Allergen specific immunotherapy: The future cure for allergic asthma

Mechanisms and improvement in a mouse model

Yousef A. Taher

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Allergeen specifieke immunotherapie: toekomstige genezing van allergisch astma. Mechanismen en verbetering in een muismodel

(met een samenvatting in het Nederlands)

Proefschrift

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Yousef A. Taher

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Promotoren:	Prof. dr. A.J.M. van Oosterhout
	Prof. dr. F.P. Nijkamp
Co-promotor:	dr. P.A.J. Henricks

To my wife **Awatef**, for her everlasting love, support and encouragement

To **Feras**, **Qussi**, **Shahd** and **Audi**, my sweet children, for making everything worthwhile

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Chapter 1

General introduction

'to know the road ahead, ask those coming back'
Chinese proverb

1. Asthma

Asthma is recognized as a chronic inflammatory disease of the airways in which many types of cells play a role, in particular mast cells, eosinophils, B- and T-lymphocytes and epithelial cells. This disease is characterized by the production of allergen-specific immunoglobulin (Ig)E antibodies, reversible airflow obstruction, airway hyperresponsiveness (AHR) to a wide variety of specific or non-specific stimuli, chronic airway inflammation and airway remodeling (1). During the past several decades, the prevalence of asthma is increasing dramatically (> 20% every 10 years), especially in children (2). Approximately, it affects up to 10-15% of the population in most developed industrial countries (2). Both genetic and environmental factors contribute to the pathogenesis of allergic asthma. Genetic studies indicate that multiple genes are linked to the onset of this disease (3-5). However, the epidemic increase of asthma cannot only be explained by genetic changes in the population. Changes in environment and lifestyle, including insulation of houses which increases numbers of house dust mite, and childhood immunizations have been postulated to contribute to the increase in several studies (6-9). In addition, epidemiological and clinical studies have suggested a link between the relative absence of infectious diseases, as a result of decreased exposure to bacteria or other pathogenic agents (such as helminths) during the first years of life, and the increase in allergic asthma (10-12); this is commonly referred to as the 'hygiene hypothesis'.

1.1. Pathogenesis of asthma

The concepts of the pathogenesis of allergic asthma are illustrated in Fig. 1. The development of allergic asthma is initiated by sensitization to an environmental allergen, which can occurs years before the onset of clinical symptoms (6, 13). For allergic sensitization allergens are taken up and processed by professional antigen-presenting cells (APCs) such as dendritic cells (DCs) and presented to allergen-specific T-cells that subsequently differentiate into T helper (Th) 2-type cells. Activation of Th2 cells leads to the production of inflammatory cytokines such as interleukin 4 (IL-4) and IL-13, thereby stimulating allergen-specific B-cells to proliferate and switch to production of IgE (14, 15). IgE binds to high affinity IgE receptor FcεRI on mast cells. Upon re-exposure, the allergen will bind to allergen-specific IgE on mast cells thereby cross-linking the IgE-FcεRI complexes which subsequently cause the release of preformed mediators like histamine and several other inflammatory mediators (16, 17). These mast cell-derived mediators cause the early asthmatic reaction which is characterized by airway smooth muscle constriction, extensive vascular leakage, mucus hypersecretion, enhanced airway responsiveness and recruitment of inflammatory cells (17-19). In approximately 50% of the asthmatic patients this early phase reaction is followed by the late phase reaction. This phase is characterized by significant involvement of infiltrated inflammatory cells, such as eosinophils, T lymphocytes and macrophages, and resident epithelial, endothelial and smooth muscle cells in promoting the chronic symptoms of airway inflammation. In addition, these cells may also be an important source of inflammatory mediators like chemokines, cytokines and leukotrienes in asthma. Thus, a variety of events are

involved in initiating and maintaining an allergic airway inflammation, leading to chronic inflammatory airway disease and increased sensitivity of the airways for different non-allergen related bronchospasmogenic stimuli.

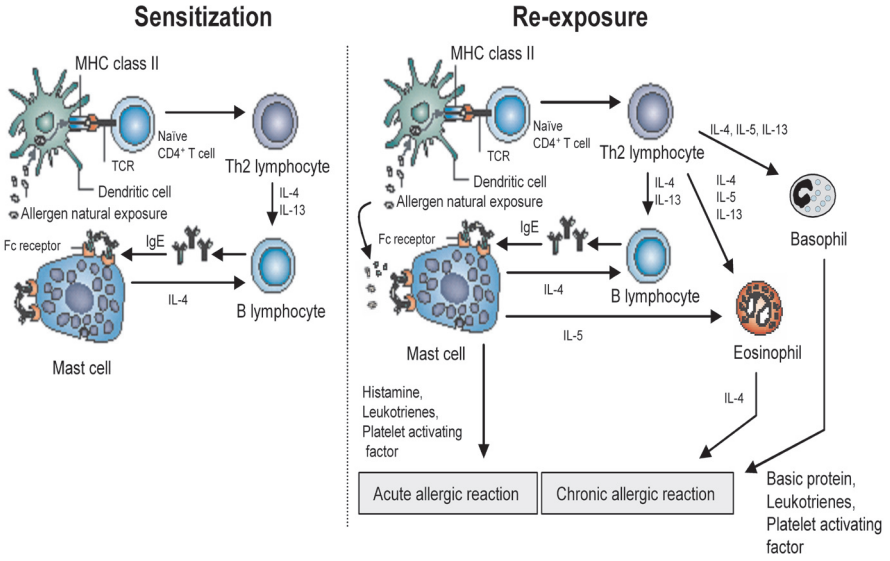

Figure 1. *Schematic representation of the pathophysiology of allergic asthma. In genetically predisposed individuals, primary exposure to an allergen leads to activation of Th2 lymphocytes and stimulation of IgE synthesis. Later on, exposure causes immediate release of the biologically active mediators (histamine, leukotrienes) via mast cell degranulation and further activation of Th2 cells with resulting eosinophil inflammation and AHR. Adapted from (20).*

1.2. Asthma: a CD4⁺ Th2-mediated disease

CD4⁺ T-cells play a crucial role in controlling inflammation in asthma. They are the predominant lymphocyte population that infiltrates the airways in asthmatics and are activated at these sites, expressing the surface activation markers class II histocompatibility antigen [HLA-DR], CD25 (IL-2R) and very late activation antigen-1 (VLA-1) (21, 22). In addition to CD4⁺ T-cells, CD8⁺ T-cells and γ/δ T-cells have been identified in the airways of allergic asthmatics (22). Studies in humans have shown that about 60% of the CD4⁺ T cells in the airways of persons with asthma are invariant natural killer T (*i*NKT) cells (23). However, this finding has recently been challenged by Mutalithas and colleagues who found that the proportion of *i*NKT cells in bronchoalveolar lavage (BAL) in asthma was very low (about 0.37%) (24). Studies in mice (25-27) showed that *i*NKT cells are required for the development of allergen-induced airway inflammation and AHR. However, there is still controversy whether *i*NKT cells can induce allergic asthma in the absence of CD4⁺ T-cells, as shown in mice (28) and the more recent human studies (24, 29).

Human and murine CD4⁺ T-cells are divided into two broad functional subsets according to their profiles of cytokine secretion (30, 31). The conditions under which CD4⁺ Th cells become activated during an immune response determine the

differentiation towards the Th1- or the Th2-phenotype (32). $CD4^+$ Th1-type cells secrete predominantly IL-2 and interferon gamma (IFN-γ), and are particularly implicated in cell-mediated immune responses against invading intracellular pathogens such as viruses (33, 34). $CD4^+$ Th2-type cells produce a different panel of cytokines including IL-4, IL-13 and IL-5 (35). IL-4 promotes the development of Th2 cells and together with other cytokines, promotes the growth of mast cells, basophils and eosinophils (36, 37). Furthermore, IL-4 and IL-13 are required for the induction of proliferation and isotype switching to IgE by B-cells that recognize the allergen (14, 38). Additionally, IL-13 has been shown to contribute to AHR in mouse models of experimental asthma (39-41) and is involved in goblet cell hyperplasia and mucus hypersecretion (39-41). IL-5 secretion by Th2 cells is critical for eosinophil differentiation and maturation (42, 43).

Over the years it became clear that allergen-induced airway inflammation is orchestrated by activated Th2 cells. Th2–type lymphocytes play a critical role in the initiation, progression and persistence of allergic asthma. Initially, a disturbed balance between Th1- and Th2-mediated immune responses has been postulated to underlie aberrant Th2 reactions to 'innocuous' environmental antigens. Indeed, allergen-specific T-cell clones isolated from the blood of allergic individuals express a Th2 cytokine profile secreting IL-4, IL-5 and minimal IFN-γ and IL-2, whereas those clones from nonatopic individuals displayed a Th1 profile (44). Furthermore, allergic asthma is associated with expression of IL-3, IL-4 and IL-5 in bronchoalveolar cells, strongly supporting Th2 activation (45). Moreover, it has been shown that when antigen-specific Th1 or Th2 cells were generated *in vitro*, transferred into recipient mice and activated in the respiratory tract with inhaled antigen, Th2 cells induced airway eosinophilia, mucus hypersecretion and AHR after short-exposure to antigen (46, 47). Transfer and activation of Th1 cells resulted in a neutrophil-predominant inflammatory response without mucus production or AHR (46), indicating that activation of a Th1 cells dominated response do not produce any of the characteristic features of asthma. However, the effects of Th1 cells on Th2-mediated AHR showed conflicting results, enhancement in some studies (48-50) but suppression in another study (51). Moreover, constitutive production of Th2 cytokines has been found to induce asthma-like symptoms. Transgenic mice that over-express IL-4, IL-5, IL-13 or IL-9 in the airway epithelium exhibit common inflammatory features in the airways including eosinophilia, mucus overproduction, collagen deposition in the airways and AHR on methacholine challenge, demonstrating that chronic exposure to Th2 cytokines can also induce airway remodeling (41, 52). In human studies, $CD4^+$ T-cells producing IL-4, IL-5 and IL-13 have been identified in bronchoalveolar lavage fluid and airway biopsies from asthmatics patients. These cytokines are secreted in the airways of patients with mild or asymptomatic disease (45). Furthermore, it has been shown that $CD4^+$ Th2 lymphocytes are increased in the airways of asthmatic patients after antigen challenge (22, 53, 54).

Therefore, considering the crucial role of $CD4^+$ Th2 cells in allergic asthma, interfering with these cells and regulation of their cytokine milieu might lead to reduced clinical symptoms to airborne antigens in allergic asthma.

2. Allergen immunotherapy (IT)

Current pharmacologic therapies for asthma, such as bronchodilators and inhaled corticosteroids, are effective in reducing and preventing symptom development, but do not reverse the progression of or cure this disease. Allergen avoidance as a first-line prevention strategy, including admission to hospital and sending children to holiday homes, can help and improve asthma control in those allergic asthmatics. For example, avoiding dust mite allergen can reduce symptoms and the need for asthma medications. However, a meta-analysis of appropriately controlled house mite avoidance trials (55) suggested that this approach was not very successful by itself in the control of asthma. There is thus need for additional approaches that could effectively improve asthma care and prevent their occurrence. So far, IT is unique in that it not only reduces symptoms, but also induces long-lasting disease remission. IT has been shown to improve allergic dysfunction and to re-direct the immune system away from the allergic response.

2.1. Definition of IT

Allergen IT involves the administration of gradually increasing amounts of allergen extracts with the aim of achieving a state of immunological tolerance and subsequently a reduction of clinical manifestations in the allergic patients. The process is specific in that the treatment is targeted to those allergen(s) recognized by the patient and physician as responsible for symptoms. Moreover, this treatment is the only intervention that offers long-lasting amelioration of clinical symptoms (56) and prevents the onset of new sensitization to different allergens (57), progression of the disease in allergic children (58) and development of asthma in patients with allergic rhinitis (59).

2.2. Development of IT

IT was first introduced at St. Mary's Hospital London at the end of the 19th century and many of the basic principles described remain valid today. In 1911, Noon carried out the first study of active immunization to prevent allergy against grass pollen using s.c. injection of a distilled water extract of the pollen of timothy grass, *Phleum pretense* (60). Patients were examined by receiving dropping dilutions of this extract in their eyes to induce conjunctival reaction. The inoculation dose increased every one to two weeks to investigate the protection against the pollens that caused allergic eye reaction. The strength of the extract required to elicit an eye reaction was increased as much as 100-fold. Noon thought that injection of small dose of a pollen toxin to hypersensitive people would induce an active immunity sufficient to protect the patients during the grass pollen season from their annual attacks of hay fever. Multiple clinical trials that were carried out later the same year by Freeman showed improvement of the allergic symptoms (61).

In the year 1918 it was generally accepted that hay fever, asthma and anaphylaxis were the result from antibodies produced after exposure to sensitizing antigen (62). For protection, it was advised to inject weekly extracts of not only airborne pollens, but also animal danders, sachets and foods. The mechanisms were assumed

to be desensitization and not a true immunity. Desensitization was described as a tolerance achieved in anaphylactically sensitized patients after further nonfatal injections of the sensitizing antigen (63). The term desensitization was changed to hyposensitization by the year 1922 since protection was less than complete.

By the year 1961, a new technique was developed to allow *in vitro* measurements of histamine release from cells in whole blood in the presence of specific allergen before and after treatment with ragweed extract (64). The findings demonstrated that after IT, histamine release was completely abolished in a few patients and was reduced in others. Five years later, a double-blind study performed by Lichtenstein and Osler (65) demonstrated that treatment with crude ragweed extract or the major allergen of ragweed "Amb a 1" resulted in reduction of cellular sensitivity in some patients. Treated patients showed little correlation between cellular sensitivity and symptom scores. This finding was accompanied with a rise in blocking antibodies. After these results, the term *immunotherapy* has been used to describe the process because it greatly deals with complex immunologic changes.

The study by Norman and Lichtenstein compared the efficacy of allergen IT in asthmatic patients sensitive to both ragweed and grass pollens (66). In this study, patients were divided into groups equally sensitive to both allergens. One group received specific IT with ragweed extract and the other group served as control. Ragweed season symptoms were significantly less severe in the specific IT group, but there was no difference in symptoms during grass pollen season. The results also showed that with continued ragweed extract treatment at high doses the same clinical results were found over two additional years, confirming the immunological specificity of IT.

2.3. IT for asthma

Allergen IT for the management of allergic asthma continues to be a matter for discussion (67-70). Multiple studies have examined the efficacy of IT in allergic asthma over the last 7 decades. These studies have generally been small, have differed in their patient selection criteria, allergen extracts and dosage regimen, and have usually compared IT with placebo. Because of conflicting outcomes from several of these studies, Abramson and colleagues (68, 71, 72) performed a meta-analysis of all trials published over the last 50 years of the last century, examining the impact of IT in patients with allergic asthma. They assessed the efficacy of allergen IT for the prevention of asthma symptoms, for medication requirement, lung function, non-specific AHR and allergen-specific AHR. Overall, IT was efficacious in decreasing asthma medication use, reducing bronchial hyperresponsiveness and improving asthma symptom scores. While there was no consistent effect on lung function, IT significantly reduced the airways response to inhalation of specific allergen, with some reduction in non-specific AHR as well. The authors concluded that the results of the studies published indicate that specific IT can contribute to improved asthma control.

However, allergen IT is less recommended in medical practice for treating asthma for several reasons. In general, indications for IT in allergic asthma are based on efficacy, safety and costs. Considering that most asthmatic patients are allergic to multiple aeroallergens, this suggest that allergen-specific IT has moderate

therapeutic efficacy since IT with a single allergen is unlikely to affect the patient's responsiveness to other allergens. Furthermore, multiple-allergen IT in allergic children with moderate to severe perennial asthma was found ineffective (73). The study by Adkinson (73) in asthmatic children receiving a broad spectrum of up to seven different allergen sources failed to demonstrate either partial or complete remission of asthmatic symptoms. The safety of specific IT in asthma limits its use. Allergen IT can induce local and systemic side effects that are estimated to occur in 5 to 35% of patients with asthma (74). Systemic responses usually start within 30 minutes of the treatment if they are severe (75, 76), and may be organ specific (rhinitis or asthma) or generalized (anaphylactic shock). In addition, allergen IT is a relatively high cost treatment, and is unlike pharmacotherapy (in particular inhaled corticosteroids) providing protection against nonallergic asthma triggers such as viral infections, irritants, and exercise. The risk of serious adverse reactions including a small number of anaphylaxis and fatalities has led some countries to restrict the use of allergen IT in the treatment of most asthmatic patients (77, 78).

3. Mechanisms involved in allergen IT

Allergen IT has been used to treat allergic disorders for more than 9 decades. However, the mechanism(s) by which it achieves beneficial effectiveness are not completely understood. Several studies have shown that allergen IT modifies the responses of T and B lymphocytes and the function of effector cells that mediate the allergic responses. The potential mechanism(s) that have been proposed to explain the beneficial effects of IT are presented in Fig. 2.

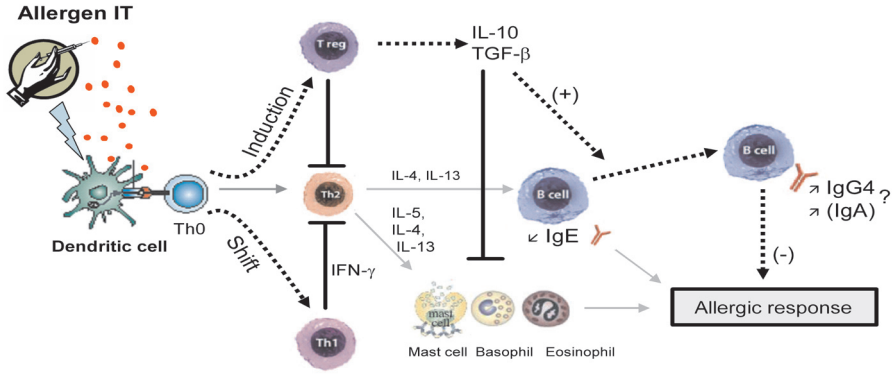

Figure 2. *Schematic representation of the potential immune deviation leading to the beneficial effects of IT. Allergen IT results in both a shift in allergen-specific T-cells from Th2 to Th0/Th1 responses and the generation of IL-10 and TGF-β producing T regulatory (Treg) cells. Allergen-specific Th1 immune responses protect against the development of allergic disorders by inducing the production of IFN-γ, which inhibits the development of Th2 cells. The regulatory cytokines IL-10 and TGF-β induce switching of B cell responses in favor of IgG4 antibodies and IgA antibodies respectively, and suppress IgE production. IL-10 and TGF-β directly or indirectly suppress effector cells of allergic inflammation such as mast cells and eosinophils thereby preventing release of mediators and late-phase inflammation. Sold gray arrows represent immune response pathway to natural exposure; dotted arrows represent immune response pathway to IT; blocked lines represent inhibition. Adapted from (79).*

3.1. Hypothesis on IgG blocking antibodies

The clinical efficacy of specific IT as treatment for IgE-mediated inhalant allergy is documented in several studies. However, although peripheral T-cell tolerance is rapidly induced during IT, there is no evidence for B-cell tolerance in the early course (80). In pollen-sensitive patients, allergen IT prevents elevation of the serum specific IgE during the pollen season (81). However, the changes in IgE levels cannot explain the diminished responsiveness to specific allergen as a result of allergen IT, because the decrease in serum IgE is relatively late and does not correlate with clinical improvement after IT.

To explain the immunological mechanisms underlying the clinical improvement, intensive research has concentrated upon the specific antibody response in serum with respect to class and subclass distribution (82). An early explanation for the benefit of allergen IT was that during treatment induction of blocking IgG antibodies, especially IgG4 isotype, is responsible for the protective effects of IT. Studies showed that allergen-specific IgE levels rise temporarily during initial phase of IT, but fall back to pre-treatment levels during maintenance therapy (83). Subsequently, it was demonstrated that IT also induces allergen-specific IgG (mostly IgG1 and IgG4) and in few reported cases IgA levels (84-86). These findings led to the hypotheses that these IgG antibodies contribute to the immunosuppressive effects of IT by blocking IgE-facilitated antigen presentation (87, 88) and that such antibodies act as blocking antibodies by engaging low-affinity Fc receptors for Ig (e.g. FcγRII) expressed by B lymphocytes, basophils and mast cells (86). In fact, a substantial number of studies demonstrated increased specific IgG4 levels associated with clinical improvement (89, 90). However, the correlation between increases in IgG antibodies and clinical improvement has not always been found (91, 92). Identical rises in IgG can be observed in responders and non-responders of IT and allergen-specific IgG levels decrease and return to pre-IT levels after several months, while the efficacy of therapy is not reduced (93). In addition, studies have shown that the rise in IgG follows rather than precedes the onset of clinical benefit. Rush IT, a treatment schedule where maintenance doses can be reached within very short time periods, is clinically effective before IgG levels start to rise (94). In this respect, the role of IgG (and IgA) in IT has been questioned and it is not clear whether the significant increase in IgG levels has a causal role in alleviating symptoms or simply represents a bystander effect, occurring as a consequence of high allergen exposure. Nevertheless, most research indicates that during IT the increase of IgG is an epiphenomenon, but the blocking antibody theory is still favored.

3.2. Hypotheses on T-cell tolerance

In addition to the effects of IT on Ig, its effect on T lymphocytes has been intensely studied.

3.2.1. T-cell anergy

The generation of an effective immune response involves antigen-specific T-cell

expansion and differentiation of effector function. T-cell activation requires at least two distinct signals, including signaling *via* the antigen-specific T-cell receptor and a costimulatory pathway (32). Antigen stimulation of T-cells can lead either to a positive immune response, characterized by proliferation, differentiation, clonal expansion and effector function, or in the absence of an appropriate co-stimulatory signal to a state of long-lasting unresponsiveness, termed anergy (95). The most important co-stimulatory molecules involved are CD28/CD152 (CTLA-4)-CD80/CD86 (B7-1/-2) and CD40-CD154 (CD40 ligand) (96-98). Anergy induction can down-regulate both cellular and humoral immune responses. The induction of T-cell non-responsiveness or anergy as the mechanism of IT is supported by the observed diminution of allergen-specific T-cell proliferation and cytokine production (99), although in some instances IL-2 was able to restore these functions (100). However, the usually increased IgG production during IT cannot be explained by the induction of T-cell anergy (101, 102). Various methods for anergy induction have been described. Stimulation of T-cells with high doses of antigen in the absence of appropriate co-stimulation or in the presence of IL-10 has been found to induce a profound form of anergy (103, 104). Under these conditions anergic T-cells showed limited peripheral expansion and a significant amount of death, with a residual subset of cells remaining that were unresponsive to antigen restimulation both *in vivo* and *in vitro*. It has been suggested that T-cells rendered anergic *in vivo* and *in vitro* become regulatory T-cells that can regulate other T-cell responses (105, 106). Therefore, anergy is now considered as a form of immunregulation rather than induction of non-responsiveness.

3.2.2. Immune deviation towards Th1 responses

In the late 1980s, a favorable explanation was based on the Th1/Th2 dichotomy in specific immune reactions (30). Mechanistic studies demonstrated that allergen IT alters the balance of cytokines released from Th-lymphocytes with a shift from Th2-cells in association with allergic inflammation toward Th1-cells that release IFN-γ, thereby inhibiting Th2 function (107-109). Early studies showed that allergen-specific T-cell clones shift from IL-4 to IFN-γ production after IT (107, 110). However, the effects of IT on Th1 cytokine secretion are less consistent (111). In humans, several clinical trials demonstrated a reduction of the allergen-specific Th2 response, whereas the Th1 response remained undetectable (108, 112) and in a separate study, IT diminished both allergen specific Th2 and Th1 responses (80). In experimental asthma models some studies have confirmed the relation between presence of Th1-associated cytokines and amelioration of disease, but others have not found such effects (113). In recent years, the concept of immune deviation toward Th1 response by IT lost favor with the alternative observation on regulatory T cells. Nowadays, Treg cells are taking the centre stage as crucial immunoregulatory cells that may offer an explanation for many of the observations that occur during IT that cannot be adequately explained by the Th2 to Th1 shift (114-116).

3.2.3. Regulatory T-cells

Several subsets of regulatory T-cells with distinct phenotypes and mechanisms of

action have been identified. These include (i) naturally occurring $CD4^+CD25^+Foxp3^+$ T-cells (nTregs) that inhibit immune responses through cell-to-cell contact, (ii) $CD4^+Foxp3^+$ iTregs induced in the periphery, in contrast to nTregs not generated in the thymus; the capacity of these cells to suppress the proliferation of naïve T cells requires cell:cell contact and (iii) cells induced in the periphery following antigen exposure (adaptive regulatory T (aTreg) cells). aTreg cells are subdivided into type 1 regulatory T (Tr1) cells which secrete high levels of IL-10 and low to moderate levels of transforming growth factor (TGF)-β and type 3 regulatory T (Th3) cells which secrete TGF-β.

Natural $CD4^+CD25^+Foxp3^+$ T regulatory cells: The natural $CD4^+CD25^+Foxp3^+$ regulatory T (nTreg) cells are generated in the thymus and represent 5-10% of the $CD4^+$ T lymphocytes both in mice and humans. These cells are thought to perform a specialized role in controlling both the innate and the adaptive immune response (117, 118). Depletion of nTreg cells in normal naïve animals leads to the spontaneous development of various autoimmune diseases, and reconstitution of those depleted cells prevents the development of autoimmunity (119, 120). Moreover, studies have revealed that lack of nTreg cell development in Foxp3 mutant mice (scrufy) results in a lethal lymphoproliferative autoimmune syndrome (121-123). Furthermore, humans suffering from immune dysregulation, polyendocrinopathy, enteropathy and X-linked syndrome (IPEX) are similarly affected and a majority of these patients lack nTreg cell development (124, 125). In addition, both scurfy mice and patients with IPEX have elevated IgE levels and eosinophilia. Although the mechanism of immune regulation mediated by nTreg cells is not well understood, these cells can both directly suppress responding T-cells and down-modulate APC function *in vitro* (126, 127). nTreg cells may also convert $CD4^+$ Th cell into regulatory cells expressing IL-10 and/or TGF-β in culture systems (128, 129). During the past few years, it has been shown that nTreg cells induce infectious tolerance *in vivo* by catalyzing the formation of IL-10 producing regulatory T-cells (130).

