedselallergie.

Tevens is onduidelijk welke omgevingsomstandigheden in de darm bepalen of het immuunsysteem op voedsel reageert met een allergische reactie. Zoals eerder vermeld zouden goede bacteriën, ook wel commensale bacteriën genoemd een belangrijke omgevingsfactor zijn. Goede bacteriën worden ook gebruikt als basis van bekende probiotica-producten, en zouden de omstandigheden in de darm zodanig kunnen veranderen dat de GALT niet reageert op ongevaarlijk voedsel.

Dit proefschrift heeft zich gericht op de mogelijkheid de immuunreactie van de GALT te beïnvloeden zodat de allergene Th2 reactie minder of niet optreedt. Het onderzoek is uitgevoerd met een bekend proefdiermodel, waarbij muizen allergisch gemaakt worden voor pinda.
In **hoofdstuk 2** is beschreven dat NK cellen inderdaad van belang zijn bij het reguleren van de reactie tegen pinda. Dit is onderzocht door de NK cellen heel gericht uit te schakelen, waarna bleek dat de muizen een sterkere reactie vertoonden tegen pinda.

Eerder onderzoek had al aangetoond dat het uitschakelen van sommige Tregs (CD25+foxp3) ook een toename van de allergische reactie veroorzaakte. Dit was aanleiding om na te gaan of deze Tregs in staat zijn een allergische reactie te voorkomen indien ze naar normale muizen worden overgebracht. **Hoofdstuk 3** laat zien dat deze Tregs inderdaad de immuunreactie als geheel beïnvloeden, maar dat ze niet beschermen tegen voedselallergie. In hetzelfde hoofdstuk wordt wel duidelijk gemaakt dat het wegvangen van bepaalde cytokines in muizen wel een zekere bescherming geeft. Cytokines die van belang lijken te zijn, zijn TGF-β (Th3) en IL-10.

De eventuele rol van Th1 en NK cellen wordt verder benaderd in **hoofdstuk 4**. In dit hoofdstuk wordt beschreven dat infectie met een Th1-stimulerend virus (reovirus) inderdaad invloed heeft op de aard van de immuunreactie tegen pinda, al wordt de typische allergische reactie niet beïnvloedt.
In **hoofdstuk 5** is nagegaan in hoeverre verschillende probiotica-stammen de allergische reactie tegen pinda beïnvloeden. Daarbij is gebruik gemaakt van reageerbuisexperimenten met cellen verkregen uit de mens en de muis en tevens van het eerder genoemde muizenmodel. Een van de probiotica-stammen, de Lactobacillus-256 (LP256), is voornamelijk onderzocht. Hoewel deze probiotica-stam duidelijke effecten had op de reactie van immuuncellen in de reageerbuis, en LP256 in de darm zelf wel TGF-β productie leek te stimuleren, bleken LP256 de allergische reactie in het proefdiermodel nauwelijks te beinvloeden, ook niet wanneer bepaalde cytokinen (onder anderen IFN-γ en TGF-β) geneutraliseerd werden.

Het werk in dit proefschrift laat zien dat het beïnvloeden van de allergische reactie middels het aanpakken van een van de regulatoire immuuncellen mogelijk een beschermend en therapeutisch effect heeft in voedselallergie. Echter dit werkt vooralsnog het beste als bepaalde cellen (NK cellen, CD25 Tregs, γδ T cellen (zie proefschrift van Bol-Schoenmakers) of cytokines geneutraliseerd worden (in dit proefschrift met specifieke anti-stoffen). Transfer van specifieke cellen of het beïnvloeden van de reactie met microorganismen zoals virussen en bacteriën levert slechts een gering en vooralsnog onduidelijk effect. Dit kan te maken hebben met het feit dat microbiële invloeden minder gericht zijn, maar ook met andere factoren, zoals dosering, kinetiek en aard van het micro-organisme. Vooralsnog lijkt het het meest voor de hand liggend om het gehele palet aan regulerende cellen (Tregs, Th1, Th3 en NK cellen) in samenhang te onderzoeken. Mogelijk leidt dit tot identificatie van een proces dat initiëel verantwoordelijk is voor het ontstaan van voedelallergie.

Allegische reacties door voedsel kunnen ernstige gezondheidsproblemen veroorzaken met soms levensbedreigende anafylactische shock als gevolg. Het doorgronden van immunologische mechanismen tijdens allergische sensibilisatie en orale tolerantie is essentieel in het ontwikkelen van effectieve therapieën voor voedselallergie. Dit proefschrift heeft inzicht verschaft in het dynamische proces van allergische sensibilisatie en orale tolerantie waarbij zowel cellen van de aangeboren als het adaptive immuunsysteem betrokken zijn. De nieuwe kennis die is vergaard met dit proefschrift kan bijdragen aan een veiliger vorm van immunotherapie. Door een op het individu toegesneden combinatie van tolerantiebevorderende en/of Th1 sturende entiteiten kan conventionele immunotherapie met een specfieke allergeen effectiever worden.

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Marisa Marcondes Rezende

Curriculum Vitae
List of Publications

Curriculum Vitae

Marisa Marcondes Rezende was born on January 23, 1979 in Campo Grande - Mato Grosso do Sul, Brazil. In 1995 she acquired 9 General Certificates of Secondary Education (GCSE's) at Tapton Secondary School in Sheffield, UK. Following her high school graduation at Central High School in Champaign, Illinois, USA in 1997, she started her academic career at the University of Illinois, Champaign, USA. A year later, she transferred to Monash University in Melbourne, Australia where she received her Bachelor's degree in biomedical sciences in February 2001. After receiving her "Nederlandse Taal II Staatsexamen" she started her Master's degree at Leiden University. During her study period in Leiden she participated in research projects at the department of immunohematology and blood transfusion under the supervision of Dr. Nynke van der Stoep and Dr. Danijela Koppers-Lalic at the department of experimental microbiology. Once she achieved her Master's degree in November 2004, Marisa began her PhD program at the Institute of Risk Assessment Sciences (IRAS) at Utrecht University, in collaboration with TNO, and under the supervision of Prof. dr. C. Bruijnzeel-Koomen, Prof. dr. W. van Eden, Dr. R. Pieters, and Dr. J. van Bilsen. She investigated mechanisms underlying the modulation of oral tolerance and allergic sensitization to peanut which may aid in the development of new therapies. The result of 4 years of research is presented in this thesis

List of Publications

Marcondes Rezende M, Bol-Schoenmakers M, Smit JJ, Hassing I, Fiechter D, Bleumink R, van Bilsen J, Pieters R. NK cells are Important Regulators of Allergic Sensitization and Oral Tolerance Induction to Peanut. *Submitted*.

Marcondes Rezende M, Hassing I, Bol-Schoenmakers M, Bleumink R, Boon L, van Bilsen J, Pieters R. $CD4^{+}CD25^{+}$ T Regulatory Cells do not Transfer Oral Tolerance to Peanut Allergens in a Mouse Model of Peanut Allergy. *Submitted.*

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