Adaptive T regulatory cells: In recent years, it has been demonstrated that IT is associated with the induction of aTreg cells. The aTreg cells are generated in the periphery from naïve T-cells after encountering antigens presented by tolerogenic DCs. In studies of IT for bee venom anaphylaxis, Akdis *et al.* (114) were the first to provide evidence for a role of IL-10 and Tr1 cells in the beneficial effects of allergen IT. They observed that the therapy-induced increase in secretion of IL-10 in T-cell cultures along with decreased allergen-driven proliferation and decreased production of Th2 and Th1 cytokines. Moreover, in clinical trials, allergen IT has been shown to increase the production of IL-10 by APCs, including B cells, monocytes and macrophages (87, 116, 131). Findings were extended by IT studies with airborne allergens that showed increases in IL-10 and TGF-β producing antigen-specific Treg cells in the blood and airway tissue (87, 114-116). Induction of IL-10 producing Tr1 cells has been shown to be associated with successful IT (114, 115). Animal studies confirmed the suppressive role of IL-10 during allergic inflammatory diseases. Intranasal administration of IL-10 simultaneously with OVA inhibited AHR, cell recruitment in the airways and Th2 cytokine production in OVA-sensitized mice (132, 133). Moreover, $IL\text{-}10^{-/-}$ mice displayed more severe inflammation, morbidity and alveolar lesions than

wild-type mice after airway challenge in a model of allergic bronchopulmonary *aspergillosis* (134). In a mouse model of allergen IT (135), Vissers *et al.* (113) recently demonstrated that blocking the IL-10 receptors largely abrogated the reduction of airway eosinophil numbers and AHR after IT. In human studies, the regulatory cytokine IL-10 is implicated in the differential regulation of IgE and IgG production (114, 136). Although not directly a class switching factor for IgG4 (137), IL-10 has been shown to increase the production of IgG4 while preventing IL-4 mediated class switching to IgE (136). Furthermore, IL-10 reduces the release of pro-inflammatory mediators by down-regulating IgE-dependent activation of basophils and mast cells (138) and by decreasing survival and activation of eosinophils. In addition, IL-10 (and TGF-β) might act on APCs. IL-10 down-regulates MHC class II expression on monocytes and reduces their antigen presentation capacity (139). Moreover, IL-10 down-regulates the CD80 expression on DCs and macrophages (140, 141). IL-10 may therefore block the APC dependent CD28-CD80 interaction and subsequent co-stimulatory signaling in T-cells (142).

The other immunoregulatory cytokine associated with regulatory T-cells is TGF-β. The Th3 cells were originally generated and identified in mice orally tolerized to myelin basic protein and suppressed the induction of experimental autoimmune encephalomyelitis by a TGF-β dependent mechanism (143). Several studies have demonstrated that TGF-β-producing Th3 cells play a crucial role in many aspects of immune regulation and T-cell homeostatis (144). In the past few years studies have provided evidence for increases in the amount of TGF-β driven allergen-specific IgA following IT, indicating other antibody classes than IgG might contribute to clinical efficacy (116). Moreover, TGF-β has been shown to induce expression of IL-10 by T-cells (145). Literature data suggest that early increase in IL-10, which in venom therapy is within 5–7 days (114), and sustained TGF-β production (over 1 year was only observed in successfully treated patients) may be required for successful IT to induce stable tolerance to non-pathogenic environmental antigens (146).

Therefore, improved knowledge of the mechanism(s) by which allergen IT induces T regulatory cells should provide a fundamental insight into the precise mechanism(s) involved in the beneficial effects of IT thereby opening avenues to improve its therapeutic efficacy in the management of allergic diseases, including asthma.

Role of dendritic cells in the generation of aTreg cells: Although there is suggestive evidence that IT achieves beneficial effectiveness by the induction of aTreg cells, the mechanism(s) by which IT induces aTreg cells is not completely understood. Over the past decade, it has become clear that DCs play a critical role in the generation of all aTreg cell subsets (147) and regulation of immune responses to a variety of antigens (148). The ability of DCs to induce immunity or tolerance depends on their maturation state. Tolerogenic DCs are semi-mature cells with increased expression of MHC II and CD86 but low levels of expression of CD40 and absence of the pro-inflammatory cytokines IL-6 and TNF-α (149). It has been shown that aTreg cells induced by immature DCs are characterized by high levels of IL-10 cytokine secretion (150, 151). The nuclear factor-κB (NF-κB) protein Re1B activity is critically required for DCs maturation (152). Re1B-deficient bone marrow-derived DCs (BMDCs) or BMDCs in which Re1B activity is inhibited have the potential to induce

antigen-specific immune tolerance *in vivo* (153). Inhibition of NF-κB signaling by various drugs (154-156) induces DCs to acquire tolerogenic properties that favor the induction of Tr1-like cells *in vitro* and tolerance in mouse models of transplantation and autoimmune diseases (157-159).

Besides this, recent findings demonstrate that DCs induce development of aTreg cells by several mechanisms including high expression of the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) (160, 161). IDO expressing DCs inhibit T-cell proliferation *in vitro* and promote tolerance *in vivo* (162, 163), including maternal tolerance during pregnancy (164), control of allograft rejection (165) and protection against autoimmunity (166). A number of studies have also demonstrated that IDO is implicated in tolerance induction by regulatory T-cells expressing cytotoxic T-lymphocyte antigen-4 (CTLA-4) (167, 168). Moreover, IDO may directly mediate inhibition of T-cells proliferation by tryptophan depletion or downstream tryptophan metabolites (169, 170).

Therefore, DCs through the expression of tolerogenic molecules like IDO, and/or its maturational state (immature/semimature), may have the potential to induce the generation of aTreg cells.

4. Improvement of allergen IT

The goal of allergen IT is the transformation of an allergic individual into one who can tolerate allergen exposure by converting deleterious allergic immune response into protective responses. Despite the impressive efficacy of allergen (injection) IT for treatment of allergic rhinitis and insect venom allergy, its efficacy in allergic asthma remains controversial (68). Furthermore, it has been reported that s.c. administration of allergens can induce severe systemic reactions due to cross-linking of allergen-specific IgE on mast cells (171, 172). Thus, for development of a more effective and safe form of IT, more insight into the underlying immunological mechanisms of allergen IT is considered necessary to improve efficacy, in particular in asthmatic patients. There is growing evidence that the positive effects of allergen IT are associated with aTreg cells (Tr1 and Th3) and the immunosuppressive cytokines that they produce. The mechanism(s) by which these cells are induced by IT are still not fully understood. Well described pathways to induce aTreg cells are antigen presentation to T-cell by immature DCs or the presence of IL-10 or TGF-β in the local microenvironment. In this regard, combination of allergen IT with administration of compounds that inhibit DCs maturation, such as the biologically active form of vitamin D_3 (1α,25-dihydroxyvitamin D_3) or glucocoticoids, might be a novel immunotherapeutic strategy to improve IT for better treatment of allergic diseases, asthma included.

5. Aim and scope of this thesis

Specific allergen IT for the treatment of atopic diseases, which was introduced almost 100 years ago, still remains controversial. Understanding of the mechanism(s) by which allergen IT produces its beneficial effects is needed to get better efficacy and high safety, in particular in asthmatic patients. Previously we developed a mouse

model of allergic asthma in which allergen IT suppressed allergen-induced airway manifestation of asthma (135). Moreover, we have shown that allergen IT induces long-lasting immune tolerance in which IL-10 is crucial, pointing to a role for Tr1 cells (113). In this thesis, the mechanism(s) underlying tolerance induction and the beneficial effects of allergen IT in ovalbumin-induced model of allergic asthma in BALB/c mice were further investigated to improve the therapeutic efficacy of allergen IT.

The observation that the beneficial effects of IT are accompanied by increases in allergen-specific IgG, in particular IgG1 (mouse equivalent of human IgG4) resulted in the hypothesis that these antibodies might contribute to the suppressive effects of IT. IgG antibodies are proposed as blocking antibodies and their effects could be mediated through co-aggregation of IgG Fcγ receptors. Interestingly, ligation of Fcγ receptor has been shown to stimulate IL-10 production by murine macrophages *in vitro* (173). Human studies have shown that IgG antibodies compete with IgE for binding to the allergen, thereby preventing effector cell degranulation (174-176), and blocking IgE-facilitated allergen presentation to T-cells (88, 177). IgG antibodies may act by engaging Fc receptors for Ig namely FcγRI, FcγRIIB and FcγRIII. FcγRIIB expressed by B cells, basophils or mast cells, contain the immunoreceptor tyrosine-based inhibition motif (ITIM) that can generate negative signals preventing cellular activation and the release of pro-inflammatory mediators following cross-linking with FcεRI (85, 86). Moreover, signaling *via* FcγRIIB on DCs has recently been shown inhibits DC maturation leading to those condition of antigen presentation that favor the induction of aTreg cells (178). By contrast, FcγRI and FcγRIII expressed by DCs, are signal-chain molecules that trigger the activation signals through the associated γ chain, which bears an immunoreceptor tyrosine-based activation motif (ITAM) (179). Signals from these receptors cause the maturation of DCs and promote efficient MHC class I and II-restricted antigen presentation (180). In the studies described in **chapter 2**, it was investigated if Fcγ receptors contribute to the induction of tolerance by allergen IT using transgenic mice deficient in γ chain, lacking the activation receptors FcγRI and FcγRIII, or mice deficient in the inhibitory receptor FcγRIIB.

The tryptophan-catabolizing enzyme, IDO has been shown to play a role in immune suppression and tolerance induction. Furthermore, studies have demonstrated that IDO expressing DCs can induce development of aTreg cells (160, 161). Therefore, in **chapter 3** we aimed to examine whether IDO activity is required during tolerance induction by allergen IT or for the suppressive effects on asthma manifestations and whether tryptophan depletion or generation of its downstream metabolites is involved.

Natural Treg cells protect against immune responses to self antigen, indicating that the immune system contains a population of professional regulatory T-cells involved in active mechanism of immune suppression (120). Moreover, passive transfer of nTreg has been shown to suppress autoimmune disorders and transplant rejection (118, 120). However, the role of nTreg cells in tolerance induced by allergen IT to inhaled antigen is presently unknown. Human studies performed so far demonstrated that co-culture of nTreg cells with conventional $CD4^+$ T-cells results in differentiation of $CD4^+$ T cells into aTreg cells that suppress Th1 or Th2 cell responses in a TGF-β and/or IL-10-dependent manner (128, 181). Animal studies have shown that nTreg can promote the formation of antigen-specific, IL-10-secreting regulatory

T-cells in experimental allergic encephalomyelitis (130). Furthermore, it has been demonstrated that murine nTreg cells that constitutively express CTLA-4 can induce activation of IDO and thereby affect tryptophan metabolism in DCs (167). These data raised the interesting possibility that nTreg cells might contribute to the induction of suppressive effects by allergen IT. In **chapter 4**, we examined this hypothesis and investigated whether induction of tolerance by allergen IT requires nTreg cells activity. This study was performed by inhibiting the functional activities of these regulatory T-cells *in vivo* using monoclonal antibodies to glucocorticoid-induced tumour necrosis factor receptor and to one of its effector molecules, CTLA- 4.

Several studies have indicated that repetitive stimulation of T-cells with immature, antigen-presenting DCs can generate IL-10 producing Tr1 cells (150, 151). The active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), a potent inhibitor of NF-κB gene expression, can prevent the maturation of DCs *in vitro* leading to tolerogenic DCs with increased potential to induce regulatory T-cells. Studies *in vivo* have demonstrated that 1,25$(OH)_2D_3$ inhibits DC maturation and promotes peripheral tolerance in mouse models of transplantation and autoimmune diseases (158, 159). Therefore, as described in **chapter 5**, it was investigated whether combination of allergen IT with 1,25$(OH)_2D_3$ potentiates the immunosuppressive effect of IT and if the immunoregulatory cytokines IL-10 and TGF-β are involved in the effector phase.

The main findings obtained in this thesis are summarized and discussed in the last chapter (**chapter 6**) of this thesis.

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Chapter 2

Fcγ-receptors are not involved in tolerance induction during allergen immunotherapy in a mouse model of allergic asthma

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Abstract

Increased serum levels of allergen-specific IgG antibodies induced by allergen immunotherapy (IT) have been postulated to be implicated in the protection against allergic disease. In the present study, we investigated whether the Fc receptors for IgG (FcγR) play a role in tolerance induction by IT in a mouse model of allergic asthma. In this model ovalbumin (OVA)-sensitization and challenge of BALB/c wild-type, FcγRIIB gene-deficient ($^{-/-}$) and FcγRI/FcγRIII$^{-/-}$ mice results in elevated OVA-specific IgE levels in serum and eosinophilic airway inflammation concomitant with increased Th2 cytokine levels in bronchoalveolar lavage (BAL) fluid. To assess the effects of IT in these mice, sensitized mice received IT with OVA (OVA-IT; 1 mg, s.c.) or sham-treatment 10 days prior to OVA inhalation challenge. Compared to sham-treatment, OVA-IT in FcγRIIB$^{-/-}$ and FcγRI/FcγRIII$^{-/-}$ mice showed equivalent suppression of airway eosinophilia and elevated serum OVA-specific IgE levels as observed in wild-type mice. These suppressive effects coincided with significant falls of IL-5 and IL-13 levels in BAL fluid in all mouse strains. Furthermore, in wild-type mice as well as in FcγRI/FcγRIII$^{-/-}$ and FcγRIIB$^{-/-}$ mice, OVA-IT induced increased OVA-specific IgG1 and IgG2a levels in serum. In sham-treated mice a similar upregulation of OVA-specific IgG1 and IgG2a levels in serum only occurred after OVA inhalation challenge. In contrast, this challenge-induced IgG1 and IgG2a upregulation was completely inhibited after OVA-IT in all mouse strains. Surprisingly, serum levels of OVA-specific IgA were significantly increased in wild-type mice but decreased in FcγRIIB$^{-/-}$ and FcγRI/FcγRIII$^{-/-}$ mice. Our data clearly demonstrate that FcγRI, -IIB and -III have no role in the induction of tolerance by allergen IT in this mouse model of allergic asthma.

Introduction

Allergen-specific immunotherapy (IT) is the only treatment shown to have long-term benefit for allergic diseases and is used most effectively in allergic reactions to insect venom and allergic rhinitis (1-3). The immunological process underlying its beneficial effects remains largely unclear, but several potential mechanisms have been postulated including the modulation of both T- and B-cell responses to allergen. One of the early observations regarding the immunological effect of IT was the shift from IgE to IgG antibodies (4-6). So far, these observations have led to the "blocking antibody hypothesis", first postulated by Lichtenstein *et al.* (7). However, the relationship between the efficacy of IT and the induction of allergen-specific IgG remains controversial with serum concentrations of allergen-specific IgG correlating with clinical improvement in some studies but not in others (8, 9).

Several mechanisms have been proposed to explain the protective effects of IgG antibodies. Cooke *et al.* (10) were the first to demonstrate that antibodies were able to prevent allergic serum-mediated skin reactions. Subsequent results obtained from numerous human studies suggest that specific IgG antibodies induced by IT can block allergen-induced IgE-mediated degranulation of mast cells (11) and may directly affect IgE production by memory B-cells through the crosslinking of FcγIIB receptors on the surface (12, 13). IgG antibodies may inhibit IgE-facilitated allergen presentation to T-cells (14, 15), a rate-limiting step in allergen-specific Th2 cell-driven allergic responses (14). Studies in mice suggest that IgG-antigen complexes may stimulate Fcγ receptor-mediated up-regulation of IL-10 production by macrophages (16), a cytokine implicated in IT (17). Moreover, recent findings demonstrate that mice deficient in signaling *via* FcγRIIB failed to develop mucosal tolerance after nasal or oral antigen administration (18). Signaling *via* FcγRIIB on dendritic cells (DCs) initiated by local IgG in the mucosa draining lymph nodes was found to down-regulate DC activation, leading to mucosal regulatory T cell induction and subsequent tolerance (18). Thus, accumulating data provides supporting evidence that IgG or IgG-antigen complexes have the potential to play an active role in immunological tolerance after IT.

We previously developed a mouse model of allergic asthma in which allergen IT in wild-type mice inhibits allergen-induced airway manifestations of asthma and significantly suppressed the elevated allergen-specific IgE levels in serum (19). Moreover, allergen IT induces IL-10 dependent long-lasting tolerance to OVA-induced asthma manifestations (17). Interestingly, IT coincides with increased serum levels of OVA-specific IgG antibodies, which could suggest that IgG mediated regulation is involved in the suppressive effects of IT and makes this a suitable model to study the role of Fcγ receptors. As IgG needs to signal *via* FcR, we questioned whether Fcγ receptors play a role in tolerance induced by allergen IT in a mouse model of allergic asthma. Three different types of FcγR for IgG, designated FcγRI (CD64), FcγRIIB (CD32) and FcγRIII (CD16) are expressed on mouse effector cells (20, 21). FcγRI and FcγRIII are activating receptors containing immune-receptor tyrosine-based activation motifs (ITAM) (22). Cross-linking of these receptors by their ligand, IgG-antigen complexes, induces intracellular signaling and various effector cell responses such as antibody-dependent cell-mediated cytotoxicity (23) and the release of inflammatory mediators (21, 24). In contrast, FcγRIIB is an inhibitory receptor

expressed by B cells, basophils, mast cells, DCs and macrophages, that signals through an immuno-receptor tyrosine-based inhibitory motif (ITIM) resulting in the inhibition of many of the functions activated by FcγRI and FcγRIII (13).

To our aim, BALB/c wild-type and FcγRI and III double knock-out or IIB knockout mice were sensitized and challenged with the experimental allergen OVA. The present findings clearly demonstrate that Fcγ receptors do not play a role in the suppression of allergen-induced allergic airway inflammation by allergen IT in this mouse model of allergic asthma.

Materials and methods

Animals

Male BALB/c mice (6-8 weeks) were obtained from Charles River (Maastricht, The Netherlands) and maintained in a pathogen-free animal facility of the Central Laboratory Animal Institute (Utrecht University). Mice deficient in the common γ chain, lacking the activation Fcγ receptors FcγRI and III (25), and mice deficient in FcγRIIB (25) (all BALB/c background) were kindly provided respectively by the Department of Human Genetics, Leiden University Medical Center, The Netherlands (S.S. Verbeek) and the Laboratory of Pediatric Gastroenterology, Erasmus University Medical Center, Rotterdam (J.N. Samsom). All mice were housed in macrolon cages in a laminar flow cabinet and provided with food and water *ad labitum*. Animal care and use were conducted in accordance with the Animal Ethics Committee of Utrecht University, The Netherlands.

Sensitization, challenge and IT protocol

The protocol used for sensitization, IT and inhalation challenge was the same as previously described (17). Mice received two i.p. injections of 10 μg OVA (chicken egg albumin, crude grade V, Sigma-Aldrich) adsorbed onto 2.25 mg alum (ImjectAlum, Pierce, Rockford, IL, USA) in 100 μl pyrogen-free saline (B. Braun, Melsungen, Germany) on days 0 and 7. Two weeks after the second sensitization, mice were treated with three s.c. injections of 1 mg OVA in 200 μl pyrogen-free saline on alternate days. The control group was sham-treated with 200 μl saline. One week after OVA or sham treatment, airways of the mice were challenged with OVA aerosols in pyrogen-free saline (1% w/v) for 20 min 3 times every third day in a Plexiglas exposure chamber (5 liter) coupled to a Pari LC Star nebulizer (PARI Respiratory Equipment, Richmond, VA, USA; particle size 2.5–3.1 μm) driven by compressed air at a flow rate of 6 l/min. Aerosol was given in groups composed of no more than 12 mice.

Serum levels of immunoglobulins, cellular infiltration and Th2-cytokine levels in BAL fluid were measures 24 h after the last OVA inhalation challenge in each mouse.

Determination of serum levels of OVA-specific immunoglobulins

Blood for assessment of serum immunoglobulin levels was recovered from each mouse by an incision made in the tail vein 7 days before the first OVA aerosol challenge and at 24 h after the last challenge upon i.p. injection of 1 ml 10% urethane by heart puncture. Blood samples were allowed to clot at room temperature for 30 min and subsequently centrifuged for 10 min at 2700 x g. Sera were collected, kept at –70°C and OVA-specific immunoglobulin levels were measured by ELISA using microtiter plates from Nunc A/S (Roskilde, Denmark), ELISA buffer for blocking and sample dilution, and PBS containing 0.05% v/v Tween-20 for washing between incubations. To determine OVA-specific IgE levels, wells were coated overnight at 4°C with 1 μg/ml of anti-mouse IgE diluted in PBS, followed by blocking with ELISA buffer (PBS containing 0.5% BSA, 2 mM EDTA, 136.9 mM NaCl, 50 mM Tris, 0.05% Tween-20 [Merck, Whitehouse Station, NJ, USA], pH 7.2) and left for 1 h at room temperature. Hereafter, diluted serum samples and duplicate dilution series of an OVA-specific IgE reference serum, prepared as described previously (26), were added and wells were incubated for 2 h. Next, after multiple washings the wells were incubated for 1.5 h with 1 μg/ml of DIG-conjugated OVA, followed by another washing and incubation for 1 h with anti-DIG Fab coupled to horseradish peroxidase, according to manufacturer's instruction (Roche Diagnostics, Basel, Switzerland).

To assess OVA-specific IgG1 or IgG2a levels, wells were coated with 10 μg/ml OVA in PBS. After blocking, diluted serum samples and duplicate dilution series of a reference standard serum obtained from multiple OVA-boosted mice were added. Hereafter, wells were washed and incubated with 1 μg/ml of biotinylated anti-mouse IgG1 or 1 μg/ml of biotinylated anti-mouse IgG2a for 1.5 h followed by another washing and incubation for 1 h with 1:10,000 diluted Poly-HRP.

For determination of OVA-specific IgA, wells were coated with 10 μg/ml OVA in PBS. After blocking, diluted serum samples and duplicate dilution series of IgA reference serum were added for 2 h. Next, after multiple washings the wells were incubated for 1 h with 2 μg/ml of biotinylated anti-mouse IgA, followed by another washing and incubation for 1 h with 1:10,000 diluted Poly-HRP.

For color development, 0.4 mg/ml of *o*-phenylenediamine-dichloride and 4 mM H_2O_2 in PBS were used, and the reaction was stopped by adding 75 μl of 4 M H_2SO_4. Optical density was read at 490 nm, using a Benchmark microplate reader (Bio-Rad, Hercules, CA, USA). Results were analyzed using Microplate Manager PC software (Bio-Rad).

Analysis of BAL fluid

BAL was performed immediately after bleeding the mice. Briefly, a midcervical skin incision was made to expose the trachea, which was cannulated with a 23-gauge blunt needle. The airways were lavaged 5 times with 1 ml aliquots of pyrogen-free saline warmed at 37°C. The first lavage was done with 1 ml saline containing BSA and protease inhibitor (Complete mini tablet [Roche Diagnostics GMbH, Penzberg, Germany] and 5% BSA). The supernatant of this first ml of recovered lavage fluid was used to measure cytokine levels. Subsequently, mice were lavaged 4 times

with 1 ml aliquots of saline at 37°C. The lavage fluid was kept on ice until further processing. Recovered lavage fluid of the second through fifth ml was pooled and cells herein (including those from the first ml) were pelleted (387 x g, 4°C, 10 min) and resuspended in 150 µl cold PBS. The total number of cells in the BAL fluid was determined using a Bürker-Türk counting chamber (Karl Hecht Assistant KG, Sondheim/Röhm, Germany). For differential cell counts, cytospin preparations were made (15 x g, 4°C, 5 min) using a cytocentrifuge (Shandon Life Science, Cheshire, UK), and cells were fixed and stained with Diff-Quick (Dade A. G., Düdingen, Switzerland). All cytospin preparations were evaluated using oil immersion microscopy (magnification: 1000x). Cells were identified and differentiated into mononuclear cells (monocytes, macrophages and lymphocytes), eosinophils and neutrophils by standard morphology and staining characteristics. Per cytospin 200 cells were counted and the absolute number of each cell type was calculated.

Cytokine levels in BAL fluid

Supernatant of the first ml BAL fluid was analyzed for IL-5, IL-13, and IL-10 contents by sandwich ELISA in Nunc-Immuno plates coated with appropriate anticytokine capture mAbs and second-step biotinylated mAbs according to the manufacturer's instructions (PharMingen, San Diego, CA, USA). Values were expressed as pg per ml deduced from standards run in parallel with recombinant cytokines. The detection limits of the ELISAs were 32 pg/ml for IL-5 and 15 pg/ml for IL-10 and IL-13.

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Statistical analysis on BAL fluid cell counts was performed using the non-parametric Mann-Whitney *U*-test. For all other parameters, results were analyzed using a Student's *t*-test (2-tailed, homosedastic). A probability value $P<0.05$ was considered significant. Analyses were performed by the usage of GraphPad Prism (GraphPad Software Inc., version 3.0, San Diego, USA).

Results

Serum levels of immunoglobulins

To investigate the efficacy of OVA-IT on the immunoglobulin levels in serum, OVA-specific immunoglobulin levels were determined in serum from all mice. In sham-treated wild-type, FcγRI/FcγRIII$^{-/-}$ or FcγRIIB$^{-/-}$ mice, OVA aerosol challenge induced significantly increased (95%, $P<0.01$; 93%, $P<0.01$ and 74%, $P<0.01$) OVA-specific IgE levels in serum compared to pre-challenge levels (fig. 1A, B and C, respectively). OVA-IT in wild-type mice completely prevented the upregulation of OVA-specific IgE levels in serum after OVA challenge (fig. 1A). Equivalent results were obtained in FcγRI/FcγRIII$^{-/-}$ and in FcγRIIB$^{-/-}$ mice. After OVA challenge, the serum antibody levels of OVA-specific IgE from IT –treated FcγRI/FcγRIII$^{-/-}$ and FcγRIIB$^{-/-}$ mice

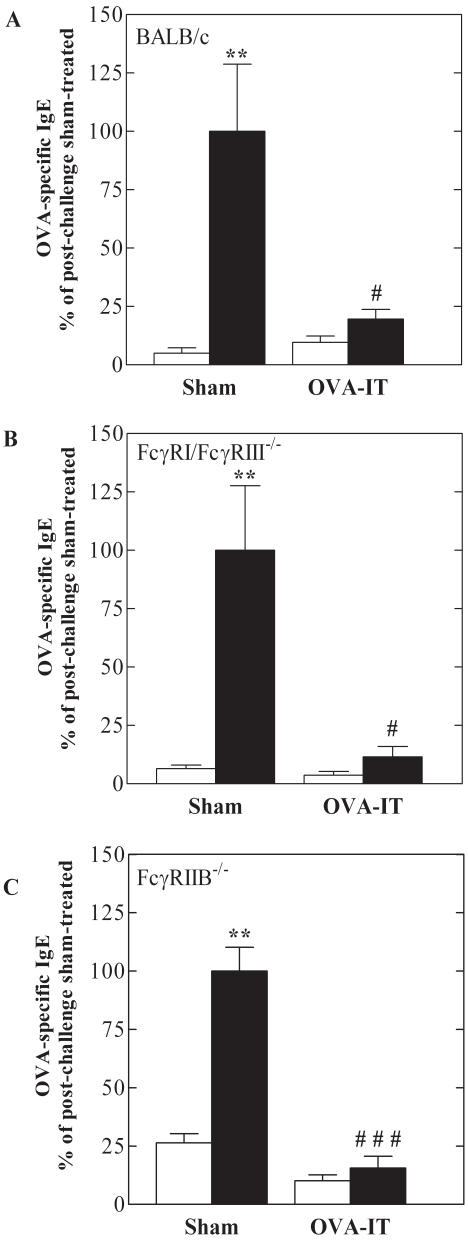

Figure 1. *OVA-specific IgE levels in serum from A) BALB/c, B) FcγRI/FcγRIII$^{-/-}$ and C) FcγRIIB$^{-/-}$ mice. OVA-sensitized mice received sham (saline) or IT treatments with 1 mg OVA. Levels of OVA-specific IgE in serum were measured just before (pre-challenge; open bars) and 1 day after (post-challenge; closed bars) OVA aerosol challenge. Values are expressed as mean percentage of sham-treated mice ± SEM (n=6). **P<0.01 compared to pre-challenge. #P<0.05, ###P<0.001 compared to post-challenge OVA-specific IgE levels of sham-treated mice.*

were significantly suppressed by 88% (P<0.05, fig. 1B) and by 84% (P<0.001, fig. 1C) respectively compared to those from sham-treated mice of the same strain.

OVA-IT induced a significant upregulation of OVA-specific IgG1 and IgG2a in serum from FcγRI/FcγRIII$^{-/-}$ mice (99%, P<0.01; 96%, P<0.05, respectively) and FcγRIIB$^{-/-}$ mice (98%, P<0.001; 76%, not significant, respectively) which was

comparable to that of wild-type mice (figs. 2A and B respectively). These levels were not further increased by OVA-challenge (figs. 2A, B). However, in all sham-treated mice, pre-challenge levels of OVA-specific IgG1 and IgG2a were low and strongly

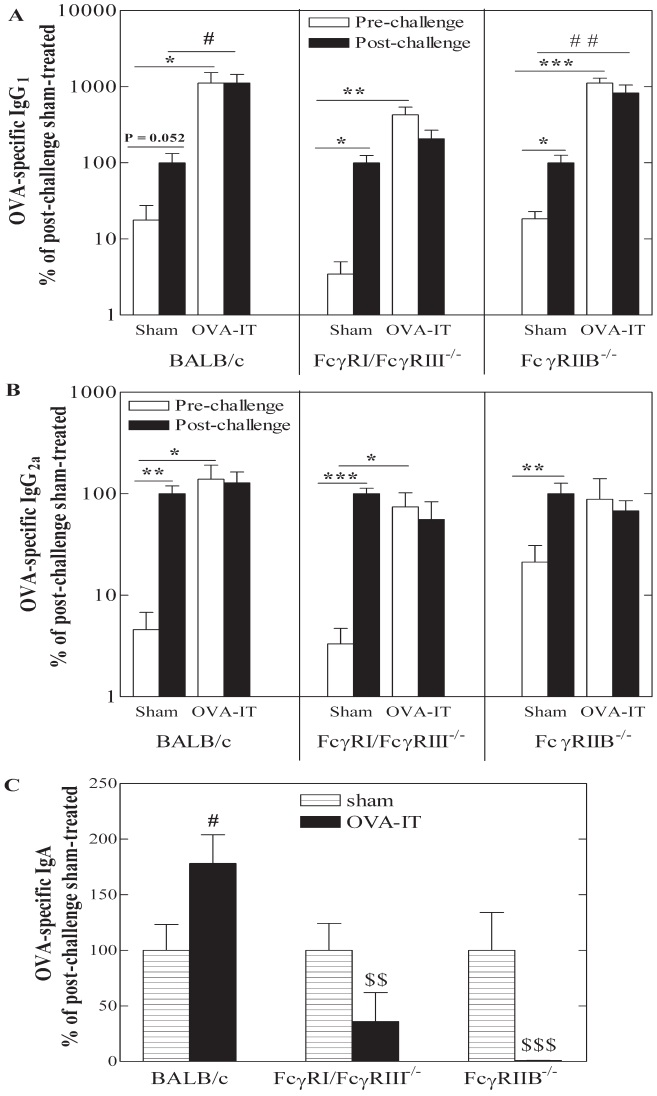

Figure 2. *OVA-specific immunoglobulins level in serum from BALB/c, FcγRI/FcγRIII$^{-/-}$ and FcγRIIB$^{-/-}$ mice. OVA-sensitized mice received sham (saline) or IT treatments with 1 mg OVA. A) Levels of OVA-specific IgG1, B) IgG2a in serum just before (pre-challenge) and 1 day after (post-challenge) OVA aerosol challenge and C) levels of OVA-specific IgA in serum 1 day after OVA aerosol challenge. Pre-challenge OVA-specific IgA levels were not determined due to insufficient amount of serum. Values are expressed as mean percentage of sham-treated mice ± SEM (n=6). *P<0.05, **P<0.01, ***P<0.001 compared to pre-challenge levels of sham-treated mice. #P<0.05, ##P<0.01 compared to post-challenge levels of sham-treated mice. $$P<0.01, $$$P<0.001 compared to OVA-IT -treated wild-type mice.*

increased after OVA-challenge (> 79% compared with pre-challenge levels) (figs. 2A and B, respectively). Interestingly, we found that in wild-type mice OVA-IT induced significantly increased OVA-specific IgA levels (78%, $P<0.05$) in serum compared with sham-treated mice (fig. 2C). In contrast, decreased levels of OVA-specific IgA were detected in serum from FcγRI/FcγRIII$^{-/-}$ and FcγRIIB$^{-/-}$ mice after OVA-IT (fig. 2C).

Thus, these data demonstrate that absence of FcγRI, -IIB and -III did not interrupted the suppressive effect of allergen IT on serum specific-IgE, -IgG1 and -IgG2a levels. Furthermore, these results suggest that FcγR are essential for the production of antigen-specific IgA in serum after IT.

Eosinophils and cytokine levels in the BAL fluid

The numbers of various leukocytes in the BAL fluid were determined at 24 h after the last OVA aerosol challenge. In wild-type mice, OVA challenge of sham-treated OVA-sensitized mice resulted in high numbers of inflammatory cells in the BAL fluid, consisting predominantly of eosinophils (fig. 3A) besides mononuclear cells

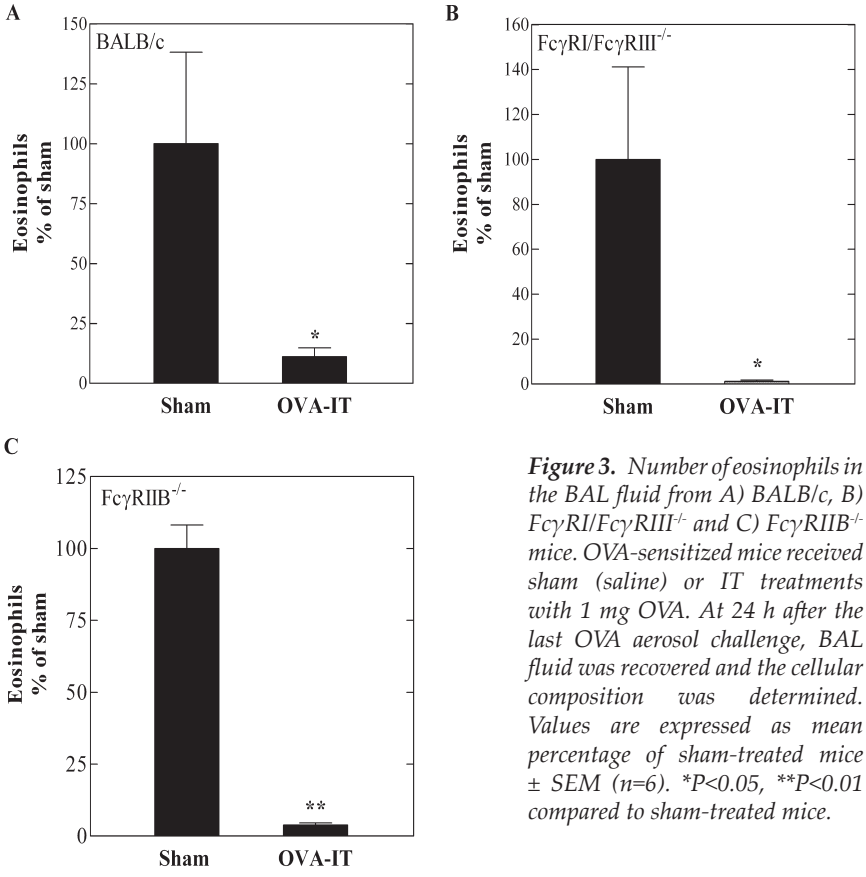

Figure 3. *Number of eosinophils in the BAL fluid from A) BALB/c, B) FcγRI/FcγRIII$^{-/-}$ and C) FcγRIIB$^{-/-}$ mice. OVA-sensitized mice received sham (saline) or IT treatments with 1 mg OVA. At 24 h after the last OVA aerosol challenge, BAL fluid was recovered and the cellular composition was determined. Values are expressed as mean percentage of sham-treated mice ± SEM (n=6). *P<0.05, **P<0.01 compared to sham-treated mice.*

and few neutrophils (data not shown). OVA-IT effectively suppressed the airway eosinophilia by 89% ($P<0.05$) compared to sham-treated mice (OVA-IT, 0.89 ± 0.30 $x10^5$ cells versus sham 7.97 ± 3.04 x 10^5 cells; fig. 3A). Similar to wild-type mice, OVA challenge induced infiltration of eosinophils in the BAL fluid from FcγRI/FcγRIII$^{-/-}$ or FcγRIIB$^{-/-}$ mice was significantly less after OVA-IT compared with OVA-sensitized and challenged sham-treated mice of the same strain (OVA-IT, 0.09 ± 0.04 $x10^5$ cells versus sham 7.66 ± 3.15 x 10^5 cells, 99% suppression, $P<0.05$; OVA-IT, 0.92 ± 0.16 x 10^5 cells versus sham 23.64 ± 1.94 x 10^5 cells, 96% suppression, $P<0.01$, figs. 3B and C, respectively).

Because Th2-type responses are crucial in the development and maintenance of allergic asthma, the levels of Th2 cytokines IL-5, IL-13 and IL-10 were assessed in BAL fluid samples obtained from sham- or OVA-IT- treated mice. The BAL fluid of OVA-sensitized wild-type sham-treated mice contained high levels of IL-5 and IL-13 after challenge (fig. 4A). OVA-IT significantly reduced the levels of IL-5 by 91% ($P<0.05$) and of IL-13 by 92% ($P<0.01$) compared with those of sham-treated mice (fig. 4A). OVA-IT also reduced the levels of IL-10 by 32% but this reduction did not reach the level of significance ($P=0.084$, data not shown). Similarly high levels of IL-5 and IL-13 were detected in the lavage fluid from sham-treated FcγRI/FcγRIII$^{-/-}$ or FcγRIIB$^{-/-}$ mice obtained 24 h after the last OVA aerosol challenge (figs. 4B and C, respectively). As shown in figs. 4B and C, OVA-IT also resulted in significant reduction of the levels of IL-5 by 89% ($P<0.001$) in FcγRI/FcγRIII$^{-/-}$ mice and by 97% ($P<0.05$) in FcγRIIB$^{-/-}$ mice and of IL-13 by 97% ($P<0.05$) in FcγRI/FcγRIII$^{-/-}$ mice and by 89%, ($P<0.05$) in FcγRIIB$^{-/-}$ mice in the BAL fluid compared with sham-treated mice (figs. 4B and C). Furthermore, upon OVA-IT, the levels of IL-10 in FcγRI/FcγRIII$^{-/-}$ were slightly reduced (31%, not significant, data not shown), and unaffected in the BAL fluid from FcγRIIB$^{-/-}$ mice (data not shown) compared with sham-treated mice. In addition, IFN-γ, a Th1 cytokine, was not detectable in the BAL fluid, irrespective of treatment and mouse strain (detection limit ELISA: 160 pg/ml, data not shown).

Together, these results suggest that absence of FcγR activity does not impair the OVA-IT induced suppression of allergen-induced airway eosinophilia as well as Th2 cytokine levels in the BAL fluid in this model.

Discussion

In the present study it is demonstrated that Fcγ receptors have no role in the beneficial effects of allergen IT in a mouse model of allergic asthma. Our findings show that allergen IT in wild-type mice significantly reduced eosinophil numbers and Th2 cytokine levels in BAL fluid and that this suppression occurred to same extent in mice deficient in FcγRI/FcγRIII or FcγRIIB. Furthermore, IT induced significant increases in serum levels of OVA-specific IgG1 and IgG2a in all mouse strains. Moreover, in all mice allergen IT completely suppressed the OVA-challenge-induced upregulation of OVA-specific IgE, IgG1 and IgG2a levels in serum that was observed in sham-treated wild-type and Fcγ receptor knockout mice. Surprisingly, serum levels of OVA-specific IgA were significantly increased after IT and inhalation challenge as compared to sham treated wild-type mice but decreased in Fcγ receptor knockout mice.

The precise mechanism(s) of allergen IT are not entirely clear, but increased

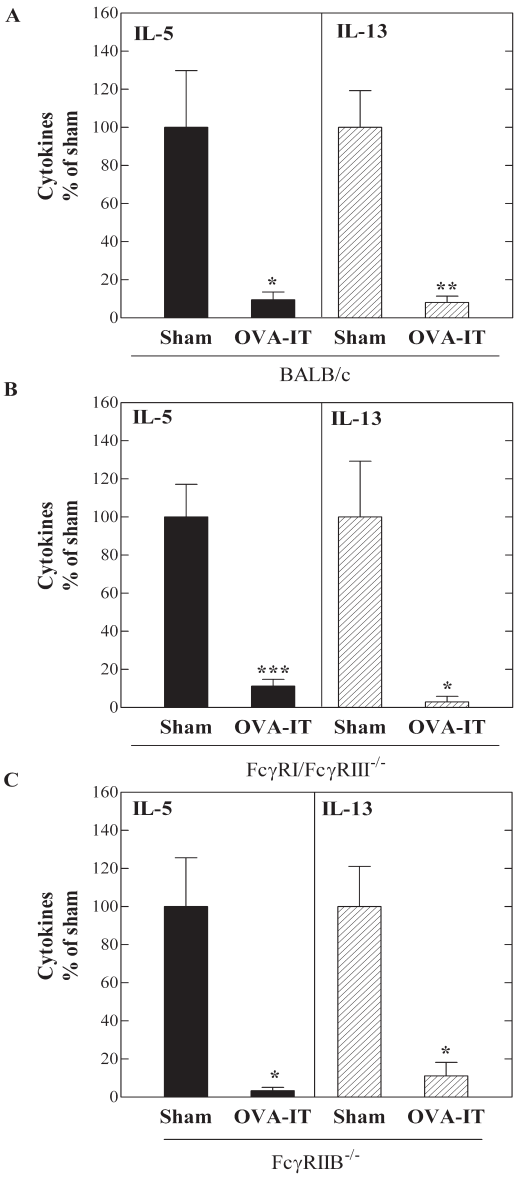

Figure 4. *Levels of IL-5 and IL-13 cytokines in BAL fluid obtained from A) BALB/c, B) FcγRI/FcγRIII$^{-/-}$ and C) FcγRIIB$^{-/-}$ mice. OVA-sensitized and challenged mice received sham (saline) or IT treatments with 1 mg OVA. At 24 h after the last OVA aerosol challenge, the cytokine levels were measured by sandwich ELISA. Values are expressed as mean percentage of sham-treated mice ± SEM (n=6). *P<0.05, **P<0.01, ***P<0.001 compared to sham-treated mice.*

serum levels of allergen-specific IgG antibodies during IT have been postulated to be implicated in the protection against allergic disease (4, 5). Likewise, serum IgG levels are also substantially increased after OVA IT in our mouse model (19). IgG-antigen immune complexes can ligate both activating (I and III) and inhibitory (IIB) Fcγ receptors and thereby modulate allergic inflammatory responses (13, 27).

First, IgG-antigen complexes can inhibit IgE-mediated leucocyte histamine release and B-cell antibody synthesis (12, 13) through FcγRIIB receptors (28-31).

Co-ligation of FcγRIIB and an ITAM-containing receptor leads to aborted FcεRII- and FcγRIII triggered activation in mast cells (29, 32) and B cell receptor-triggered activation in B cells (33, 34). Interestingly, a role for FcγRIIB in nasal eosinophilia and IgE production has been demonstrated in a murine model of allergic rhinitis (27). Watanabe *et al*. showed that FcγRIIB$^{-/-}$ mice displayed severe eosinophilia compared with that of wild-type counterparts. However, FcγRIIB$^{-/-}$ mice conversely produced less allergen-specific IgE levels (27). Although the chronic mouse model is not very suitable to evaluate mast-cell activation, we did observe a complete inhibition of the upregulation of IgE and IgG antibodies upon OVA inhalation challenge. This may point to suppression of B-cell responses to recall antigen mediated by inhibitory FcγRIIB receptors. However, in FcγRIIB$^{-/-}$ mice, OVA IT still completely suppressed the upregulation of IgE and IgG upon OVA challenge, ruling out a role for this receptor in this phenomenon. Interestingly, the upregulation of serum OVA-specific IgA antibodies after IT and inhalation challenge was completely reversed in FcγRIIB$^{-/-}$ mice suggesting the possibility that IgG-FcγRIIB might be involved in Ig isotype switch to IgA antibodies after IT.

Second, IgG antibodies can inhibit IgE facilitated allergen presentation by B-cells to T cell clones (14, 15). However, mouse models are not suitable to study this mechanism since their antigen presenting cells do not express FcεRI receptors (35, 36). Therefore, we cannot draw any conclusion regarding this potential mechanism of action of allergen IT.

Third, some studies have shown a role of FcγRIIB in tolerance induction in response to specific antigen (18, 37, 38). A recent study with FcγRIIB knockout mice showed that these animals failed to develop mucosal tolerance to OVA *via* nasal or oral routes (18). It has been demonstrated that after mucosal OVA application IgG-FcγRIIB signaling plays a role in inhibiting DC maturation, leading to mucosal regulatory T cell induction and subsequent tolerance to a systemic delayed type hypersensitivity response. However, we observed that OVA IT-induced tolerance to antigen-induced allergic asthma manifestations was not hampered in FcγRIIB$^{-/-}$ mice. Although Samsom *et al*. (18) used the same strains of mice and the same antigen as in our model, there are important differences in the experimental protocol. Tolerance induction *via* different routes may be regulated by different mechanisms. Upon mucosal tolerance induction the antigen is carried to the mucosa draining lymph nodes where mucosal regulatory T-cells are formed (39, 40). In many aspects the microenvironment in these nodes is different from that of peripheral lymph nodes (41), in particular they contain more IgG2b producing plasma cells when compared to peripheral nodes (42), the site to which s.c. IT antigen will drain. It can be envisaged that due to these micoenvironmental differences mucosal tolerance responses may depend on FcγRIIB whereas peripheral tolerogenic responses do not. The fact that different administration routes can lead to different mechanisms of tolerance is best illustrated by comparing oral and intranasal tolerance (43, 44). TGF-β producing Th3 cells were shown to play a role in oral tolerance, whereas IL-10 producing Tr1 cells appear to be involved intranasal tolerance. Although we previously showed a crucial role for IL-10 in the beneficial effects of s.c. OVA IT (17), we have not yet been able to identify whether the IL-10 is produced by T-cells. Furthermore, another factor that may explain the different roles for FcγRIIB in mucosal tolerance and s.c. OVA IT is the time of intervention. In our model OVA IT was carried out after

parenteral sensitization of mice, a time at which OVA-specific IgGs were present, whereas in the mucosal tolerance studies mucosal OVA application was performed prior to parenteral sensitization, a time before OVA-specific IgG induction may have occurred .

The contribution of allergen-specific IgA in the protection against allergic disease by IT has been controversial. In human asthmatics, a significant increase in serum allergen-specific IgA was noted after 70 days of IT using house dust mite (5), while Lack *et al*. (45) found no consistent change in serum allergen-specific IgA after IT. The present study demonstrates that in wild-type mice, allergen IT and inhalation challenge induced significantly increased serum OVA-specific IgA levels compared to sham-treated OVA-challenged mice. Binding of IgA to FcαRI on leukocytes may have potent anti-inflammatory effects mediated by intracellular recruitment of the tyrosine phosphatase SHP-1 (46). Moreover, it has been previously reported that airway delivery of allergen-specific IgA mAb attenuates pulmonary hypersensitivity responses in Th2-sensitized mice (47). Therefore, the increment of IgA in response to OVA IT in wild-type mice may contribute to its effectiveness in protecting Th2-sensitized mice from airway allergen challenges. However, our findings show that the beneficial effects of IT in mice deficient in FcγRI/FcγRIII or FcγRIIB is not related to serum OVA-specific IgA levels, indicating that another mechanism is involved.

In summary, our findings are the first to clearly demonstrate that FcγR are not implicated in the efficacy of allergen IT in a mouse model of allergic asthma. After IT and inhalation challenge, serum IgA levels were elevated in wild-type mice but not in FcγR$^{-/-}$ mice which may indicate that FcγR are essential for the production of antigen-specific IgA. Furthermore, serum OVA-specific IgA levels do not appear to correlate with the beneficial effect of IT e.g. suppression of IgE upregulation and allergic inflammation. Further experiments are needed to elucidate the mechanism(s) involved in tolerance induction by allergen IT.

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Chapter 3

Indoleamine 2,3-dioxygenase-dependent tryptophan metabolites mediate tolerance induction during allergen immunotherapy in a mouse asthma model

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Abstract

The tryptophan-catabolizing enzyme, indoleamine 2,3-dioxygenase, has been shown to be involved in immune suppression and tolerance induction. Herein, we examined (i) whether indoleamine 2,3-dioxygenase activity is required during tolerance induction by allergen immunotherapy or for the suppressive effects on asthma manifestations and (ii) whether tryptophan depletion or generation of its downstream metabolites is involved. Ovalbumin-sensitized and -challenged BALB/c mice, that display increased airway responsiveness to methacholine, serum ovalbumin-specific IgE levels, bronchoalveolar eosinophilia, and Th2 cytokine levels were used as a model of allergic asthma. Sensitized mice received subcutaneous immunotherapy with optimal (1 mg) or suboptimal (100 μg) doses of ovalbumin 10 days prior to inhalation challenge. Inhibition of indoleamine 2,3-dioxygenase by 1-methyltryptophan during immunotherapy, but not during inhalation challenge, reversed the suppressive effects of immunotherapy on airway hyperresponsiveness, bronchoalveolar eosinophilia and Th2 cytokine levels, while serum ovalbumin-specific IgE levels remained suppressed. In subsequent experiments, administration of tryptophan during immunotherapy failed to abrogate its beneficial effects. Interestingly, administration of the tryptophan metabolites, kynurenine, 3-hydroxykynurenine, or xanthurenic acid, but not 3-hydroxyanthranilinic acid, quinolinic acid and kynurenic acid, during suboptimal immunotherapy potentiated the reduction of airway hyperresponsiveness and eosinophilia. These effects coincided with reduced Th2 cytokine levels in bronchoalveolar lavage fluid, but no effects on IgE levels were detected. The tryptophan metabolites, kynurenine, 3-hydroxykynurenine, and xanthurenic acid generated *via* indoleamine 2,3-dioxygenase play an important role in the induction of tolerance by immunotherapy in this mouse asthma model.

Introduction

Allergen immunotherapy (IT) conducted by s.c. administration of allergen extract is used for treating allergic diseases (1). The therapy is allergen-specific and is effective in allergic rhinitis and insect venom allergy. Its efficacy in allergic asthma, however, remains controversial (2). More insight into the underlying immunological mechanisms of allergen IT is needed to improve efficacy, in particular in asthmatic patients. The beneficial effects of allergen IT are presumed to be mediated through reduction of allergen-induced inflammation. A variety of immunological processes underlying these effects have been reported. Induction of blocking IgG antibodies particularly of the IgG4 isotype (3), downregulation of Th2 lymphocytes and/or upregulation of Th1 lymphocytes (4), and induction of $CD8^+$ T-cells (5) were claimed to be responsible for successful allergen IT. Recent data suggest an important role for IL-10-producing type 1 regulatory T (Tr1) cells and TGF-β-producing Th3 type cells in IT against bee venom, house dust mite, grass pollen, and other airborne allergens (6-8).

Exposure to antigen leads to its uptake, processing and presentation by dendritic cells (DCs) that initiate and regulate T-cell responses (9). Besides skewing T-cells towards Th1 or Th2, DCs have been shown to mediate the induction of adaptive regulatory T (aTreg) cells, like Th3 cells and Tr1 cells (9, 10). DCs induce development of aTreg cells by several mechanisms, including production of IL-10 or TGF-β (11, 12) and expression of indoleamine 2,3-dioxygenase (IDO) (13, 14). IDO is the rate-limiting enzyme that converts tryptophan into kynurenine and other down-stream metabolites (15). The main metabolites resulting from IDO-induced degradation of tryptophan, along the so-called 'kynurenine pathway', are generally known as kynurenines and comprise kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, anthranilic acid and quinolinic acid (Fig. 1). The major enzymes and substrates of the kynurenine metabolic pathway are reviewed in details elsewhere. Several studies have demonstrated that IDO is expressed in DCs, inhibits T-cell proliferation, and promotes tolerance (16, 17), including maternal tolerance toward an allogeneic fetus (18). Moreover, suppression of T-cell responses to MHC-mismatched allografts (19), control of T-cells in autoimmune disorders (20), and suppression of immune response to tumors (21) have been attributed to IDO activation. IDO may mediate inhibition of T-cell proliferation by starvation due to tryptophan depletion and by the antiproliferative and pro-apoptotic effects of its downstream kynurenine metabolites (22, 23).

In the present study, the role of IDO in tolerance induction by experimental allergen IT was examined using a mouse model of allergic asthma (24). In this model, we demonstrated earlier that allergen IT by s.c. administration of ovalbumin (OVA) between sensitization and challenge inhibits development of airway hyperresponsiveness (AHR) and eosinophilia (24). Furthermore, we recently demonstrated that the beneficial effects of allergen IT were mediated by IL-10 since blocking of the IL-10 receptor completely negate the suppression of asthma manifestations (25). Our present results clearly demonstrate that tryptophan metabolites generated by IDO during IT are crucial in the suppression of allergen-induced allergic airway eosinophilia and AHR in this mouse model of allergen IT.

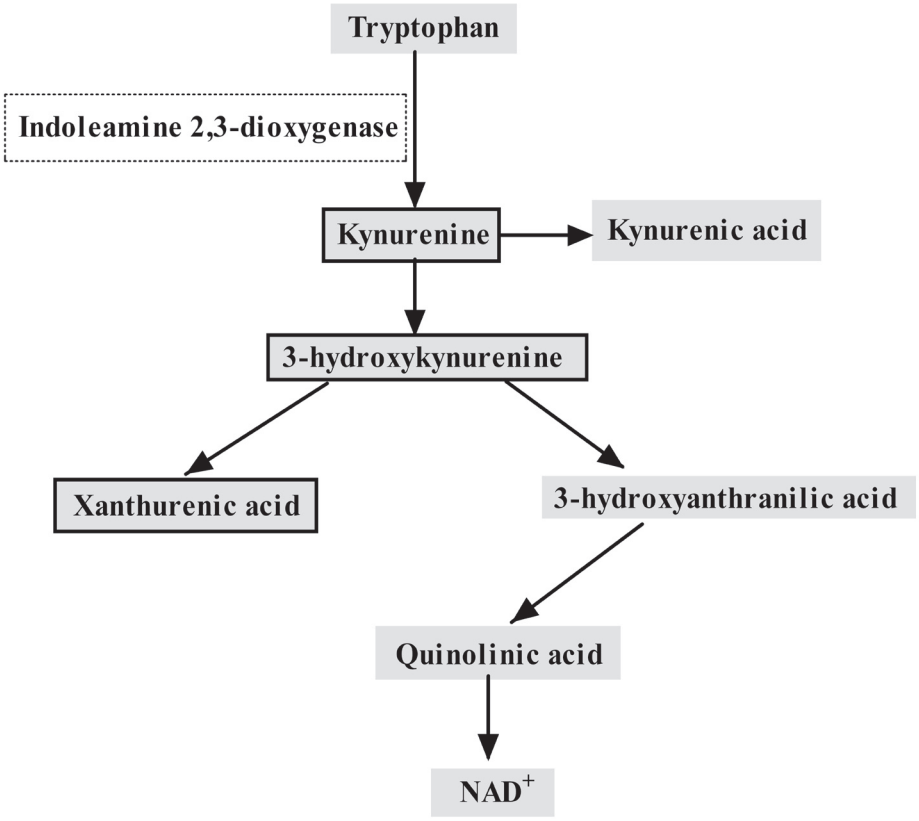


Figure 1. *Tryptophan degradation along the kynurenine pathway with the rate-limiting enzyme IDO. Alternative pathways and downstream enzyme activities are not depicted. The active metabolites kynurenine, 3-hydroxykynurenine and xanthurenic acid involved in the efficacy of IT were marked.*

Material and methods

Animals

Specified pathogen-free (according to the Federation of European Laboratory Animal Science Associations) (26) male BALB/c mice (6-8 weeks) were obtained from Charles River (Maastricht, The Netherlands). The mice were housed in macrolon cages in a laminar flow cabinet and provided with OVA-free food and water *ad libitum.* Animal care and use were conducted in accordance with the Animal Ethics Committee of Utrecht University, The Netherlands. No obvious signs of discomfort were noted in any mice regardless of treatment.

Sensitization, challenge and IT protocol

The protocol used for sensitization, IT and inhalation challenge was the same as

previously described (fig. 2A) (25). Mice received two intraperitoneal (i.p.) injections of 10 μg OVA (chicken egg albumin, crude grade V, Sigma-Aldrich) adsorbed onto 2.25 mg alum (ImjectAlum, Pierce, Rockford, IL, USA) in 100 μl pyrogen-free saline (B. Braun, Melsungen, Germany) on days 0 and 7. Two weeks after the second sensitization, mice were treated with 3 subcutaneous (s.c.) injections of 100 μg or 1 mg OVA in 200 μl pyrogen-free saline on alternate days. The control group was sham-treated with 200 μl saline. One week after OVA or sham treatment, airways of the mice were challenged with OVA aerosols in pyrogen-free saline (1% w/v) for 20 min 3 times every third day in a Plexiglas exposure chamber (5 liter) coupled to a Pari LC Star nebulizer (PARI Respiratory Equipment, Richmond, VA, USA; particle size 2.5–3.1 μm) driven by compressed air at a flow rate of 6 l/min. Aerosol was given in groups composed of no more than twelve mice.

Intervention studies

To examine the role of IDO in IT (fig. 2B), the IDO inhibitor, 1-methyl-DL-tryptophan (1MT, Sigma-Aldrich) was used. It was dissolved in a small volume of 1 N NaOH and further diluted with phosphate-buffered saline (PBS). The pH was adjusted to 7.1 with 1 N HCl before injection. In experiment B1, mice of the intervention groups were daily (day 21-26) injected i.p. with 1MT (10 mg/mouse/day in 1 ml PBS; dose was based on preliminary results and literature data (20)) starting 1 h before the first s.c. injection of IT. Controls received 1 ml PBS i.p. In experiment B2, intervention and control groups were treated with 1MT or PBS, respectively as in study B1, but during the OVA challenge period (day 35-41) starting 1 h before the first OVA aerosol challenge. In both studies (B1 and B2), airway responsiveness to methacholine, OVA-specific IgE levels in serum, cellular infiltration and Th2-cytokine levels in the bronchoalveolar lavage (BAL) fluid were measured 24 h after OVA aerosol challenge.

Since the studies above showed that IDO inhibition during IT interfered with its beneficial effects, we next determined whether depletion of tryptophan (TRP) or particular TRP metabolites mediated the effects of IT. Therefore, in study B3, mice were treated i.p. with either TRP (100 mg/kg) (27), kynurenine (KYN, 900 mg/kg) (28) or saline during the entire period of IT or sham-IT, starting 1 hour before (sham-)IT.

The next series of experiments was aimed to analyze which TRP metabolite was involved in IT since KYN is further metabolized to kynurenines (29). To this end, effects of the following IDO-dependent TRP metabolites, kynurenic acid (KA; 300 mg/kg, (30)), 3-hydroxykynurenine (3-OH-KYN; 50 mg/kg, (31)), xanthurenic acid (XA; 300 mg/kg, (30)), 3-hydroxyanthranilic acid (3-OH-AA; 50 mg/kg, (31)) and quinolinic acid (QUINA; 300 mg/kg, (30)) (Fig. 1, reviewed in details elsewhere (29)) were tested (studies B4 and B5). Compounds (all from Sigma-Aldrich) were dissolved in saline and daily injected i.p. during IT, starting 1 h before IT. Control mice received saline under the same conditions.

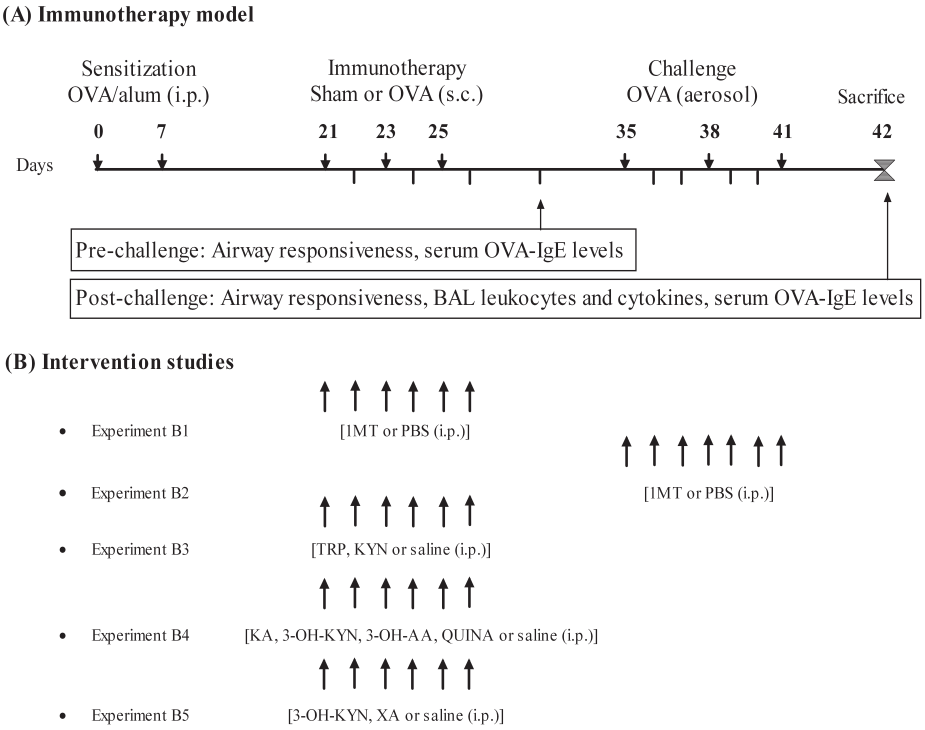


Figure 2. *Outline of the IT protocol in a murine model of allergic asthma and the IT intervention studies. A) OVA-sensitized mice received IT with an optimal (1 mg) or suboptimal (100 μg) dose of OVA s.c. on days 21, 23 and 25, and were challenged with OVA aerosols on days 35, 38 and 41. Airway responsiveness and serum levels of OVA-specific IgE were measured just before as well as 1 day after OVA aerosol challenge and leukocyte numbers and cytokine levels in BAL fluid after sacrifice immediately after challenge. B) daily intervention with the IDO inhibitor 1-methyltryptophan (1MT) or PBS during IT (days 21-26; B1) or during challenge (days 35-41; B2) or daily intervention with tryptophan (TRP), its metabolites kynurenine (KYN), kynurenic acid (KA), 3-hydroxykynurenine (3-OH-KYN), 3-hydroxyanthranilic acid (3-OH-AA), qunolinic acid (QUINA), xanthurenic acid (XA) or saline (days 21-26; B3-B5). For details, see Materials and Methods.*

Evaluation of airway responsiveness

Airway responsiveness to inhaled methacholine (acetyl-β-methylcholine chloride, Sigma-Aldrich) was measured twice (6 days before the first OVA aerosol challenge and at 24 h after the last OVA aerosol challenge) in conscious, unrestrained mice using barometric whole-body plethysmography by recording respiratory pressure curves (Buxco, EMKA Technologies, Paris, France) as described in details elsewhere (32). Briefly, mice were placed in a whole-body chamber and baseline values were measured and averaged for 3 min. Hereafter, mice were exposed for 3 min to a saline aerosol and aerosols with increasing concentrations of methacholine (solution doubling in concentrations, ranging from 3.13 to 50 mg/ml in saline). Aerosols

were generated by a Pari LC Star nebulizer and each aerosol was followed by 3 min of recording to assess average values. As a parameter of airway responsiveness, increases in enhanced pause (Penh), an index of airway obstruction as described in details previously (33), were determined. Airway responsiveness was expressed as the Penh per dose methacholine. Herein, we would like to stress that Penh values may not always correlate with changes in pulmonary resistance (34).

Determination of serum levels of OVA-specific IgE

Blood for assessment of serum IgE levels was obtained by incision of the tail vein (250 μl) 7 days before the first OVA challenge and directly after assessment of airway responsiveness upon i.p. injection of 1 ml 10% urethane by heart puncture. Sera were collected after clotting at room temperature for 30 min and subsequent centrifugation, and kept at –70°C until determination of OVA-specific IgE levels by ELISA as described in details previously (35). In short, 96-well microtiter plates from Nunc A/S (Roskild, Denmark) were coated overnight at 4°C with 1 μg/ml of anti-mouse IgE diluted in PBS, followed by blocking with ELISA buffer (PBS containing 0.5% BSA, 2 mM EDTA, 136.9 mM NaCl, 50 mM Tris, 0.05% Tween-20 [Merck, Whitehouse Station, NJ, USA], pH 7.2) and left for 1 h at room temperature. After multiple washings with PBS containing 0.05% v/v Tween-20, diluted serum samples and OVA-specific IgE reference serum were added to the wells and incubated for 2 h. The OVA-specific IgE reference serum was obtained by immunization of mice with OVA as described above and arbitrarily assigned a value of 1,000 experimental units/ml (EU/ml) (33). Next, after multiple washings the wells were incubated for 1.5 h with 1 μg/ml of DIG-conjugated OVA, followed by another washing and incubation for 1 h with anti-DIG Fab coupled to horseradish peroxidase, according to manufacturer's instructions (Roche Diagnostics, Basel, Switzerland). For color development, 0.4 mg/ml of *o*-phenylenediamine and 4 mM H_2O_2 in PBS were used, and the reaction was stopped by adding 75 μl of 4 M H_2SO_4. O.D. was read at 490 nm, using a Benchmark microplate reader (Bio-Rad, Hercules, CA, USA). Results were analyzed using Microplate Manager PC software (Bio-Rad). IgE concentration was calculated with reference to the standard curve. The lower detection limit of the ELISA was 0.5 EU/ml IgE.

Analysis of the BAL fluid

BAL was performed immediately after bleeding the mice. Briefly, a midcervical skin incision was made to expose the trachea, which was cannulated with a 23-gauge blunt needle. The airways were lavaged 5 times with 1 ml aliquots of pyrogen-free saline warmed at 37°C. The first lavage was done with 1 ml saline containing BSA and protease inhibitor (Complete mini tablet [Roche Diagnostics GMbH, Penzberg, Germany] and 5% BSA). The supernatant of this first ml of recovered lavage fluid was used to measure cytokine levels. Subsequently, mice were lavaged 4 times with 1 ml aliquots of saline at 37°C. The lavage fluid was kept on ice until further processing. Recovered lavage fluid of the second through fifth ml was pooled and cells herein (including those from the first ml) were pelleted (387 x g, 4°C, 10 min)

and resuspended in 150 µl cold PBS. The total number of cells in the BAL fluid was determined using a Bürker-Türk counting chamber (Karl Hecht Assistant KG, Sondheim/Röhm, Germany). For differential cell counts, cytospin preparations were made (15 x g, 4°C, 5 min) using a cytocentrifuge (Shandon Life Science, Cheshire, UK), and cells were fixed and stained with Diff-Quick (Dade A. G., Düdingen, Switzerland). All cytospin preparations were evaluated using oil immersion microscopy (magnification: 1000x). Cells were identified and differentiated into mononuclear cells (monocytes, macrophages and lymphocytes), eosinophils and neutrophils by standard morphology and staining characteristics. Per cytospin 200 cells were counted and the absolute number of each cell type was calculated.

Cytokine levels in BAL fluid

The levels of IL-5, IL-10, and IL-13 in the BAL fluid were determined by sandwich ELISA in Nunc-Immuno plates coated with appropriate anticytokine capture monoclonal antibodies (mAbs) and second-step biotinylated mAbs according to the manufacturer's instructions (PharMingen, San Diego, CA, USA). Values were expressed as pg per ml deduced from standards run in parallel with recombinant cytokines. The detection limits of the ELISAs were 32 pg/ml for IL-5 and 15 pg/ml for IL-10 and IL-13.

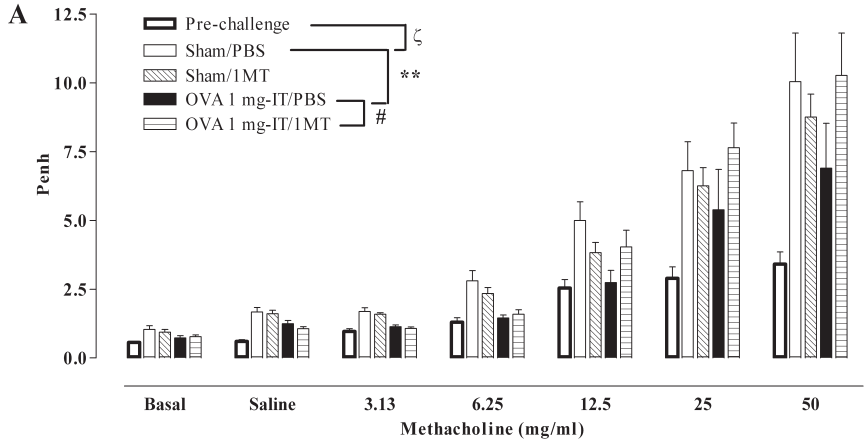
Statistical analysis

Data were expressed as mean ± SEM. A general linear model of repeated measurements followed by *post-hoc* comparison between groups statistically analyzed the airway dose-response curves to methacholine. Data were log transformed before analysis to equalize variance in all groups. Statistical analysis on BAL fluid cell counts was performed using the non-parametric Mann-Whitney *U* test. For all ELISA, results were analyzed using a Student's *t* test (2-tailed, homosedastic). A probability value $P<0.05$ was considered significant.

Results

Effects of IDO inhibition on the efficacy of IT

Airway responsiveness. In all treatment groups, OVA-sensitized mice demonstrated a similar dose-dependent increase in Penh values to increasing concentrations of methacholine before OVA challenge, irrespective of sham or optimal IT (fig. 3A and data not shown). One day after the last respiratory challenge with OVA, mice having received sham-IT showed significant AHR as judged by the increase in dose-response curve (DRC) to methacholine compared to that before challenge ($P<0.001$). Compared to sham-IT, optimal IT significantly suppressed development of OVA-induced AHR ($P<0.01$) (fig. 3A). Interestingly, treatment with 1MT during IT completely counteracted the beneficial effects of the IT, while 1MT treatment did not change AHR of mice having received sham-IT (fig. 3A). Treatment of mice with 1MT



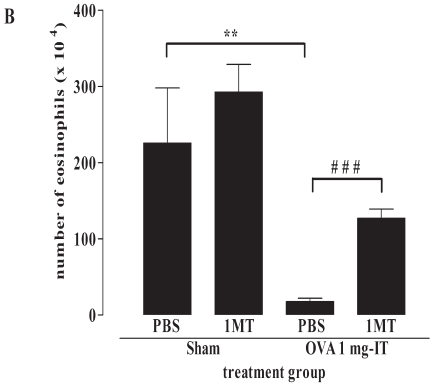
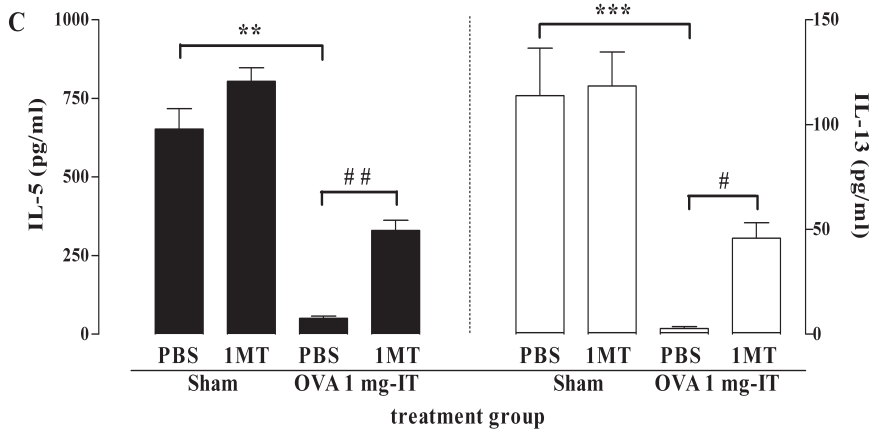


Figure 3. *Effects of IDO inhibition on the efficacy of IT. OVA-sensitized mice received IT with 1 mg OVA and daily treatment with the IDO inhibitor, 1MT or with PBS during IT. (A) airway responsiveness to methacholine just before (pre-challenge) and 1 day after OVA aerosol challenge, (B) eosinophil numbers and (C) IL-5 and IL-13 levels in BAL fluid. Values are expressed as mean ± SEM (n=7-8). Penh, enhanced pause. ζP<0.001 compared to pre-challenge. **P<0.01, ***P<0.001 compared to sham/PBS treated mice. #P<0.05, ##P<0.01, ###P<0.001 compared to OVA 1 mg-IT/PBS treated mice.*



during the challenge period (experiment B2) did not reverse the decreased airway responsiveness after IT (data not shown).

Eosinophils and cytokine levels in the BAL fluid. Respiratory OVA challenge of sham-treated, OVA-sensitized mice resulted in high numbers of inflammatory cells in the BAL fluid, consisting predominantly of eosinophils (fig. 3B) besides mononuclear cells and few neutrophils (data not shown). IT effectively suppressed the airway eosinophilia by 93% ($P<0.01$) compared to sham-treated mice (fig. 3B). Importantly, inhibition of IDO during IT (experiment B1) significantly antagonized the suppression in airway eosinophilia induced by IT (56% reversal, $P<0.001$) compared to mice treated only with IT (fig. 3B). Treatment of mice with 1MT during the challenge period (experiment B2) did not affect the IT-induced reduction in number of eosinophils (data not shown). Inhibition of IDO during sham-IT (fig. 3B) or during the subsequent challenge (study B2; data not shown) did not influence the number of eosinophils.

Assessment of Th2 cytokine levels in the BAL fluid of sham-treated mice revealed high levels of IL-5 and IL-13 24 h after challenge. IT significantly reduced the levels of IL-5 by 92% ($P<0.01$) and of IL-13 by 97% ($P<0.001$) compared to sham-treated mice (fig. 3C). Inhibition of IDO during IT considerably abrogated the suppression in IL-5 levels (51% reversal, $P<0.01$, fig. 3C) and IL-13 levels (40% reversal, $P<0.05$, fig. 3C). IT also reduced the levels of IL-10 by 73% but this reduction did not reach statistical significance ($P=0.12$) and this effect of IT was not influenced by inhibition of IDO (data not shown). The BAL fluid from sham-treated mice showed no changes in IL-5 and IL-13 levels after inhibition of IDO (fig. 3C). The Th1-cytokine IFN-γ was not detectable in the BAL fluid obtained from sham- or IT-treated mice (data not shown).

IDO inhibition during the challenge period did not affect the decrease in Th2-cytokines due to the IT (experiment B2, data not shown).

OVA-specific IgE levels in serum. Sham-treated mice displayed significantly increased (96%, $P<0.05$) OVA-specific IgE levels in serum after OVA challenge compared to pre-challenge levels (table I). IT significantly suppressed the increase in IgE by 79% ($P<0.05$) compared to sham-IT (table I). Neither inhibition of IDO during the time of IT (experiment B1; table I) nor during the time of OVA challenge (experiment B2; data not shown) influenced the reduction in OVA-specific IgE levels by IT. OVA-specific IgE levels in serum of sham-treated mice were not affected by inhibition of IDO in both experiments (B1, table I and B2, data not shown, respectively).

Table I. *Serum levels of OVA-specific IgE.*

OVA-specific IgE (x 10^3 EU/ml) Mean ± SEM		
Treatment group	Pre-challenge	Post-challenge
Sham/PBS	1.36 ± 0.37	32.66 ± 10.12 *
Sham/1MT	2.41 ± 0.55	49.17 ± 07.99 ***
OVA-IT/PBS	7.93 ± 2.41	6.73 ± 01.11 #
OVA-IT/1MT	4.80 ± 1.47	6.03 ± 00.43 ###

*OVA sensitized BALB/c mice were treated with 1MT or PBS during the entire period of OVA-IT (day 21–26). Levels of OVA-specific IgE in serum were measured before (pre-challenge) and 1 day after (post-challenge) OVA aerosol challenges. Values are expressed as mean ± SEM (n=7-8). *P<0.05, ***P<0.001 compared to pre-challenge OVA-specific IgE levels. #P<0.05, ###P<0.001 compared to post-challenge OVA-specific IgE levels of sham-treated mice.*

Effects of TRP and KYN on IT

To determine whether IDO affected the induction of tolerance by depleting TRP or by producing KYN or other downstream metabolites, mice were treated with TRP during optimal IT with 1 mg OVA or with KYN during suboptimal IT with 100 μg OVA (study B3).

Airway responsiveness. Treatment of OVA-sensitized and challenged mice with TRP did not influence the suppression of AHR due to IT with 1 mg OVA (fig. 4A). In contrast, administration of KYN during IT with a suboptimal dose of 100 μg OVA significantly suppressed the AHR to methacholine compared to sham-treated mice ($P<0.05$) and mice treated with suboptimal IT only ($P<0.01$) (fig. 4A). TRP and KYN had no significant effect on the development of AHR in sham-treated mice (fig. 4A).

Eosinophils and cytokine levels in the BAL fluid. Optimal IT with 1 mg OVA significantly reduced the airway eosinophilia by 88% ($P<0.01$), whereas suboptimal IT with 100 μg did not influence the number of eosinophils (fig. 4B). Administration of TRP did not affect the reduction in airway eosinophilia by optimal IT. However, administration of KYN during suboptimal IT with 100 μg OVA successfully suppressed the influx of eosinophils by 68% ($P<0.05$) compared to sham-treated mice and by 69% ($P<0.05$) compared to mice received suboptimal IT only (fig. 4B). Remarkably, in sham-treated mice TRP administration caused a significant further increase in eosinophil numbers in the BAL fluid by 57% ($P<0.05$) (fig. 4B), but numbers of neutrophils and mononuclear cells were not changed (data not shown). Administration of TRP during optimal IT did not influence the IT-induced reduction of IL-5 (90% decrease, $P<0.01$), IL-13 (95% decrease, $P<0.05$) and IL-10 (91% decrease, $P<0.001$) levels in the BAL fluid (figs. 4B and 4C). In contrast, administration of KYN during suboptimal IT significantly decreased the levels of IL-5 by 64% ($P<0.05$, fig. 4B), of IL-13 by 75% ($P<0.05$, fig. 4C) and of IL-10 by 53% ($P<0.05$, fig. 4C) compared with sham-treated mice and levels of IL-5 (67%, $P<0.01$), of IL-13 (72%, $P<0.05$) and of IL-10 (57%, $p=0.066$) compared with mice treated only with suboptimal IT (figs. 4B and C).

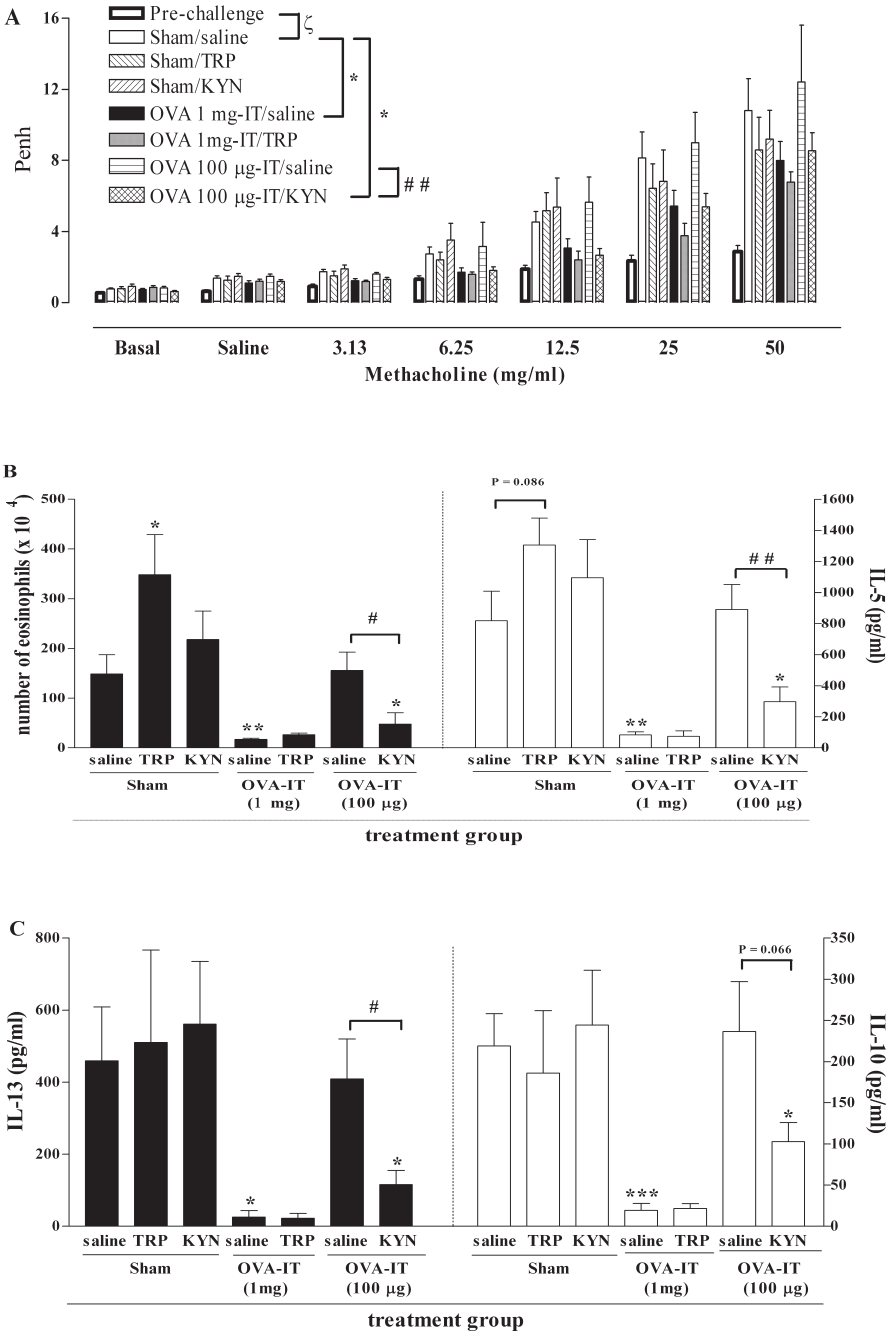


Figure 4. *Effects of TRP or its metabolite, KYN, on the efficacy of optimal or suboptimal IT. OVA-sensitized mice received IT with 1 mg or 100 µg OVA and daily treatment with the TRP, KYN or with saline during IT. (A) airway responsiveness to methacholine just before (pre-challenge) and 1 day after*

*OVA aerosol challenge, (B) eosinophil numbers and IL-5 levels and (C) IL-13 and IL-10 levels in BAL fluid. Values are expressed as mean ± SEM (n=6). Penh, enhanced pause. ζP<0.001 compared to pre-challenge. *P<0.05, **P<0.01, ***P<0.001 compared to sham/saline-treated mice. #P<0.05, ##P<0.01 compared to OVA 100 μg-IT/saline treated mice.*

OVA-specific IgE levels in serum. The down-regulation of OVA-specific IgE levels in serum induced by optimal IT was not changed by treatment with TRP (Table II). Also, KYN administration did not affect the OVA-specific IgE levels in serum from sensitized mice receiving suboptimal IT or sham-IT (Table II).

***Table II**. Serum levels of OVA-specific IgE.*

	OVA-specific IgE (x 10^3 EU/ml) Mean ± SEM	
Treatment group	Pre-challenge	Post-challenge
Sham/saline	2.56 ± 0.89	69.01 ± 14.85 **
Sham/TRP	4.86 ± 2.48	67.56 ± 18.90 *
Sham/KYN	2.24 ± 0.65	76.62 ± 18.63 **
OVA 1 mg-IT/saline	12.77 ± 2.72	12.41 ± 02.28 ##
OVA 1 mg-IT/TRP	14.79 ± 4.98	13.40 ± 06.28 #
OVA 100 μg-IT/saline	7.73 ± 0.82	69.92 ± 14.11 **
OVA 100 μg-IT/KYN	5.78 ± 1.45	64.93 ± 11.27 **

*OVA-sensitized BALB/c mice received IT with optimal (1 mg; s.c.) or suboptimal (100 μg) doses of OVA. TRP (100 mg/kg), KYN (900 mg/kg) or saline treatment was administered i.p. once a day at the time of OVA-IT. Levels of OVA-specific IgE in serum were measured before (pre-challenge) and 1 day after (post-challenge) OVA aerosol challenges. Values are expressed as mean ± SEM (n=6). *P<0.05, **P<0.01 compared to pre-challenge OVA-specific IgE levels. #P<0.05, ##P<0.01 compared to post-challenge OVA-specific IgE levels of sham-treated mice.*

Effects of KYN metabolites on the efficacy of IT

As *in vivo* KYN is quickly degraded to kynurenines, we aimed to determine whether KYN itself or one of its metabolites that are physiologically generated downstream of the initial and rate-limiting step mediated by IDO in TRP degradation (fig. 1) mediated the beneficial effects of IT. Mice were treated with suboptimal IT and saline or one of the metabolites KA, 3-OH-KYN, 3-OH-AA, QUINA (study B4).

Eosinophils and cytokine levels in BAL fluid. Suboptimal IT suppressed the airway eosinophilia, although not significantly (fig. 5A) and this was not changed by administration of 3-OH-AA, KA or QUINA during the IT. Interestingly, administration of 3-OH-KYN effectively suppressed the number of eosinophils in the BAL fluid by 62% (P<0.01) compared to sham-treated mice and by 45% (P<0.05) compared to mice receiving suboptimal IT only (fig. 5A). The suppression was paralleled by reduction in BAL fluid levels of IL-5 by 34% (P<0.05, fig. 5A), IL-13 by 51% (P<0.05, fig. 5B) and IL-10 by 41% (P=0.09, fig. 5B) compared to sham-treated mice and by 31% (P<0.05), 37% (P=0.12) and 30% (P=0.055) for IL-5, IL-13 and IL-10 respectively compared with

mice received suboptimal IT only (figs. 5A and B). Levels of these cytokines were not changed by administration of KA, 3-OH-AA or QUINA during suboptimal IT (figs. 5A, and B).

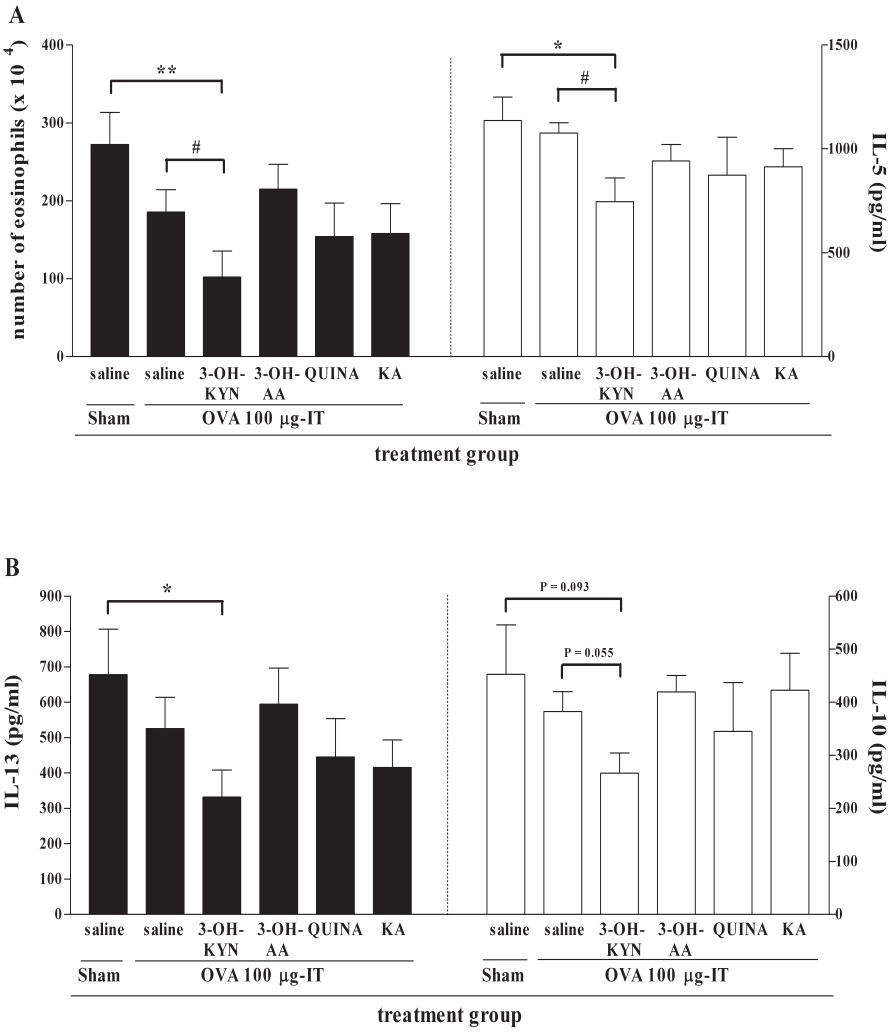


Figure 5. *Effects of kynurenines on the efficacy of suboptimal IT. OVA-sensitized mice received IT with 100 µg OVA and daily treatment with 3-OH-KYN, 3-OH-AA, QUINA, KA or with saline during IT. (A) eosinophil numbers and IL-5 levels and (B) IL-13 and IL-10 levels in BAL fluid 1 day after OVA aerosol challenge. Values are expressed as mean ± SEM (n=6). *P<0.05, **P<0.01 compared to sham/saline treated mice. #P<0.05 compared to OVA 100 µg-IT/saline treated mice.*

OVA-specific IgE levels in serum. Suboptimal IT alone or in combination with the KYN metabolites did not affect OVA-specific IgE levels in serum compared to those upon sham-IT (data not shown), as had been shown for KYN itself in table II.

Effects of 3-OH-KYN and XA on the efficacy of IT

Herein, we wanted to determine the effects of combination of 3-OH-KYN and the direct downstream metabolite of kynurenine aminotransferase, XA on the beneficial effects of IT responses since under physiological conditions TRP metabolites probably not act as single substance and to answer an interesting question whether the combination of active metabolites is more effective than a single substance. Mice were treated with 3-OH-KYN, XA or both during suboptimal IT (study B5).

Airway responsiveness. Suboptimal IT did not affect OVA challenge-induced AHR to methacholine, but administration of either 3-OH-KYN or XA or the combination during therapy significantly suppressed the development of AHR compared to sham-treated mice and to mice treated with suboptimal IT only (fig. 6A). Notably, the administration of both 3-OH-KYN and XA did not have additive effects.

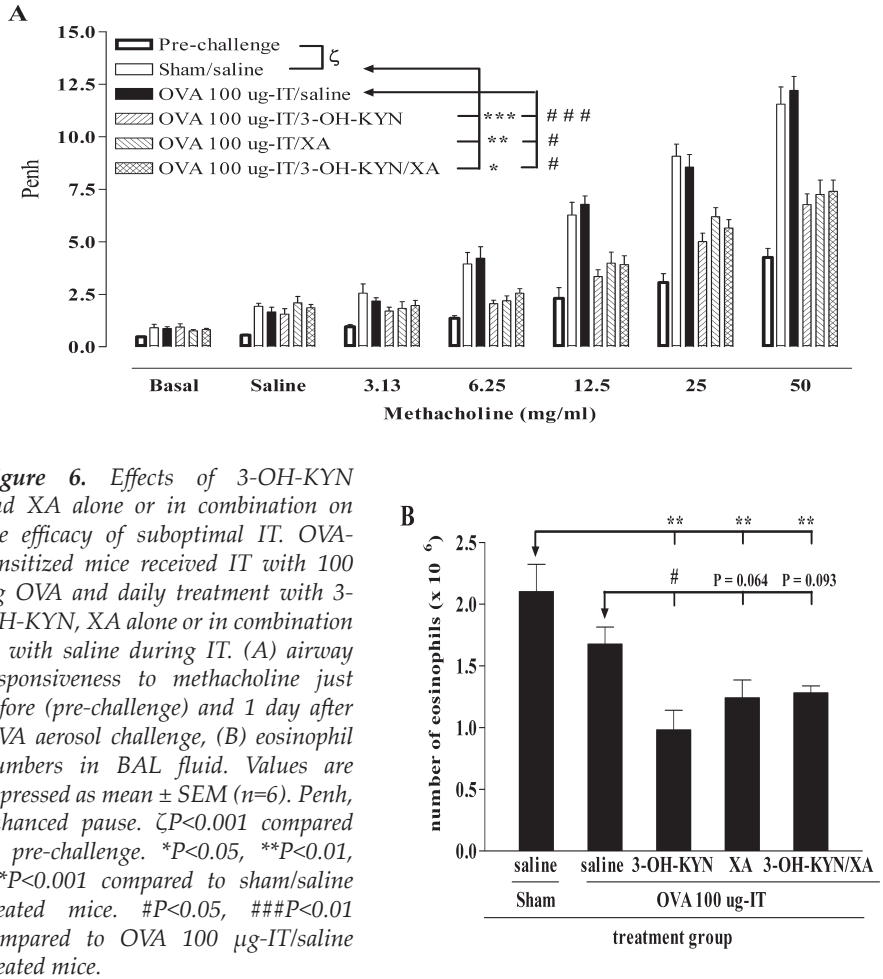


Figure 6. *Effects of 3-OH-KYN and XA alone or in combination on the efficacy of suboptimal IT. OVA-sensitized mice received IT with 100 μg OVA and daily treatment with 3-OH-KYN, XA alone or in combination or with saline during IT. (A) airway responsiveness to methacholine just before (pre-challenge) and 1 day after OVA aerosol challenge, (B) eosinophil numbers in BAL fluid. Values are expressed as mean ± SEM (n=6). Penh, enhanced pause. ζP<0.001 compared to pre-challenge. *P<0.05, **P<0.01, ***P<0.001 compared to sham/saline treated mice. #P<0.05, ###P<0.01 compared to OVA 100 μg-IT/saline treated mice.*

Eosinophils and cytokine levels in BAL fluid. Suboptimal IT did not significantly affect OVA challenge-induced eosinophilia. Administration of 3-OH-KYN, XA or the combination during the therapy significantly reduced airway eosinophilia compared to sham-treated mice (fig. 6B), but when compared to mice receiving suboptimal IT, only 3-OH-KYN significantly suppressed eosinophilia.

Suboptimal IT caused no significant changes of cytokine levels in BAL fluid, but when combined with 3-OH-KYN, XA or the combination levels of IL-5, IL-10 and IL-13 were significantly lower than in sham-treated mice. Compared to mice merely receiving suboptimal IT, levels of IL-5 and IL-13 were significantly lower upon co-treatment with 3-OH-KYN or XA, and IL-13 levels also upon co-treatment with the combination (Table III).

***Table III**. Th2 cytokine levels IL-5, IL-10 and IL-13 in BAL fluid as determined by means of ELISA 1 day after OVA aerosol challenges.*

Treatment group	BAL fluid cytokine levels (pg/ml) Mean ± SEM		
	IL-5	IL-10	IL-13
Sham + saline	1720 ± 170	420 ± 50	690 ± 150
OVA 100 μg-IT + saline	1580 ± 80 (8%) n.s.	370 ± 30 (12%) n.s.	520 ± 60 (25%) n.s.
OVA 100 μg-IT + 3-OH-KYN	950 ± 230 (44%) *, #	240 ± 60 (44%) *	300 ± 40 (57%) *, ##
OVA 100 μg-IT + XA	960 ± 250 (44%) *, #	270 ± 40 (35%) *	330 ± 20 (52%) *, #
OVA 100 μg-IT + 3-OH-KYN + XA	1180 ± 170 (31%) *	300 ± 30 (29%) *	300 ± 60 (57%) *, #

*Mice were sensitized, treated and challenged as described in experiment B5 (Materials and Methods). Values are expressed as mean ± SEM in pg per ml. *P<0.05 compared to sham/saline-treated mice. #P<0.05, ##P<0.01 compared to OVA-IT/saline-treated mice. Percent reduction in cytokine levels in BAL fluid relative to sham/saline treated mice is shown between brackets. n.s.=non significant. (n=6).*

Discussion

The present study demonstrates that IDO plays an essential role in the efficacy of allergen IT. Our observation that inhibition of IDO with 1MT interfered with IT when it was given during IT, but not during challenge, demonstrates that tolerance induction is in part mediated by IDO activation during IT and that activity of IDO is irrelevant to maintenance of tolerance thereafter. This and the observation that TRP, when given during IT, appeared not to inhibit the efficacy of IT, indicate that formation of TRP metabolites, rather than TRP depletion is a mechanism by which IT induces tolerance to the induction of airway manifestations of asthma. This conclusion is further supported by our findings that the TRP metabolites, KYN, 3-

OH-KYN, and XA potentiated the efficacy of suboptimal IT. Although it can not be excluded that the effects of 1MT are related to the recently described interference with toll-like receptor signaling in DCs (36), this appears rather unlikely considering the effects TRP metabolites on IT.

Till now, IDO was shown to be involved in maternal tolerance during pregnancy (18), control of allograft rejection (19), and protection against autoimmunity (37), and experimental colitis (38). This is to our best knowledge the first study showing that IDO plays at least a partial role in tolerance induction by allergen IT in a mouse model of allergic asthma, but not as to all parameters measured. IDO-dependent TRP metabolites appeared involved in tolerance induction by IT as to AHR, eosinophilia and Th2 cytokines. The IT-induced reduction in allergen-specific IgE, however, appeared not mediated by an IDO-dependent mechanism, since the efficacy of optimal IT to reduce IgE levels was not affected by 1MT, and since TRP metabolites did not potentiate reduction of IgE levels by suboptimal IT. These data demonstrate that IT differentially regulates the pathways leading to allergen-induced asthma manifestation and those increasing serum IgE levels. In addition, these data support earlier observations that production of allergen-specific IgE and development of AHR and airway eosinophilia can be dissociated in mouse asthma models (39). Moreover, our data are in agreement with the observation that B cells, unlike T cells, are insensitive to the cytotoxic action of TRP metabolites (22) and with studies showing that tolerizing B cells is T cell-independent (40). The mechanisms by which IDO-dependent TRP metabolites mediate the IT-induced suppression of AHR and eosinophilia are not known at present. Since IFN-γ levels remained below the detection limit after IT, a shift from Th2 to Th1 responses is probably not at play. Therefore, it is not unlikely that one or more subsets of regulatory T cells are implicated since a role for IDO in the generation of aTreg cells has been suggested (14). Particularly Tr1 cells may be involved, since efficacy of IT in our model was earlier shown to involve IL-10 (25) and since Tr1 cells are potent producers of this immunoregulatory cytokine (41). Moreover, in human studies, it was clearly demonstrated that allergen IT against bee venom, house dust mite, and grass pollen is associated with increased numbers of IL-10 and/or TGF-β producing regulatory T cells (6-8).

The fact that not all kynurenines are active and no additive or synergistic effects between 3-OH-KYN and XA were found when given together during suboptimal IT may suggest that one of these two substances is responsible for the induction of immune-tolerance mediated by IDO. Interestingly, 3-OH-KYN was recently found to inhibit proliferation, to increase IL-10 production by murine splenocytes stimulated with a Th1- response-inducing peptide antigen, and to stimulate IL-10 production *in vivo* (42). It can be questioned if the suppressive action of 3-OH-KYN in our *in vivo* model is mediated *via* this mechanism, since 3-OH-AA that was inactive in our model, acted similarly on the antigen-stimulated splenocytes (42). However, this may merely be a matter of different pharmacokinetic profiles *in vivo*. Therefore, although 3-OH-AA, QUINA and KA were not active in potentiating the effect of IT we can not completely exclude that they do play a role in IT.

Even though in the current study, identity of cells expressing IDO is not yet known, several mouse and human studies showed that macrophages and DCs are the cells with most prominent IDO-activity (16, 22, 43). Since human macrophages

can generate 3-OH-AA, but not 3-OH-KYN, on stimulation with IFN-γ (44) and since 3-OH-KYN, but not 3-OH-AA, was active in our study, IT-induced IDO-expression by DCs rather than macrophages may be involved in our model of IT. Further studies are needed to address the antigen presenting cell type(s) that express IDO and generate kynurenines during IT.

Our data are not completely in line with the hypothesis that the combined effects of TRP depletion and KYN production are required for the generation of IL-10 and TGF-β producing regulatory T-cells (13, 14). Although we observed potentiation of immune-tolerance using the specific kynurenines 3-OH-KYN and XA, TRP administration did not reverse this potentiation and even induced stronger suppression of AHR. One likely explanation for this discrepancy may be that Belladonna *et al.* (13) and Fallarino *et al.* (14) used *in vitro* T-cell activation whereas we used an *in vivo* model.

In the present study, we observed that inhibition of IDO during the effector phase did neither antagonize the beneficial effects of allergen IT nor affect the development of AHR and eosinophilia after allergen inhalation. In agreement with the latter, both Hessel *et al.* (45) and Hayashi *et al.* (46) did not observe an effect of IDO inhibition by 1MT during allergen inhalation challenge in previously sensitized sham-treated mice. However, Hayashi *et al.* (46) did observe a role for IDO during allergen inhalation challenge in mice treated systemically with immunostimulatory oligodeoxynucleotide sequences (ISS-ODN). This indicates that the mechanism(s) of suppression after allergen-IT is different from that after ISS-ODN treatment.

In summary, we clearly demonstrated that IDO activity is essential for induction of tolerance towards airway manifestations of asthma during allergen IT, and that generation of TRP metabolites rather than TRP depletion is involved in promoting this type of tolerance. These findings provide further understanding of the complex mechanisms that may contribute to IT intervention and may be helpful to enhance the prospects for successful IT in allergic asthma.

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Chapter 4

Role for natural regulatory T cells in specific allergen immunotherapy in a mouse model of asthma

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Abstract

Regulatory $CD4^+CD25^+Foxp3^+$ T (nTreg) cells have recently been shown to be involved in tolerance induction and the generation of adaptive regulatory T-cells. Glucocorticoid-induced tumour necrosis factor receptor (GITR) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) are expressed on nTreg cells and regulate their immunosuppressive activities. Herein, we examined whether induction of immune tolerance by allergen immunotherapy (IT) requires nTreg cell activity, by blocking the suppressive activities of these nTreg cells *in vivo*. We used a mouse model of allergic asthma in which ovalbumin (OVA)-sensitized BALB/c mice receive IT consisting of three OVA injections (1 mg, s.c.) 10 days prior to OVA inhalation challenge. The functional activities of nTreg cells were inhibited *in vivo* by i.v. administration of either anti-GITR mAb or anti-CTLA-4 mAb one hour before the first IT injection. After allergen IT, mice displayed a significant ($P<0.05$) reduction in airway hyper-responsiveness (AHR) to methacholine, airway eosinophilia in bronchoalveolar lavage (BAL) fluid (83%, $P<0.01$) and OVA-specific IgE levels in serum (93%, $P<0.01$) compared to sham treatment. Suppression coincided with significant falls of Th2 cytokine levels in BAL fluid. Interestingly, functional inactivation of nTreg cells by anti-GITR mAb or anti-CTLA-4 mAb treatment counteracted the suppressive effects of OVA-IT on AHR (100% reversal, $P<0.05$), OVA-specific IgE levels in serum (31% reversal, $P<0.05$; 35% reversal, $P<0.001$, respectively), airway eosinophilia (17% reversal, $P<0.01$; 31% reversal, $P<0.01$, respectively) and Th2 cytokine levels in BAL fluid. Our data strongly indicate that nTreg cells partially contribute to tolerance induction by allergen IT in a murine model of allergic asthma.

Introduction

Allergen-specific immunotherapy (IT) is effective in modulating allergic disorders, in particular allergic rhinitis (1, 2) and insect venom allergy (3, 4). However, its efficacy in allergic asthma remains controversial (5, 6). Elucidation of the immunologic mechanisms by which this intervention achieves beneficial effectiveness may be helpful to improve this treatment in a way that is effective and acceptable not only for allergic rhinitis, but also for asthmatic patients.

It has been shown that successful IT is associated with induction of blocking IgG antibodies (7), induction of T-cell anergy (8), and deviation of Th2 cytokine responses to allergens in favour of Th1 responses (4, 9). Recently, human studies provide evidence for a role of IL-10 producing type 1 regulatory T (Tr1) cells and TGF-β producing Th3 type cells in the beneficial effects of allergen IT in bee-venom and airborne allergen atopic patients (10-12).

Studies so far have shown that regulatory T (Treg) cells effectively participate in suppression of Th1- and Th2-mediated immune disorders, but the role of Treg cells in allergic diseases have primarily focused on adaptive Treg cells (Tr1 and Th3). Natural occurring $CD4^+CD25^+Foxp3^+$ regulatory T (nTreg) cells are a thymus-derived population of regulatory $CD4^+$ T-cells that suppress conventional T-lymphocyte activation *in vitro* (13), and promote tolerance *in vivo* in different models of autoimmune diseases (14, 15) and allograft rejection (16). Unlike adaptive Treg cells, nTreg cells exert their immunosuppressive effects *in vitro* through cell-to-cell contact (13, 17). However, *in vivo* nTreg cells can also function through the inhibitory cytokines TGF-β (18) and IL-10 (19), although in a separate study these cytokines were shown not to be required for the function of nTreg cells (20). GITR (TNFRSF18) and CTLA-4 (CD152) were originally identified as markers for nTreg cells and are involved in their immunosuppressive function (21, 22). GITR, a member of the TNF receptor family, is expressed on nTreg cells in the thymus and periphery (22). High expression levels of GITR on nTreg cells compared to other activated T-cells suggest an important role for GITR in their function (22, 23). Stimulation of GITR by activating antibodies abrogates suppressor activity of nTreg cells *in vitro* and breaks immunological self-tolerance *in vivo* (22, 23). CTLA-4, a homolog of CD28 that binds with high affinity to B7 molecules, is constitutively expressed on nTreg cells and functions as a negative regulator of immune responses (21, 24). It has been shown that CTLA-4 blockade dose dependently abrogates nTreg cell-mediated peripheral tolerance (24) and allograft survival (16) and exacerbates autoimmune diseases *in vivo* (25, 26). Additionally, CTLA-4 ligation of B7 on dendritic cells (DCs) was found to result in the induction of the enzyme indoleamine 2,3-dioxygenase (IDO) in DCs (27, 28). IDO catalyzes the initial and rate-limiting step of tryptophan degradation into metabolic products collectively known as kynurenines (29). Many studies have demonstrated that IDO contributes to tolerance induction (28, 30) and the generation of adaptive Treg cells (31).

In the present study, we investigated whether nTreg cells play a role in tolerance induction by allergen IT by blocking the suppressive activities of these cells *in vivo*. To this end, we utilized a murine model of allergen IT to address the role of GITR activation and CTLA-4 blockade at the time of allergen IT injections. In this model, we have demonstrated previously that allergen IT inhibits development of AHR

and eosinophilic airway inflammation (32). Furthermore, we have recently shown that allergen IT induces long-lasting immune tolerance and its beneficial effects were mediated by IL-10 since blocking of the IL-10 receptor completely reversed the suppression of asthma manifestations (33). Our present findings show that functional inactivation of nTreg cells through GITR activation or CTLA-4 blockade counteracts the suppressive effects of IT. These data indicate that nTreg cells partially contribute to the induction of tolerance by allergen IT in this mouse model of IT.

Materials and methods

Animals

Animal care and use were conducted in accordance with the Animal Ethics Committee of Utrecht University, The Netherlands. Specific pathogen-free (according to the Federation of European Laboratory Animal Science Associations) (34) male BALB/c mice (6-8 weeks) were purchased from Charles River (Maastricht, The Netherlands). The mice were housed in macrolon cages in a laminar flow cabinet and provided with food and water *ad libitum*.

Sensitization, challenge and IT protocol

The protocol used for sensitization, IT and inhalation challenge was the same as previously described (33). In short, mice were sensitized to OVA (chicken egg albumin, crude grade V, Sigma-Aldrich) by 2 i.p. injections (days 0 and 7) of 0.1 ml alum-precipitated antigen composed of 10 μg OVA absorbed onto 2.25 mg alum (ImjectAlum, Pierce, Rockford, IL, USA). Two weeks after the second sensitization, mice were treated with three s.c. injections of 1 mg OVA in 200 μl pyrogen-free saline (B. Braun, Melsungen, Germany) on alternate days. The other group was sham-treated with 200 μl saline. One week after OVA-IT or sham treatment, mice were challenged with OVA aerosols in pyrogen-free saline (10 mg/ml) for 20 min 3 times every third day in a Plexiglas exposure chamber (5 liter) coupled to a Pari LC Star nebulizer (PARI Respiratory Equipment, Richmond, VA, USA; particle size 2.5–3.1 μm) driven by compressed air at a flow rate of 6 l/min. Aerosol was given in groups composed of no more than twelve mice.

Experimental treatment protocol

To examine the role of GITR and CTLA-4 pathway in the efficacy of allergen IT (table I), mice received either 600 μg rat mAb to mouse GITR (DTA-1; kindly provided by S. Sakaguchi, Department of Experimental Pathology, Institute for Frontier Medical Sciences, Kyoto University, Japan) or 250 μg hamster anti-murine CTLA-4 mAb 4F10 as a single i.v. administration one hour before the first s.c. injection of OVA-IT (day 21). Doses used were based on efficacy in other studies (35-37). Control mice received an equivalent dose of control antibodies Armenian hamster IgG (Ha4/8; kindly provided by Biogen (Cambridge, MA, USA)) and rat IgG (rIgG) under the same conditions.

Table I. *Experimental design to determine the effects of anti-GITR and anti-CTLA-4 on the efficacy of allergen IT in a mouse model of allergic asthma.*

Group	Sensitization (i.p.) (days 0 and 7)	Treatment (i.v.) (day 21)	OVA-IT (s.c.) (days 21, 23, 25)	Challenge (aerosol) (days 35, 38, 41)
1	OVA/alum	Ha4/8 + rIgG	Sham (saline)	OVA
2	OVA/alum	Ha4/8 + rIgG	1 mg	OVA
3	OVA/alum	anti-GITR	1 mg	OVA
4	OVA/alum	anti-CTLA-4	1 mg	OVA

Ha4/8 + rIgG are control antibody treatments. Mice were sensitized, challenged and treated as described in Materials and Methods. Each experimental group consists of six animals.

Ha4/8 and DTA-1 antibodies were generated as described previously (22) and purified using thiophilic agarose (Kem-En-Tec cat. no. 1340F, BIOzym, The Netherlands). rIgG was purchased from ICN Pharmaceuticals (Cosa Mesa, CA, USA). The hybridoma cell line which produces hamster anti-CTLA-4 mAb 4F10 was obtained from American Type Culture Collection and purified using a protein G column (Pharmacia, Peapack, NJ, USA). These antibodies were treated with 10% v/v polymyxin B-agarose (Sigma; washed twice with saline) for 1 h at 4°C to remove lipopolysaccharide. After incubation, agarose beads were removed by centrifugation (1700 x g, 4°C, 15 min), and the supernatants were sterilized by using a 0.22-μm filter (Omnilabo, Breda, The Netherlands). The endotoxin level in all treatment preparations was less than 1 endotoxin U/mg.

Airway responsiveness to methacholine, levels of OVA-specific immunoglobulins in serum, cellular infiltration and Th2-cytokine levels in the BAL fluid were measured 24 h after the last OVA inhalation challenge in all mice.

Evaluation of airway responsiveness

Airway responsiveness to inhaled methacholine (acetyl-β-methylcholine chloride, Sigma-Aldrich) was measured twice (6 days before the first OVA aerosol challenge and at 24 h after the last challenge) in conscious, unrestrained mice using barometric whole-body plethysmography by recording respiratory pressure curves (Buxco, EMKA Technologies, Paris, France) as described in details previously (38). Briefly, mice were placed in a whole-body chamber and basal values were measured and averaged for 3 min. Next, mice were exposed for 3 min to a saline aerosol and aerosols with increasing concentrations of methacholine (solution doubling in concentrations, ranging from 3.13 to 50 mg/ml in saline). Aerosols were generated by a Pari LC Star nebulizer and each aerosol was followed by 3 min of recording to assess average values. Airway responsiveness was expressed in enhanced pause (Penh), an index of airway obstruction as described in detail previously (39). Herein, we would like to stress that Penh values may not always correlate with changes in pulmonary resistance (40).

Determination of serum levels of OVA-specific immunoglobulins

Approximately 250 μl of pre-challenge blood (7 days before the first OVA aerosol challenge) was recovered from each mouse by an incision made in the tail vein. Directly after assessment of airway responsiveness *in vivo* (24 h after the last OVA aerosol challenge (day 42)), mice were sacrificed by i.p. injection of 1 ml 10% urethane. Post-challenge blood was withdrawn by heart puncture. Blood samples were allowed to clot at room temperature for 30 min and subsequently centrifuged for 10 min at 2700 x g. Sera were collected, kept at –70°C and antibodies levels were measured by ELISA using microtiter plates from Nunc A/S (Roskilde, Denmark), ELISA buffer for blocking and sample dilution, and PBS containing 0.05% v/v Tween-20 for washing between incubations. To determine OVA-specific IgE levels, wells were coated overnight at 4°C with 1 μg/ml of anti-mouse IgE diluted in PBS, followed by blocking with ELISA buffer (PBS containing 0.5% BSA, 2 mM EDTA, 136.9 mM NaCl, 50 mM Tris, 0.05% Tween-20 [Merck, Whitehouse Station, NJ, USA], pH 7.2) and left for 1 h at room temperature. Hereafter, diluted serum samples and duplicate dilution series of an OVA-specific IgE reference serum, prepared as described previously (41), were added and wells were incubated for 2 h. Next, after multiple washings the wells were incubated for 1.5 h with 1 μg/ml of DIG-conjugated OVA, followed by another washing and incubation for 1 h with anti-DIG Fab coupled to horseradish peroxidase, according to manufacturer's instruction (Roche Diagnostics, Basel, Switzerland).

To assess OVA-specific IgG1 or IgG2a levels, wells were coated with 10 μg/ml OVA in PBS. After blocking, diluted serum samples and duplicate dilution series of a reference standard serum obtained from multiple OVA-boosted mice were added. Hereafter, wells were washed and incubated with 1 μg/ml of biotinylated anti-mouse IgG1 or 1 μg/ml of biotinylated anti-mouse IgG2a for 1.5 h followed by another washing and incubation for 1 h with 1:10,000 diluted Poly-HRP. For color development, 0.4 mg/ml of *o*-phenylenediamine-dichloride and 4 mM H_2O_2 in PBS were used, and the reaction was stopped by adding 75 μl of 4 M H_2SO_4. Optical density was read at 490 nm, using a Benchmark microplate reader (Bio-Rad, Hercules, CA, USA). Results were analyzed using Microplate Manager PC software (Bio-Rad).

Analysis of BAL fluid

BAL was performed immediately after bleeding the mice. A midcervical skin incision was made to expose the trachea, which was cannulated with a 23-gauge blunt needle. The airways were lavaged 5 times with 1 ml aliquots of pyrogen-free saline warmed at 37°C. The first lavage was done with 1 ml saline containing BSA and protease inhibitor (Complete mini tablet [Roche Diagnostics GMbH, Penzberg, Germany] and 5% BSA). The supernatant of this first ml of recovered lavage fluid was used to measure cytokine levels. Subsequently, mice were lavaged 4 times with 1 ml aliquots of saline at 37°C. The lavage fluid was kept on ice until further processing. Recovered lavage fluid of the second through fifth ml was pooled and cells herein (including those from the first ml) were pelleted (387 x g, 4°C, 10 min) and resuspended in 150 μl cold PBS. The total number of cells in the BAL fluid

was determined using a Bürker-Türk counting chamber (Karl Hecht Assistant KG, Sondheim/Röhm, Germany). For differential cell counts, cytospin preparations were made (15 x g, 4°C, 5 min) using a cytocentrifuge (Shandon Life Science, Cheshire, UK), and cells were fixed and stained with Diff-Quick (Dade A. G., Düdingen, Switzerland). All cytospin preparations were evaluated using oil immersion microscopy (magnification: 1000x). Cells were identified and differentiated into mononuclear cells (monocytes, macrophages and lymphocytes), eosinophils and neutrophils by standard morphology and staining characteristics. Per cytospin 200 cells were counted and the absolute number of each cell type was calculated.

Cytokine levels in BAL fluid

The levels of IL-5, IL-10, IL-13 and IL-4 in the BAL fluid were determined by sandwich ELISA in Nunc-Immuno plates coated with appropriate anticytokine capture mAbs and second-step biotinylated mAbs according to the manufacturer's instructions (PharMingen, San Diego, CA, USA). Values were expressed as pg per ml deduced from standards run in parallel with recombinant cytokines. The detection limits of the ELISAs were 32 pg/ml for IL-5, 15 pg/ml for IL-10 and IL-13 and 8 pg/ml for IL-4.

Statistical analysis

All data are expressed as mean ± SEM. The airway response curves to methacholine were statistically analysed by a general linear model of repeated measurements followed by *post hoc* comparison between groups. Data were log transformed before analysis to equalize variances in all the groups. Cell counts were statistically analysed using the Mann–Whitney *U*-test. All other analyses were performed using Student's *t*-test (2-tailed, homosedastic). A probability value $P<0.05$ was considered significant.

Results

Effects of anti-GITR and anti-CTLA-4 on the efficacy of IT

Airway responsiveness. Airway responsiveness was measured in conscious, unrestrained mice by barometric whole-body plethysmography. At day 28, after sensitization and treatment but before challenge, OVA-sensitized mice demonstrated a dose-dependent increase in Penh values in response to methacholine. The complete methacholine dose-response curves (DRCs) did not differ between different groups. DRC of the sham-treated group is depicted as representative of pre-challenge measurements (fig. 1). Airway responsiveness *in vivo* was measured again at 24 h after the last OVA aerosol on day 42. After OVA aerosol challenge, sham-treated mice developed AHR as the second methacholine DRC was significantly ($P<0.01$) different from the DRC obtained before challenge (fig. 1). Mice treated with OVA-IT displayed significantly ($P<0.05$) suppressed development of OVA-induced AHR *in*

vivo compared to sham-treated mice (fig. 1). Interestingly, the suppression of this nonspecific AHR after IT was completely reversed in animals treated at time of IT with anti-GITR (P<0.05, fig. 1). Similar results were obtained in mice treated with anti-CTLA-4 mAb before the first s.c. injection with OVA-IT (100% reversal, P<0.05, fig. 1).

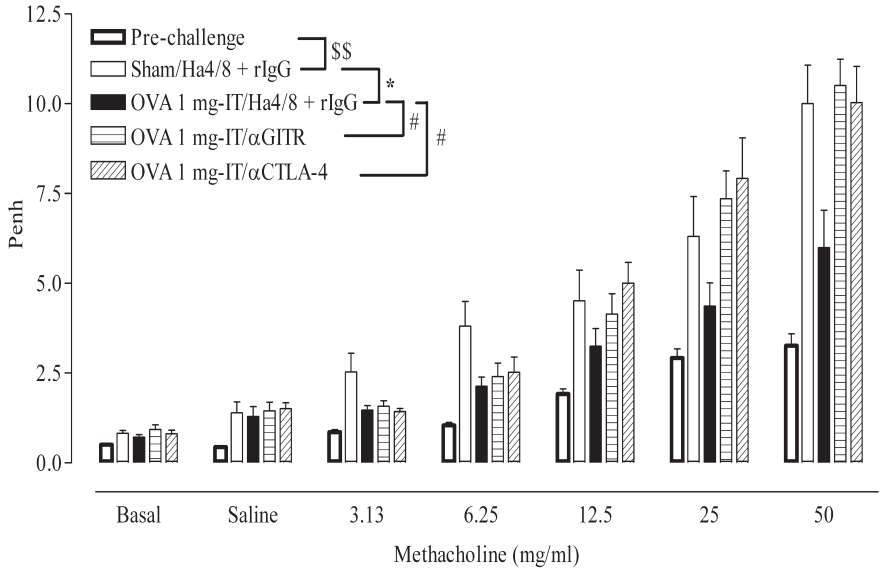

Figure 1. *Effects of treatment with anti-GITR and anti-CTLA-4 on the efficacy of OVA-IT. OVA-sensitized mice received a single i.v. treatment with anti-GITR or anti-CTLA-4 1 h before the first IT with OVA. Airway responsiveness to aerosolized methacholine just before (pre-challenge) and 1 day after OVA aerosol challenge. Values are expressed as mean ± SEM. Penh, Enhanced pause. $$P<0.01 compared to pre-challenge. *P<0.05 compared to sham-treated mice. #P<0.05 compared to OVA-IT/Ha4/8 + rIgG- treated mice.*

Serum levels of immunoglobulins. We further examined whether treatment with anti-GITR or anti-CTLA-4 could influence the efficacy of OVA-IT on immunoglobulins levels in serum. In sham and control antibody-treated mice, OVA challenge displayed significantly increased (98%, P<0.01) OVA-specific IgE levels in serum compared with pre-challenge levels (fig. 2). OVA-IT significantly suppressed the increase in OVA-specific IgE levels in serum by 83% (P<0.01) compared to sham-treated mice (fig. 2). Interestingly, a partial reversal in OVA-specific IgE levels in serum was observed after treatment with anti-GITR (31% reversal, P<0.05) or anti-CTLA-4 (35% reversal, P<0.001) compared to mice treated with OVA-IT and control antibodies (fig. 2).

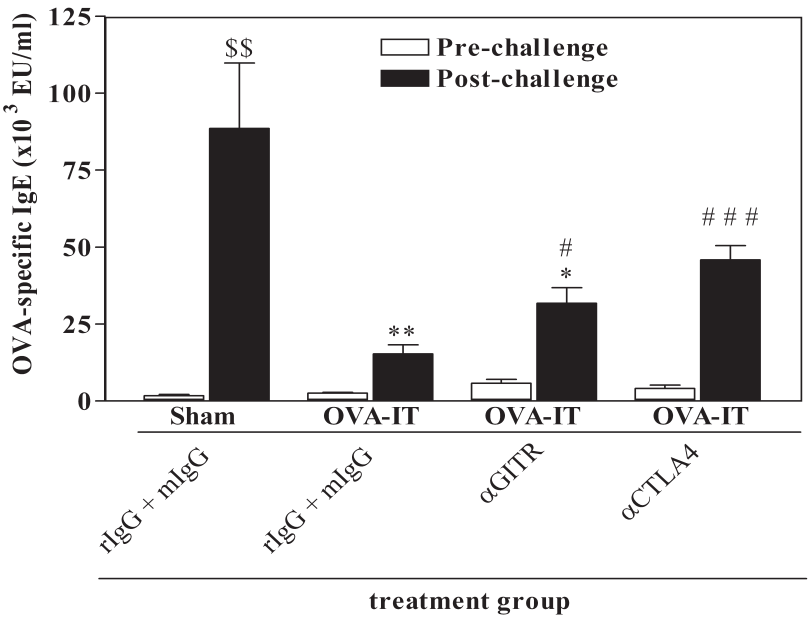

Figure 2. *Effects of treatment with anti-GITR and anti-CTLA-4 on the efficacy of OVA-IT. OVA-sensitized mice received a single i.v. treatment with anti-GITR or anti-CTLA-4 1 h before the first IT with OVA. Levels of OVA-specific IgE in serum just before (pre-challenge) and 1 day after (post-challenge) OVA aerosol challenge. Values are expressed as mean ± SEM. $$P<0.01 compared to pre-challenge. *P<0.05, **P<0.01 compared to sham-treated mice. #P<0.05, ###P<0.001 compared to OVA-IT/Ha4/8 + rIgG- treated mice.*

OVA-IT and control antibody-treatment induced a significant upregulation in serum OVA-specific IgG1 but not IgG2a levels. Anti-GITR and anti-CTLA4 treatments at the time of OVA-IT significantly potentiated the increase in IgG1, whereas IgG2a was only potentiated by anti-CTLA4 treatment (table II). Furthermore, in sham and control antibody-treated mice, OVA challenge induced an increase of OVA-specific IgG1 (95%, P<0.01) and IgG2a (95%, P<0.01) levels in serum compared with pre-challenge levels (table II). OVA-IT and control antibody treatment prevented the upregulation of OVA-specific IgG1 levels and largely reduced the upregulation of OVA-specific IgG2a levels upon OVA inhalation challenge (table II). Treatment with anti-GITR at the time of OVA-IT did not significantly affect the abrogation of IgG1 upregulation but partially reversed the IgG2a upregulation by OVA inhalation challenge. Treatment with anti-CTLA4 at the time of OVA-IT did not affect the abrogation of IgG1 and IgG2a upregulation by OVA inhalation challenge.

Thus, OVA-IT significantly increases serum levels of the Th2-dependent isotype IgG1 but not the Th1-dependent IgG2a levels. Treatment with either anti-CTLA4 or anti-GITR potentiated the IgG1 increases by OVA-IT. Importantly, OVA-IT abrogates the upregulation of serum IgG1 and IgG2a levels after OVA inhalation challenge

as observed in sham-treated mice. Only anti-GITR treatment partially reverses the abrogation of IgG2a upregulation.

Table II. *Serum OVA-specific antibody levels.*

Treatment group	Ig Isotype			
	OVA-specific IgG1 Mean ± SEM (x 10^6 EU/ml)		OVA-specific IgG2a Mean ± SEM (x 10^5 EU/ml)	
	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge
Sham/Ha4/8 + rIgG	0.73 ± 0.32	15.45 ± 6.63 $$	0.68 ± 0.46	14.89 ± 4.82 $$
OVA-IT/Ha4/8 + rIgG	54.23 ± 22.36 *	23.58 ± 8.02	1.06 ± 0.56	2.42 ± 0.60
OVA-IT/anti-GITR	140.35 ± 11.35 #	57.82 ± 14.44 $$	1.08 ± 0.32	7.29 ± 1.21 $$
OVA-IT/anti-CTLA-4	105.35 ± 11.35 #	60.71 ± 11.41 $	3.59 ± 2.56 #	8.49 ± 1.94 $

*OVA-sensitized mice received IT with 1 mg OVA. Intervention with anti-GITR or anti-CTLA-4 was 1 hr before the first OVA-IT. Levels of OVA-specific IgG1 and IgG2a in serum were measured before (pre-challenge) and 1 day after (post-challenge) OVA aerosol challenge. Values are expressed as mean ± SEM (n=6). $P<0.05, $$P<0.01 compared to pre-challenge levels of the same group. *P<0.05 compared to pre-challenge levels of sham-treated mice. #P<0.05 compared to pre-challenge levels of OVA-IT/Ha4/8 + rIgG-treated mice.*

Eosinophils and cytokine levels in the BAL fluid. Eosinophil infiltration into airways of sensitized mice was induced by allergen inhalation challenge. The BAL fluid contained high numbers of inflammatory cells consisting predominantly of eosinophils (fig. 3) besides mononuclear cells and few neutrophils (data not shown). OVA-IT effectively suppressed the airway eosinophilia as the number of eosinophils in BAL fluid was significantly reduced by 93% (P<0.01) compared with sham-treated mice (fig. 3). Activation of GITR before the first OVA-IT resulted in partial reversal of the number of eosinophils in BAL fluid by 17% (P<0.01) compared with mice treated only with OVA-IT (fig. 3). Similar results were obtained when CTLA-4 was blocked with anti-CTLA-4 before the first IT treatment with OVA (31% reversal, P<0.01, fig. 3).

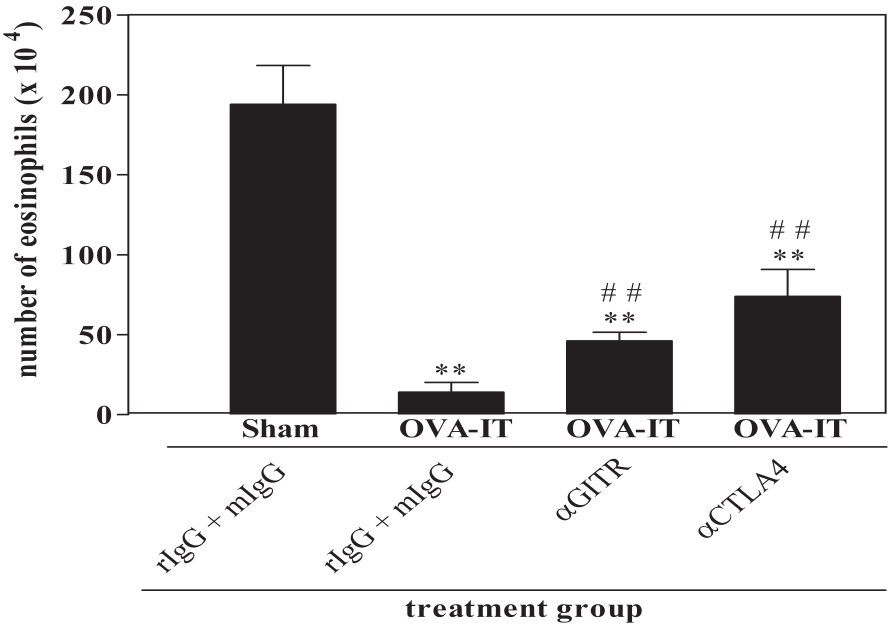

Figure 3. *Effects of treatment with anti-GITR and anti-CTLA-4 on the efficacy of OVA-IT. OVA-sensitized mice received a single i.v. treatment with anti-GITR or anti-CTLA-4 1 h before the first IT with OVA. Eosinophil numbers in BAL fluid were measured 1 day after OVA aerosol challenge. Values are expressed as mean ± SEM. **P<0.01 compared to sham-treated mice. ##P<0.01 compared to OVA-IT/Ha4/8 + rIgG- treated mice.*

Since Th2-type responses are crucial in the development and maintenance of allergy and allergic asthma, Th2 cytokines levels in the BAL fluid were analyzed. Mice treated with OVA-IT displayed significant falls in IL-5 levels by 86% (P<0.01, fig. 4A), IL-13 levels by 75% (P<0.01, fig. 4B), IL-10 levels by 82% (P<0.01, fig. 4C) and IL-4 levels by 43% (P<0.05, fig. 4D) in BAL fluid compared with sham-treated mice. Activation of GITR before the first s.c. OVA-IT partially reversed the suppression in IL-5 levels (17%, P<0.01, fig. 4A), IL-13 levels (16%, P<0.05, fig. 4B), IL-10 levels (15%, P=0.052, fig. 4C) and IL-4 (9%, P<0.05, fig. 4D) compared with only OVA-IT-treated mice. Treatment of mice with anti-CTLA-4 partially reversed the suppression in levels of the Th2-associated pro-inflammatory cytokines IL-5 (23%, P<0.01, fig. 4A), IL-13 (29%, P<0.05, fig. 4B) and IL-4 (14%, P<0.001, fig. 4D) in BAL fluid induced by OVA-IT. IL-10 levels were also partially reversed by 16% (P=0.056) in BAL fluid obtained from mice treated with anti-CTLA-4 compared with mice merely receiving OVA-IT and control antibody treatment (fig. 4C). The Th1-cytokine IFN-γ was not detectable in the BAL fluid obtained from sham- or IT-treated mice (data not shown).

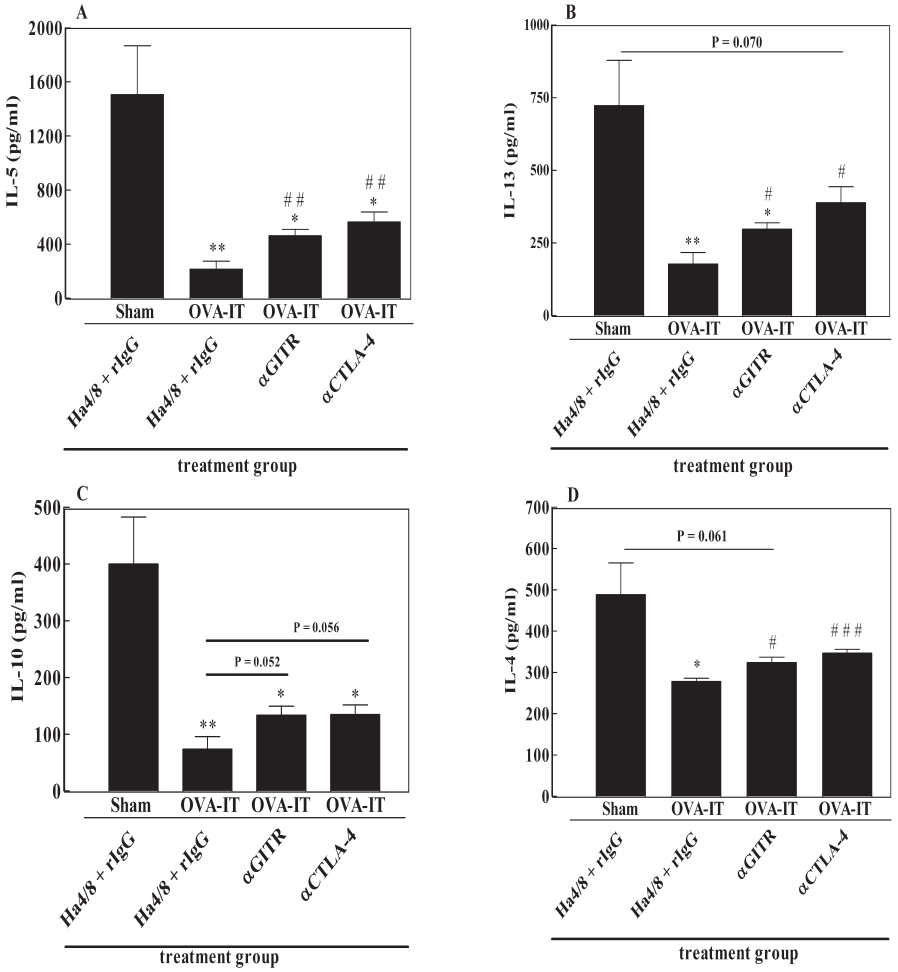

Figure 4. *Effects of treatment with anti-GITR and anti-CTLA-4 on the efficacy of OVA-IT. OVA-sensitized mice received a single i.v. treatment with anti-GITR or anti-CTLA-4 1 h before the first IT with OVA. A) IL-5 levels, B) IL-13 levels, C) IL-10 levels and D) IL-4 levels in BAL fluid 1 day after OVA aerosol challenge. Values are expressed as mean ± SEM. *P<0.05, **P<0.01 compared to sham-treated mice. #P<0.05, ##P<0.01, ###P<0.01 compared to OVA-IT/Ha4/8 + rIgG- treated mice.*

Discussion

Although the role of $CD4^+CD25^+Foxp3^+$ naturally occurring regulatory T (nTreg) cells in immunoregulation becomes increasingly apparent, their potential involvement in tolerance induction during allergen IT has not yet been evaluated. In the present study, we show that functional inhibition of nTreg cells *via* GITR activation at the time of IT reduced the efficacy of allergen IT, in particular the suppression of AHR.

Similarly, anti-CTLA-4 mAb treatment partially reversed the induction of tolerance when it was given during allergen IT. These data indicate that induction of tolerance by allergen IT partially requires nTreg cells suppressive activity.

Most functional studies on nTreg cells have demonstrated that these cells regulate the expansion of conventional $CD4^+$ T-cells (19) and can promote tolerance in different models of autoimmune diseases (14) and allograft rejection (16). This study is the first to show that nTreg cells contribute to tolerance induction during allergen IT. Of particular interest are our findings that ligation of GITR by an activating antibody which has previously been shown to abrogate nTreg cell suppressor function (22), results in complete reversal of OVA-induced AHR in allergen IT-treated mice. Our findings also showed partial reversal of tolerance induction after IT with respect to suppression of serum IgE levels, BAL eosinophilia and Th2-type cytokine levels in anti-GITR treated mice. These observations suggest that allergen IT partially achieves beneficial effectiveness *via* nTreg-dependent tolerance induction. Although GITR was originally shown to be constitutively expressed on nTreg cells but not on conventional naive T-cells (22, 23), recent evidence showed that it is also expressed by activated conventional T-cells (42, 43). GITR stimulation leads to enhanced activation of conventional T-cells (42). Therefore, it can not be excluded that the observed effects of anti-GITR treatment during IT are related to an effect on conventional OVA-specific T-cells.

CTLA-4 is constitutively expressed on nTreg cells (24). Previous studies have shown that CTLA-4 is critically involved in the immunosuppressive effects of nTreg cells *in vitro* and that nTreg cells from CTLA-4 deficient mice display impaired suppressive activity (24). Moreover, it has been demonstrated in a murine model of $CD4^+$ T-cell induced colitis that the capacity of nTreg cells to inhibit pathogenic $CD4^+$ T-cells was abolished by administration of CTLA-4 blocking antibody (21). In the present study the abrogation of IT effects by anti-CTLA-4 treatment during IT qualitatively and quantitatively resembled those observed after anti-GITR treatment with complete reversal of AHR and partial reversal of the other asthma manifestations. However, anti-CTLA-4 can also stimulate activation of effector T-cells by blocking negative signaling provided by CTLA-4:CD80/CD86 interaction (44). It can be argued that it is unlikely that stimulation of Th2 effector cell activation by anti-GITR or anti-CTLA4 during IT is the explanation for the partial reversal of IT effects. First, we have not observed a potentiation of asthma manifestations when OVA sensitized mice were treated with anti-CTLA4 during OVA inhalation challenge (Van Oosterhout *et al*., unpublished observations and (45)). Second, abrogation by IT of OVA-challenge induced upregulation of the Th2-dependent IgG1 level in serum was not reversed by anti-CTLA-4 or anti-GITR treatment.

In the present study, different asthma manifestations show differential sensitivity to reversal of allergen IT induced tolerance by functional inhibition of nTreg cells. Whereas AHR can be completely reversed by anti-GITR or anti-CTLA4 treatment, IgE upregulation, BAL eosinophilia and Th2-type cytokine levels are only partially reversed. This is in line with previous studies (Taher *et al*., unpublished observations) showing that the sensitivity for down-regulation by different doses of OVA-IT follows the rank order of IgE > BAL eosinophils > AHR. Hence, as it can be expected the least sensitive characteristic to IT-induced downregulation, AHR, is readily reversed.

A number of characteristics have been described for the murine nTreg cells

that may provide insight into their mechanism of action during allergen IT. First, nTreg cells may promote the generation of antigen-specific Treg cells that secrete the immunosuppressive cytokines IL-10 and TGF-β. Natural Treg cells have been shown to promote the formation of antigen-specific, IL-10-secreting regulatory T-cells in experimental allergic encephalomyelitis (46). *In vitro* studies using human nTreg cells demonstrate that these cells are able to induce an IL-10 (47) and TGF-β (48) -dependent regulatory phenotype in naïve T-cells, a phenomenon known as infectious tolerance. Therefore, the present findings suggest that induction of adaptive Treg cells producing IL-10 and TGF-β could be one mechanism whereby nTreg cells contribute to tolerance induction by allergen IT in our model. In agreement herewith, we were able to completely reverse the beneficial effects of IT by treatment with antibodies to IL-10R (33).

Second, several studies have postulated that engagement of CTLA-4 on nTreg cells is essential for their regulatory function (21) and for promoting induction of adaptive Treg cells (49). It remains to be elucidated whether nTreg cells induce adaptive Treg cells indirectly *via* a mechanism that involves antigen presenting cells, or directly *via* interaction with $CD4^+$ T-cell. Interestingly, nTreg cells have recently been shown to trigger the induction of IDO in DCs by engagement of B7 through CTLA-4, leading to the degradation of tryptophan into kynurenines and subsequent inhibition of T-cell proliferation (28, 50) and to tolerance induction by promoting the generation of adaptive Treg cells (31). Interestingly, we recently showed that inhibition of IDO with the competitive inhibitor 1-methyltryptophan at time of IT partially abrogated its beneficial effects, e.g. reduction of BAL eosinophil numbers and AHR to methacholine (chapter 3). Thus, it can be speculated that tolerance induction by IT is partially mediated by a nTreg – CTLA-4 – IDO axis.

In summary, our data strongly suggest that nTreg cells partially contribute to tolerance induction by allergen IT in a mouse model of allergic asthma. We show for the first time that functional inhibition of nTreg cells through GITR activation as well blockade of CTLA-4 as effector molecule for the immunosuppressive activity of nTreg cells completely reversed the suppressive effects of IT on AHR whereas suppressions of BAL eosinophilia, Th2 cytokines and OVA-specific IgE levels are partially reversed. Although further studies are needed, the present findings suggest that modulation of nTreg cells may enhance the prospects for successful IT and may provide an effective way for controlling aberrant immune responses to airborne allergens in asthma.

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Chapter 5

1α,25-dihydroxyvitamin D_3 potentiates the beneficial effects of allergen immunotherapy in a mouse model of allergic asthma: role for IL-10 and TGF-β

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Abstract

The active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), a potent inhibitor of nuclear factor-κB protein expression, can prevent the maturation of dendritic cells *in vitro* leading to tolerogenic dendritic cells with increased potential to induce regulatory T-cells. Herein, we investigated whether combination of allergen immunotherapy with 1,25$(OH)_2D_3$ potentiates the suppressive effects of immunotherapy and if the immunoregulatory cytokines IL-10 and TGF-β are involved in the effector phase. OVA-sensitized and challenged BALB/c mice, displayed increased airway responsiveness, serum OVA-specific IgE levels and bronchoalveolar lavage (BAL) eosinophilia and Th2 cytokine levels. In this model, the dose-response relation of allergen immunotherapy 10 days prior to OVA inhalation challenge shows strong suppression of all asthma manifestations at 1 mg OVA, but partial suppression of BAL eosinophilia, IgE upregulation and no reduction of airway hyperresponsiveness (AHR) at 100 μg. Interestingly, co-administration of 10 ng 1,25$(OH)_2D_3$ with 100 μg OVA immunotherapy significantly inhibited AHR and potentiated the reduction of serum OVA-specific IgE levels, BAL eosinophilia and Th2-related cytokines concomitant with increased TGF-β and OVA-specific IgA levels in serum. The suppressive effects of this combined immunotherapy were partially reversed by treatment with mAb to either IL-10R or to TGF-β before the first OVA inhalation challenge but completely abrogated when both antibodies were given simultaneously. These data demonstrate that 1,25$(OH)_2D_3$ potentiates the efficacy of immunotherapy and that the regulatory cytokines IL-10 and TGF-β play a crucial role in the effector phase of this mouse model.

Introduction

Allergen-specific immunotherapy (IT) has proven to be beneficial and is recommended as an alternative for treating allergic disorders (1). Although administration of specific allergens is effective in rhinitis and insect venom allergy, the role of this intervention in allergic asthma remains controversial (2, 3). Furthermore, the major drawbacks of this treatment are the risk of eliciting rare but life-threatening systemic reactions in patients receiving build-up and maintenance doses of IT (3). Improvement of IT is needed to get better efficacy before widespread application in asthmatic patients can be considered. However, for rational improvement of IT elucidation of the precise underlying mechanisms is required. The efficacy of IT has been related to the induction of blocking IgG antibodies (4), the induction of anergy in T-cells (5), or to a shift of cytokine expression from Th2 cytokines which are associated with an asthmatic phenotype toward Th1 cytokines (6, 7). Recently, induction of regulatory T (Treg) cells secreting the immunoregulatory cytokines IL-10 and TGF-β has been implicated in the down-regulation of allergen-specific T-cell proliferation *in vitro* after IT (8-10).

Dendritic cells (DCs) are the most potent antigen presenting cells, and play a key role in the generation of adaptive T-cell subsets (11). DCs are not only immunogenic but also tolerogenic, depending on their maturation state (12). Although several studies have suggested that mature DCs can induce $CD4^+$ T-cell tolerance (13, 14), tolerogenic DCs generally are semi-mature with increased expression of major histocompatibility complex class II and B7-2, but low expression of CD40 and no production of the pro-inflammatory cytokines IL-6 and TNF-α (15). Likewise, incubation of T-cells with semi-mature or immature DCs has been shown to induce antigen-specific Treg cells, like $CD4^+$ Th3 cells and $CD4^+$ T regulatory 1 (Tr1) cells (16, 17). Although direct evidence is lacking, we assume that immature tolerogenic DCs can be instrumental in the generation of Treg cells during allergen IT.

Previously, we developed a mouse model of allergic asthma in which allergen IT suppressed allergen-induced airway manifestations of asthma (18). Moreover, allergen IT induces IL-10 dependent long-lasting tolerance to OVA-induced asthma manifestations, pointing to a role for Tr1 cells (19). Since many of the observed immunological changes following allergen IT may be mediated by the induction of Treg cells, and since immature tolerogenic DCs play a critical role in Treg cell generation and peripheral tolerance, we were interested if allergen IT could be improved using a compound that inhibits DC maturation. The nuclear factor-κB (NF-κB) protein Re1B is essential for DC differentiation and maturation (20, 21). Inhibition of NF-κB signaling by various drugs has been shown to generate immature tolerogenic DCs (22-24). A number of studies have consistently shown that treatment of human DCs or murine bone marrow-derived DCs with $1,25(OH)_2D_3$ inhibits expression of Re1B, costimulatory molecules and IL-12 secretion but enhances IL-10 production (20, 25, 26). Moreover, $1,25(OH)_2D_3$ appears to generate tolerogenic DCs *in vivo*, as demonstrated in models of transplantation and autoimmune diseases (27, 28).

In this study, we investigated whether co-injection of $1,25(OH)_2D_3$ potentiates the suppressive effects of IT in our mouse model. In addition, to investigate whether the regulatory cytokines IL-10 and TGF-β are involved in tolerance to OVA-induced

airway manifestations of asthma, we functionally blocked IL-10 receptors and TGF-β *in vivo*.

Materials and methods

Animals

Animal care and use were conducted in accordance with the Animal Ethics Committee of Utrecht University, The Netherlands. Specific pathogen-free (according to the Federation of European Laboratory Animal Science Associations) (29) male BALB/c mice (6-8 weeks) were purchased from Charles River (Maastricht, The Netherlands). The mice were housed in macrolon cages in a laminar flow cabinet and provided with food and water *ad libitum*.

Antibodies

Mouse IgG (mIgG) was purchased from Sigma (St. Louis, MO, USA). Rat IgG (rIgG) was purchased from ICN Pharmaceuticals (Cosa Mesa, CA, USA). Rat mAb to mouse IL-10 receptor (anti-IL-10R) and mouse mAb to mouse TGF-β (anti-TGF-β) were purified from culture supernatant of hybridomas (HB-12538 and HB-9849 respectively, American Type Culture Collection, Manassas, VA, USA) by using a protein G column (Pharmacia, Peapack, NJ, USA). All antibodies were treated with 10% v/v polymyxin B-agarose (Sigma; washed twice with saline) for 1 h at 4°C to remove lipopolysaccharide. After incubation, agarose beads were removed by centrifugation (1700 x g, 4°C, 15 min), and the supernatants were sterilized by using a 0.22-μm filter (Omnilabo, Breda, The Netherlands). The endotoxin level in all treatment preparations was less than 1 endotoxin U/mg.

Sensitization, challenge and IT protocol

The protocol used for sensitization, IT and inhalation challenge (fig. 1) was the same as previously described (19). Mice were sensitized i.p. on days 0 and 7 with 10 μg OVA (chicken egg albumin, crude grade V, Sigma-Aldrich) adsorbed onto 2.25 mg alum (ImjectAlum, Pierce, Rockford, IL, USA) in 0.1 ml pyrogen-free saline (B. Braun, Melsungen, Germany). To study the efficacy of allergen IT, we performed a dose-response relation of OVA-IT (fig. 1A). For that purpose 0.5% w/v OVA in saline was prepared and freshly diluted before injection. In experiment A, mice were treated with 3 s.c. injections of either a low (10 μg), a suboptimal (100 μg) or optimal (1 mg) dose OVA in 0.2 ml pyrogen-free saline on alternate days two weeks after the second sensitization. The control group was sham-treated with 0.2 ml saline. 10 days after OVA-IT or sham treatment, mice were challenged with OVA aerosols in pyrogen-free saline (10 mg/ml) for 20 min 3 times every third day in a Plexiglas exposure chamber (5 liter) coupled to a Pari LC Star nebulizer (PARI Respiratory Equipment, Richmond, VA, USA; particle size 2.5–3.1 μm) driven by compressed air at a flow rate of 6 l/min. Aerosol was given in groups composed of no more than

twelve mice.

Since the dose-response relation of OVA-IT showed that IT with 100 μg OVA partially suppressed asthma like symptoms in this model, we next determined whether inhibition of NF-κB activation potentiates the suppressive effects of allergen IT (fig. 1B). Therefore, the active form of vitamin D_3, $1,25(OH)_2D_3$ (Fluka, Switzerland) was dissolved in 96% v/v ethanol and freshly diluted in OVA solution before s.c. injection. Based on preliminary results (data not shown) and literature data (30), we chose the dose 10 ng $1,25(OH)_2D_3$ for our *in vivo* experiments. In experiment B, mice received 100 μg OVA-IT alone or in combination with 10 ng $1,25(OH)_2D_3$. Control mice were sham-treated with saline alone or in combination with 10 ng $1,25(OH)_2D_3$. In both experiments (A and B), airway responsiveness to methacholine, serum levels of immunoglobulins, cellular infiltration and Th2-cytokine levels in the BAL fluid were measured 24 h after the last OVA inhalation challenge.

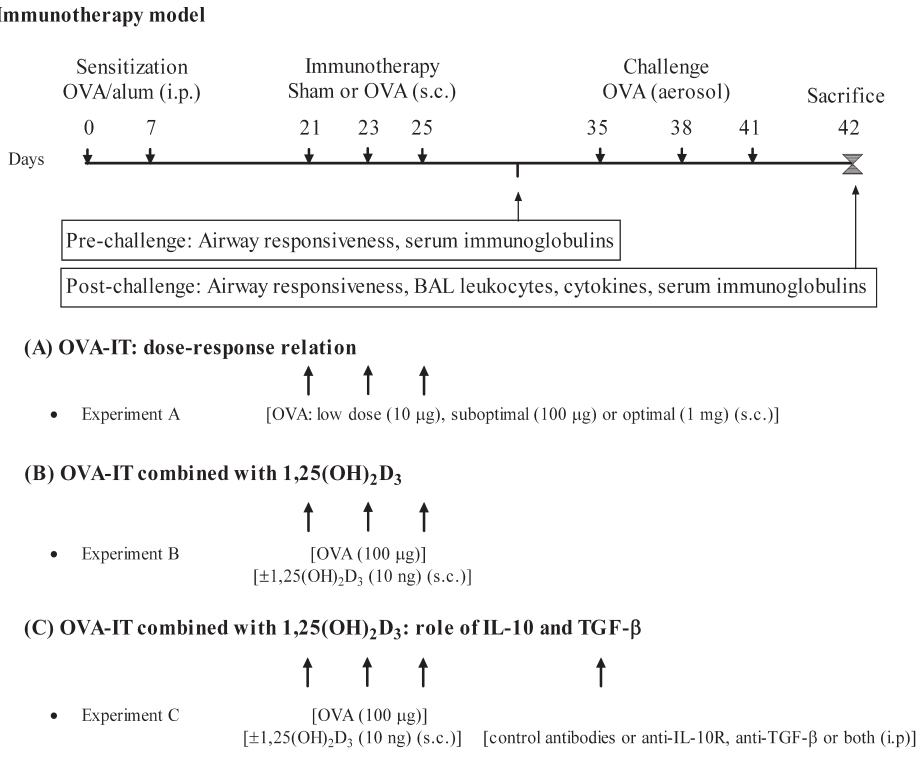

Figure 1. *Outline of the IT protocol in a murine model of asthma and the intervention studies. OVA-sensitized mice received A) IT with 10 μg (low), 100 μg (suboptimal) or 1 mg (optimal) dose OVA or, B) 100 μg OVA combined with 10 ng $1,25(OH)_2D_3$ s.c. on days 21, 23 and 25. All mice were challenged with OVA aerosols on days 35, 38 and 41. Airway responsiveness to methacholine and serum levels of OVA-specific immunoglobulins were measured just before as well as 1 day after last challenge and leukocyte numbers in BAL fluid and cytokine levels in BAL fluid or TLN-cell cultures 1 day after challenge. C) OVA-sensitized mice received IT with suboptimal (100 μg) OVA combined with 10 ng $1,25(OH)_2D_3$ s.c. (days 21, 23 and 25). Intervention with anti-IL-10R, anti-TGF-β or both or, control antibodies (rIgG + mIgG) was just before the first OVA aerosol challenge (day 35).*

The third series of experiments was aimed to analyze whether the immunoregulatory cytokines IL-10 and TGF-β are involved in the effector phase of immunmodulation (fig. 1C). In experiment C, mice were injected i.p. with 0.5 mg anti-IL-10R mAb (19), 2.5 mg anti-TGF-β neutralizing mAb (31) or both one hour before the first OVA aerosol challenge. Control mice were treated with 0.5 mg rIgG plus 2.5 mg mIgG.

Evaluation of airway responsiveness

Airway responsiveness to inhaled methacholine (acetyl-β-methylcholine chloride, Sigma-Aldrich) was measured twice (6 days before the first OVA aerosol challenge and at 24 h after the last challenge) in conscious, unrestrained mice using barometric whole-body plethysmography by recording respiratory pressure curves (Buxco, EMKA Technologies, Paris, France) as described in details previously (32). Briefly, mice were placed in a whole-body chamber and basal values were measured and averaged for 3 min. Next, mice were exposed for 3 min to a saline aerosol and aerosols with increasing concentrations of methacholine (solution doubling in concentrations, ranging from 3.13 to 50 mg/ml in saline). Aerosols were generated by a Pari LC Star nebulizer and each aerosol was followed by 3 min of recording to assess average values. Airway responsiveness was expressed in enhanced pause (Penh), as described in detail previously (33). Herein, we would like to stress that Penh values may not always correlate with changes in pulmonary resistance (34).

To determine airway resistance, we measured respiratory system resistance changes in response to methacholine as described by Kang *et al.* For details, see **Supplementary Method**.

Determination of serum levels of OVA-specific immunoglobulins

Approximately 0.25 ml of pre-challenge blood (7 days before the first OVA aerosol challenge) was recovered from each mouse by an incision made in the tail vein. Directly after assessment of airway responsiveness *in vivo* 24 h after the last OVA aerosol challenge, mice were sacrificed by i.p. injection of 1 ml 10% urethane. Post-challenge blood was withdrawn by heart puncture. Blood samples were allowed to clot at room temperature for 30 min and subsequently centrifuged for 10 min at 2700 x g. Sera were collected, kept at –70°C and antibodies levels were measured by ELISA using microtiter plates from Nunc A/S (Roskilde, Denmark), ELISA buffer for blocking and sample dilution, and PBS containing 0.05% v/v Tween-20 for washing between incubations. To determine OVA-specific IgE levels, wells were coated overnight at 4°C with 1 μg/ml of anti-mouse IgE diluted in PBS, followed by blocking with ELISA buffer (PBS containing 0.5% BSA, 2 mM EDTA, 136.9 mM NaCl, 50 mM Tris, 0.05% Tween-20 [Merck, Whitehouse Station, NJ, USA], pH 7.2) and left for 1 h at room temperature. Hereafter, diluted serum samples and duplicate dilution series of an OVA-specific IgE reference serum were added and incubated for 2 h. An OVA-specific IgE reference serum was obtained by sensitization and challenge of mice with OVA and arbitrarily assigned a value of 1000 experimental units/ml (EU/ml). Next, after multiple washings the wells were incubated for 1.5 h with 1

μg/ml of DIG-conjugated OVA, followed by another washing and incubation for 1 h with anti-DIG Fab coupled to horseradish peroxidase, according to manufacturer's instruction (Roche Diagnostics, Basel, Switzerland).

To assess OVA-specific IgG1 or IgG2a levels, wells were coated with 10 μg/ml OVA in PBS. After blocking, diluted serum samples and duplicate dilution series of a reference standard serum obtained from multiple OVA-boosted mice were added. Hereafter, wells were washed and incubated with 1 μg/ml of biotinylated anti-mouse IgG1 or 1 μg/ml of biotinylated anti-mouse IgG2a for 1.5 h followed by another washing and incubation for 1 h with 1:10,000 diluted Poly-HRP.

For determination of OVA-specific IgA levels, wells were coated with 0.5 μg/ml anti-mouse IgA mAb (PharMingen, San Diego, CA, USA) in PBS. After blocking, diluted serum samples and duplicate dilution series of an IgA reference serum were added for 2 h. Next, after multiple washings the wells were incubated for 1 h with 2 μg/ml of biotinylated anti-mouse IgA, followed by another washing and incubation for 1 h with 1:10,000 diluted Poly-HRP.

For color development, 0.4 mg/ml of *o*-phenylenediamine-dichloride and 4 mM H_2O_2 in PBS were used, and the reaction was stopped by adding 75 μl of 4 M H_2SO_4. Optical density was read at 490 nm, using a Benchmark microplate reader (Bio-Rad, Hercules, CA, USA). Results were analyzed using Microplate Manager PC software (Bio-Rad).

Analysis of BAL fluid

BAL was performed immediately after bleeding the mice. Briefly, a midcervical skin incision was made to expose the trachea, which was cannulated with a 23-gauge blunt needle. The airways were lavaged 5 times with 1 ml aliquots of pyrogen-free saline warmed at 37°C. The first lavage was done with 1 ml saline containing BSA and protease inhibitor (Complete mini tablet [Roche Diagnostics GMbH, Penzberg, Germany] and 5% BSA). The supernatant of this first ml of recovered lavage fluid was used to measure cytokine levels. Subsequently, mice were lavaged 4 times with 1 ml aliquots of saline at 37°C. The lavage fluid was kept on ice until further processing. Recovered lavage fluid of the second through fifth ml was pooled and cells herein (including those from the first ml) were pelleted (387 x g, 4°C, 10 min) and resuspended in 0.15 ml cold PBS. The total number of cells in the BAL fluid was determined using a Bürker-Türk counting chamber (Karl Hecht Assistant KG, Sondheim/Röhm, Germany). For differential cell counts, cytospin preparations were made (15 x g, 4°C, 5 min) using a cytocentrifuge (Shandon Life Science, Cheshire, UK), and cells were fixed and stained with Diff-Quick (Dade A. G., Düdingen, Switzerland). All cytospin preparations were evaluated using oil immersion microscopy (magnification: 1000x). Cells were identified and differentiated into mononuclear cells (monocytes, macrophages and lymphocytes), eosinophils and neutrophils by standard morphology and staining characteristics. Per cytospin 200 cells were counted and the absolute number of each cell type was calculated.

Determination of cytokine production by OVA-restimulated thoracic lymph node cells *in vitro*

Cytokines production by antigen re-stimulated T-cells in thoracic lymph nodes (TLN) were determined as described previously (35). Briefly, 24 h after the last OVA aerosol challenge, TLN-cells derived from the paratracheal and parabronchial region were collected and transferred to cold sterile PBS. TLN-cells were pooled, gently minced and filtered through a 70 μm nylon cell strainer (Becton Dickinson Labware, Franklin, Lakes, NJ, USA) with 15 ml PBS to obtain a single-cell suspension. The TLN-cell suspension was washed, and cells were pelleted (180 x g, 4°C, 10 min), resuspended in culture medium (RPMI 1640 [Cambrex Bio Science, Verviers, Belgium] containing 10% FCS, 0.1% gentamicin and 20 μm β-mercaptoethanol [Sigma]). The total number of TLN-cells was determined using a Bürker-Türk counting-chamber. TLN-cells (2 x 10^5 cells/well) were cultured in triplicate in 96-well round-bottomed plates (Greiner Bio-One, GmbH, Kremsmuenster, Austria) in the absence (medium only) or presence of OVA (10 μg/ml). To determine their capacity to produce cytokines, TLN-cells were polyclonally stimulated with 50 μg/ml immobilized anti-CD3 mAb (clone 17A2, coated overnight at 4°C). The hybridoma for the 17A2 mAb to CD3 was obtained from the American Type Tissue Collection (Manassas, VA, USA) and purified antibody was used. After 5 days of culture at 37°C in 5% CO_2, the supernatants were harvested, pooled and stored at –70°C until determination of cytokine levels.

Measurement of cytokines

Supernatant of the first ml BAL fluid and TLN-cell cultures were analyzed for Th2 cytokines contents. The levels of IL-5, IL-10 and IL-13 were determined by sandwich ELISA using antibody pairs and standards purchased from PharMingen, according to the manufacturer's instructions. A commercially available ELISA kit was used to assess levels of TGF-β in the BAL fluid and serum (Biosource, Etten-Leur, The Netherlands). Values are expressed as pg per ml deduced from standards run in parallel with recombinant cytokines. The detection limits of the ELISAs for IL-5, IL-10, IL-13 and TGF-β were 32, 15, 15 and 15 pg/ml respectively.

Statistical analysis

All data are expressed as mean ± standard error of mean (SEM). A general linear model of repeated measurements followed by *post-hoc* comparison between groups statistically analyzed the airway dose-response curves to methacholine. Data were log transformed before analysis to equalize variance in all groups. Statistical analysis on BAL fluid cell counts was performed using the non-parametric Mann-Whitney *U* test. For all other parameters, results were analyzed using a Student's *t* test (2-tailed, homosedastic). A probability value $P<0.05$ was considered significant.

Results

OVA-IT: dose-response relation

To evaluate the efficacy of allergen IT, we first examined the dose-response relation of OVA- IT. OVA-sensitized mice were treated with 10 μg, 100 μg or 1 mg OVA.

Airway responsiveness. Airway responsiveness was measured in conscious, unrestrained mice by barometric whole-body plethysmography. At day 28, after sensitization and treatment but before challenge, OVA-sensitized mice demonstrated a dose-dependent increase in Penh values in response to methacholine. The complete methacholine dose-response curves (DRCs) did not differ between different groups. DRC of the sham-treated group is depicted as representative of pre-challenge measurements (fig. 2A). Airway responsiveness *in vivo* was measured again at 24 h after the last OVA aerosol on day 42. After OVA aerosol challenge, sham-treated mice developed AHR as the second methacholine DRC was significantly ($P<0.05$) different from the DRC obtained before challenge (fig. 2A). Mice treated with 1 mg OVA-IT displayed significantly ($P<0.05$) suppressed development of OVA-induced AHR *in vivo* compared to sham-treated mice (fig. 2A). Neither 10 μg nor 100 μg OVA-IT influenced AHR to methacholine (fig. 2A).

BAL eosinophilia. To determine the efficacy of OVA-IT on airway eosinophilia, we examined the amount of inflammatory cells present in the airway lumen 24 h after the last OVA aerosol challenge. The BAL fluid of OVA-sensitized and challenged mice contained high numbers of eosinophils (fig. 2B) besides mononuclear cells and few neutrophils (data not shown). 1 mg OVA-IT effectively suppressed the airway eosinophilia as the number of eosinophils in the BAL fluid was significantly reduced by 87% ($P<0.01$) compared with sham-treated mice (fig. 2B). 100 μg OVA-IT partially suppressed the influx of airway eosinophilia but this reduction did not reach the level of significance (44%, $P=0.93$, fig. 2B). No reduction was found in number of eosinophils in BAL fluid from mice treated with 10 μg OVA-IT (fig. 2B).

OVA-specific IgE levels in serum. We further investigated the efficacy of OVA-IT on the levels of OVA-specific IgE in serum. In sham-treated mice, OVA challenge displayed significantly increased (89%, $P<0.05$) OVA-specific IgE levels in serum compared with pre-challenge levels (fig. 2C). 1 mg OVA-IT significantly suppressed the OVA-specific IgE levels in serum by 81% ($P<0.01$) compared with sham-treated mice (fig. 2C). IT with 100 μg OVA also suppressed the levels of OVA-specific IgE in serum by 48% but this reduction did not reach the level of significance ($P=0.09$, fig. 2C). The up-regulation of OVA-specific IgE levels in serum were slightly changed (22% decrease, not significant) by treatment of mice with 10 μg OVA-IT (fig. 2C).

So, low dose of OVA-IT (10 μg) failed to counteract the asthma like symptoms after OVA inhalation challenge. On the other hand, suboptimal OVA-IT (100 μg) partially suppressed the airway eosinophilia in the BAL fluid and OVA-specific IgE levels in serum but not the AHR. Treatment with optimal OVA-IT (1 mg) effectively suppressed AHR, airway eosinophilia and OVA-specific IgE levels in serum.

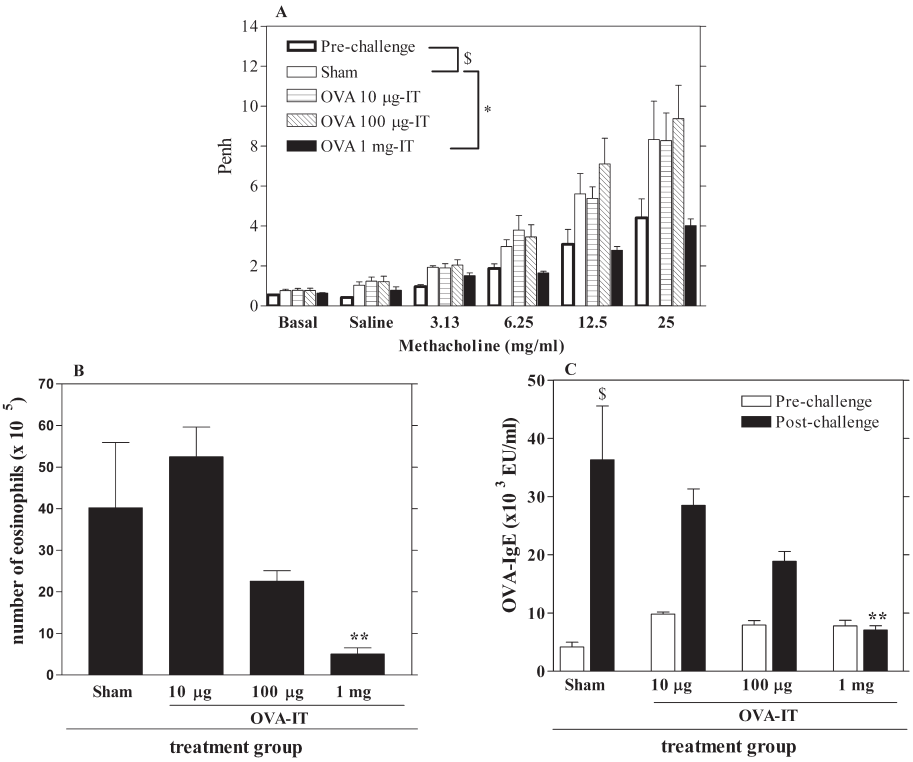

Figure 2. *Dose-response relation of OVA-IT. OVA-sensitized mice received IT with 10 µg, 100 µg or 1 mg OVA. (A) airway responsiveness to methacholine just before (pre-challenge) and 1 day after the last OVA aerosol challenge, (B) eosinophil numbers in BAL fluid and (C) levels of OVA-specific IgE in serum just before (pre-challenge) and 1 day after (post-challenge) OVA aerosol challenge. Values are expressed as mean ± SEM (n=6). Penh, enhanced pause. $P<0.05 compared to pre-challenge. *P<0.05, **P<0.01 compared to sham-treated mice.*

Effects of co-administration 10 ng 1,25(OH)$_2$D$_3$ on suboptimal OVA-IT

Subsequently, we aimed to determine whether co-administration of 10 ng 1,25(OH)$_2$D$_3$ could potentiate suboptimal IT. For this purpose, mice were treated with 100 µg OVA-IT combined with 10 ng 1,25(OH)$_2$D$_3$.

Airway responsiveness. In fig. 3A only the responses to 25 and 50 mg/ml methacholine of the complete DRC are shown. Compared to sham treatment, IT with suboptimal (100 µg) OVA failed to reduce development of OVA-induced AHR to methacholine (fig. 3A). Interestingly, co-administration of 10 ng 1,25(OH)$_2$D$_3$ with 100 µg OVA-IT significantly suppressed AHR to methacholine compared with sham-treated mice (P<0.05, fig. 3A and **Supplemenatry fig. S1**). 10 ng 1,25(OH)$_2$D$_3$ by itself did not change AHR in sham-treated mice (fig. 3A).

Eosinophils and cytokine levels in the BAL fluid. 100 μg OVA-IT partially suppressed (31%, not significant) the influx of airway eosinophilia in the BAL fluid (fig. 3B). Importantly, co-administration of 10 ng $1,25(OH)_2D_3$ with 100 μg OVA-IT effectively reduced airway eosinophilia by 68% ($P<0.05$) compared with sham-treated mice and by 53% ($P<0.01$) compared with mice merely receiving 100 μg OVA-IT (fig. 3B). The BAL fluid from sham-treated mice showed no changes in number of eosinophils after treatment with 10 ng $1,25(OH)_2D_3$ (fig. 3B).

In parallel with the reduction in airway eosinophilia, there was a significant fall in IL-5 levels by 58% ($P<0.05$, fig. 3C) and IL-13 levels by 76% ($P<0.05$, data not shown) in BAL fluid after co-administration of 10 ng $1,25(OH)_2D_3$ with 100 μg OVA-IT compared to sham-treated mice. 100 μg OVA-IT partially reduced (37%, not significant) the levels of IL-10 in BAL fluid compared with sham-treated mice and this effect was not influenced by co-administration of 10 ng $1,25(OH)_2D_3$ (data not shown). No reduction was found in IL-5, IL-13 and IL-10 levels in BAL fluid from sham and 10 ng $1,25(OH)_2D_3$-treated mice (fig. 3C and data not shown, respectively).

TGF-β levels in BAL fluid and serum. We next examined whether co-administration of 10 ng $1,25(OH)_2D_3$ influenced the levels of the regulatory cytokine TGF-β. High levels of TGF-β were present in BAL fluid of sham-treated mice (fig. 3D). 100 μg OVA-IT significantly reduced the levels of TGF-β by 38% ($P<0.05$) in BAL fluid compared with sham-treated mice (fig. 3D). Co-administration of 10 ng $1,25(OH)_2D_3$ did not influence the reduction in TGF-β levels in BAL fluid induced by 100 μg OVA-IT (fig. 3D). Interestingly, we found that co-administration of 10 ng $1,25(OH)_2D_3$ with 100 μg OVA-IT displayed markedly increased serum levels of TGF-β compared with sham-treated mice and with mice treated only with 100 μg OVA-IT ($P<0.001$, fig. 3E). In addition, treatment with 10 ng $1,25(OH)_2D_3$ did not influence the levels of TGF-β in the BAL fluid and serum obtained from sham-treated mice (figs. 3D and E, respectively).

Serum levels of immunoglobulins. We further examined whether co-administration of 10 ng $1,25(OH)_2D_3$ with 100 μg OVA-IT could influence the levels of immunoglobulins in serum. In sham-treated mice, OVA challenge displayed significantly increased (92%, $P<0.01$) OVA-specific IgE levels in serum compared with pre-challenge levels (fig. 3F). IT with 100 μg OVA partially down-regulated (41%, not significant) the OVA-specific IgE levels in serum (fig. 3F). Interestingly, co-administration of 10 ng $1,25(OH)_2D_3$ with 100 μg OVA-IT displayed significantly suppressed OVA-IgE levels in serum by 61% ($P<0.05$) compared to sham-treated mice and by 34% ($P<0.05$) compared to mice treated with 100 μg OVA-IT alone (fig. 3F). In contrast, we found that, combination of 10 ng $1,25(OH)_2D_3$ with 100 μg OVA-IT caused significantly increased OVA-specific IgA (55%, $P<0.05$) levels in serum compared with sham-treated mice (fig. 3G). In addition, 10 ng $1,25(OH)_2D_3$ by itself had no significant effect on OVA-specific IgE and IgA levels in serum of sham-treated mice (figs. 3F and G, respectively). Furthermore, in sham-treated mice, OVA challenge induced an increase of OVA-specific IgG1 (99%, $P<0.05$) and IgG2a (97%, $P<0.05$) levels in serum compared with pre-challenge levels (table I). In OVA-sensitized mice, suboptimal OVA-IT markedly increased the serum OVA-specific IgG1 (99%, $P<0.05$) and of IgG2a

(98%, P<0.01) levels compared with pre-challenge levels of sham-treated mice (table I). Co-administration of 10 ng $1,25(OH)_2D_3$ with 100 µg OVA-IT caused a further increase in levels of OVA-specific IgG1 (10%, P=0.81) and of IgG2a (68%, P<0.05) in serum compared to mice treated with 100 µg OVA-IT alone (table I). These levels did not increase further on OVA-challenge, except for the IgG1 serum levels (table I).

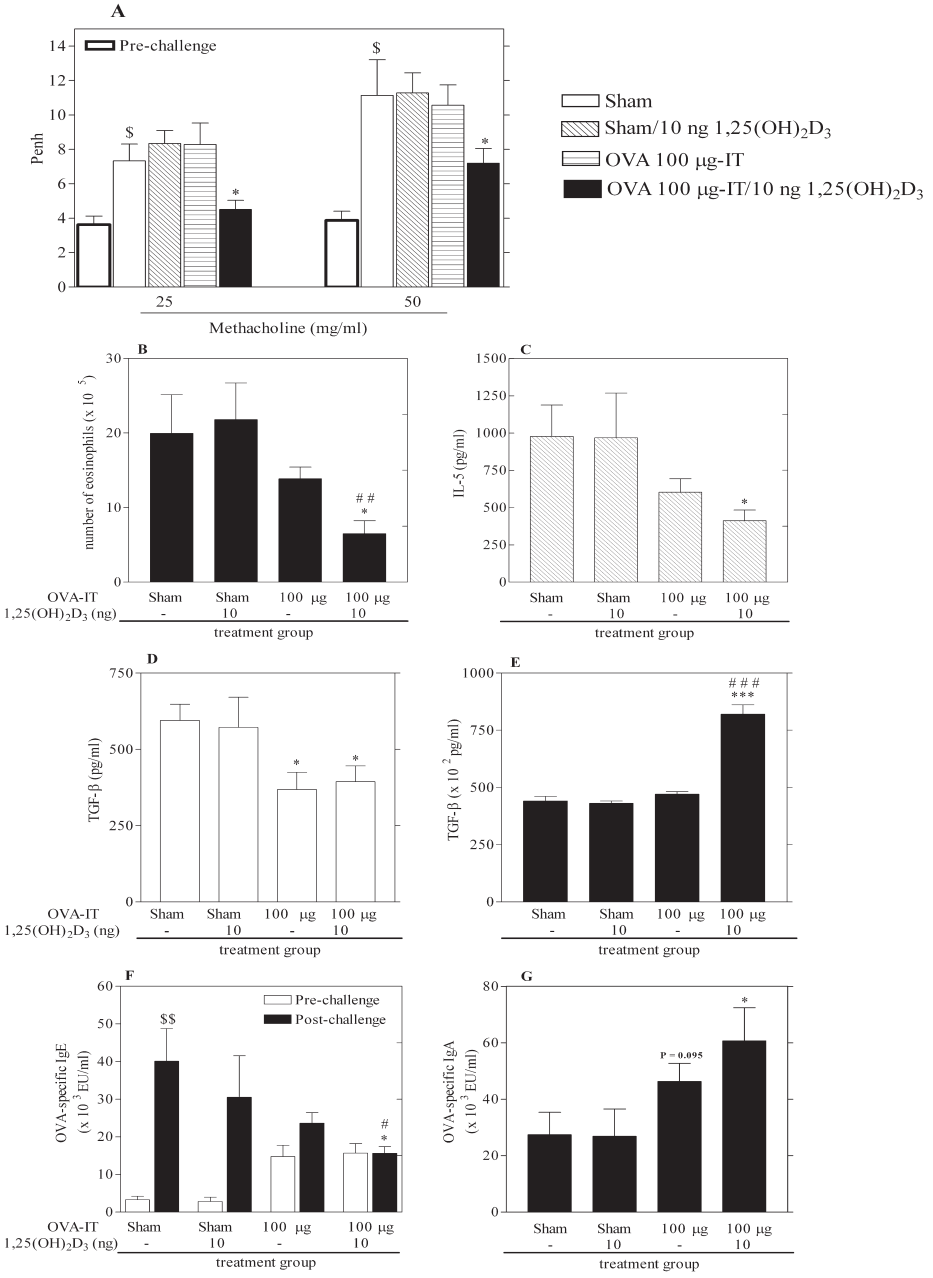

Figure 3. *Effects of co-administration of $1,25(OH)_2D_3$ on the efficacy of suboptimal IT. OVA-sensitized*

*mice received IT with 100 μg OVA combined with 10 ng 1,25(OH)$_2$D$_3$. (A) airway responsiveness to methacholine just before (pre-challenge) and 1 day after OVA aerosol challenge, (B) eosinophil numbers, (C) IL-5 levels and (D) TGF-β levels in BAL fluid, (E) TGF-β levels in serum, (F) levels of OVA-specific IgE in serum just before (pre-challenge) and 1 day after (post-challenge) OVA aerosol challenge and (G) levels of OVA-specific IgA in serum 1 day after OVA aerosol challenge. Values are expressed as mean ± SEM (n=6). Penh, enhanced pause. $P<0.05 and $$P<0.01 compared to pre-challenge. *P<0.05, ***P<0.001 compared to sham-treated mice. #P<0.05, ##P<0.01,###P<0.001 compared to OVA 100 μg-IT treated mice.*

Table I. *Serum OVA-specific antibody levels.*

Treatment group	Ig Isotype			
	OVA-specific IgG1 Mean ± SEM (x 10^6 EU/ml)		OVA-specific IgG2a Mean ± SEM (x 10^5 EU/ml)	
	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge
Sham	0.38 ± 0.18	26.49 ± 9.56 #	0.17 ± 0.13	6.44 ± 2.07 #
Sham + 10 ng 1,25(OH)$_2$D$_3$	0.26 ± 0.13	21.48 ± 6.31 $	0.56 ± 0.29	7.16 ± 3.91
OVA 100 μg-IT	48.26 ± 17.67 #	39.12 ± 6.99	11.12 ± 2.91##	8.27 ± 1.36
OVA 100 μg-IT + 10 ng 1,25(OH)$_2$D$_3$	53.73 ± 13.87 ##	61.73 ± 11.85 *	34.18 ± 9.81## €	22.92 ± 6.83 *

*OVA-sensitized mice received IT with 100 μg OVA combined with 10 ng 1,25(OH)$_2$D$_3$. Levels of OVA-specific IgG1 and IgG2a in serum were measured before (pre-challenge) and after (post-challenge) OVA aerosol challenge. Values are expressed as mean ± SEM (n=6). #P<0.05, ##P<0.01 compared to pre-challenge levels of sham-treated mice. $P<0.05 compared to pre-challenge levels of same treated mice. €P<0.05 compared to pre-challenge levels of OVA 100 μg-IT treated mice. *P<0.05 compared to post-challenge levels of sham-treated mice.*

Determination of cytokine production by OVA-restimulated TLN cells in vitro. To examine T-cell responses on antigen-specific re-stimulation, single-cell suspensions of TLN of each mouse were prepared 24 h after the last OVA aerosol challenge. OVA re-stimulated TLN-cell cultures derived from sham-treated mice produced significantly (P<0.001) higher amounts of IL-5, IL-13 and IL-10 (figs. 4A, B and C respectively), than those from non-restimulated TLN-cell cultures. TLN-cell cultures derived from mice treated only with 100 μg OVA-IT or only with 10 ng 1,25(OH)$_2$D$_3$ produced similar levels of these cytokines after OVA re-stimulation *in vitro* as those of sham-treated mice. However, the amount of IL-5 in TLN-cell cultures from mice that received the combination of 100 μg OVA-IT with 10 ng 1,25(OH)$_2$D$_3$ was reduced (79%, P<0.001) compared with those from sham-treated mice and by 65% (P<0.05) compared with those from mice treated only with 100 μg OVA-IT (fig. 4A). Also, the levels of IL-13 and IL-10 were significantly reduced in the OVA-restimulated TLN-cell cultures from mice treated with 100 μg OVA-IT combined with 10 ng 1,25(OH)$_2$D$_3$ compared with those from sham-treated mice (57%, P<0.05 and 72%, P<0.01, figs. 4B and C respectively) and by 60% (P<0.05) and by 58% (P<0.05) for IL-13 and IL-10 respectively compared with those from mice merely received 100 μg

OVA-IT (figs. 4B and C). These reductions of IL-5, IL-13 and IL-10 levels were also observed when TLN-cell cultures were re-stimulated with anti-CD3 antibody (figs. 4A, B and C respectively).

So, co-administration of 10 ng $1,25(OH)_2D_3$ potentiated the immunosuppressive effects of suboptimal IT with 100 μg OVA on AHR, OVA-specific IgE levels in serum, BAL eosinophilia and Th2 cytokine levels in BAL fluid and on *in vitro* allergen re-stimulated TLN-cell cultures.

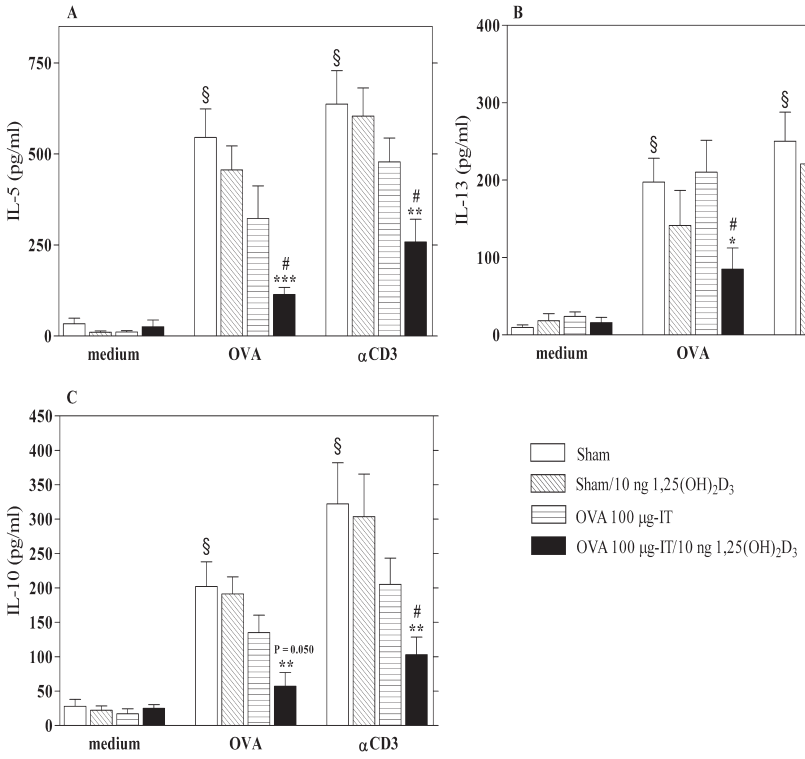

Figure 4. *Effects of co-administration of $1,25(OH)_2D_3$ on the efficacy of suboptimal IT. OVA-sensitized mice received IT with 100 μg OVA combined with 10 ng $1,25(OH)_2D_3$. (A) IL-5, (B) IL-13 and (C) IL-10 levels in OVA-restimulated TLN-cell cultures 1 day after OVA aerosol challenge. Values are expressed as mean ± SEM (n=6). §P<0.001 compared to non-stimulated (medium) sham-treated mice. *P<0.05, **P<0.01, ***P<0.001 compared to sham-treated mice. #P<0.05 compared to OVA 100 μg-IT treated mice.*

IL-10 and TGF-β are crucial in tolerance induction by co-administration of 10 ng $1,25(OH)_2D_3$ with suboptimal IT

We next investigated the role of IL-10 and TGF-β in the observed immunosuppressive effects induced by combination of 100 μg OVA-IT with 10 ng $1,25(OH)_2D_3$. OVA-sensitized mice were treated with 100 μg OVA-IT combined with 10 ng $1,25(OH)_2D_3$

and, IL-10R and TGF-β were functionally blocked using mAbs *in vivo* on the first day of OVA aerosol challenge.

Eosinophils and cytokine levels. We determined the number of eosinophils in BAL 24 h after the last OVA aerosol challenge. In mice that received control antibodies, 100 μg OVA-IT combined with 10 ng $1,25(OH)_2D_3$ readily suppressed OVA-induced influx of eosinophils into the BAL fluid by 74% ($P<0.05$) compared with sham-treated mice (fig. 5A). In contrast, mice treated with anti-IL-10R mAb, 100 μg OVA-IT combined with 10 ng $1,25(OH)_2D_3$ failed to suppress OVA-induced influx of eosinophils into the BAL fluid (fig. 5A). Similar results were obtained with anti-TGF-β (fig. 5A). However, blocking of IL-10R together with anti-TGF-β showed a further increase in number of eosinophils in BAL fluid compared with blocking of IL-10R or anti-TGF-β alone implying an additive effect between both cytokines IL-10 and TGF-β (not significant, fig. 5A).

In parallel with airway eosinophilia, treatment with anti-IL-10R or anti-TGF-β alone partially reversed the suppression in levels of the Th2-associated asthmatogenic cytokines IL-5 (fig. 5B) and IL-13 (fig. 5C) in BAL fluid induced by 100 μg OVA-IT combined with 10 ng $1,25(OH)_2D_3$. IL-5 and IL-13 levels were completely reversed when both IL-10R and TGF-β were functionally blocked *in vivo* (figs. 5B and C respectively). In addition, IL-10 levels were completely reversed in BAL fluid obtained from mice treated with either anti-IL-10R, anti-TGF-β or both (fig. 5D). Furthermore, the increased TGF-β levels in serum of 100 μg OVA-IT combined with 10 ng $1,25(OH)_2D_3$-treated mice were not affected by treatment with anti-IL-10R or anti-TGF-β (fig. 5E), while it was significantly suppressed in mice treated with both anti-IL-10R and anti-TGF-β ($P<0.05$, fig. 5E).

Serum levels of immunoglobulins. The serum antibody levels of OVA-specific IgE from mice that received 100 μg OVA-IT combined with 10 ng $1,25(OH)_2D_3$ and control antibodies were significantly lower by 67% ($P<0.05$) than those from sham and control antibodies-treated mice (fig. 5F). As fig. 5F shows, a partial recovery in OVA-specific IgE levels in serum was observed after treatment with either anti-IL-10R or anti-TGF-β alone. Treatment of mice with both anti-IL-10R and anti-TGF-β completely reversed the suppression of OVA-specific IgE levels in serum (fig. 5F). Interestingly, the increased OVA-specific IgA levels in serum from mice treated with 100 μg OVA-IT combined with 10 ng $1,25(OH)_2D_3$ were remarkably down-regulated after treatment with anti-IL-10R (57% suppression, $P=0.08$), anti-TGF-β (71% suppression, $P<0.05$) or both (70% suppression, $P<0.05$) (fig. 5G). Further, treatment with anti-IL-10R, anti-TGF-β or both did not change the IgG1 levels in serum of 100 μg OVA-IT combined with 10 ng $1,25(OH)_2D_3$ and control antibodies-treated mice (table II). Compared to mice merely receiving 100 μg OVA-IT combined with 10 ng $1,25(OH)_2D_3$ and control antibodies, IgG2a levels were partially lower (not significant) after treatment with anti-IL-10, anti-TGF-β or both (table II).

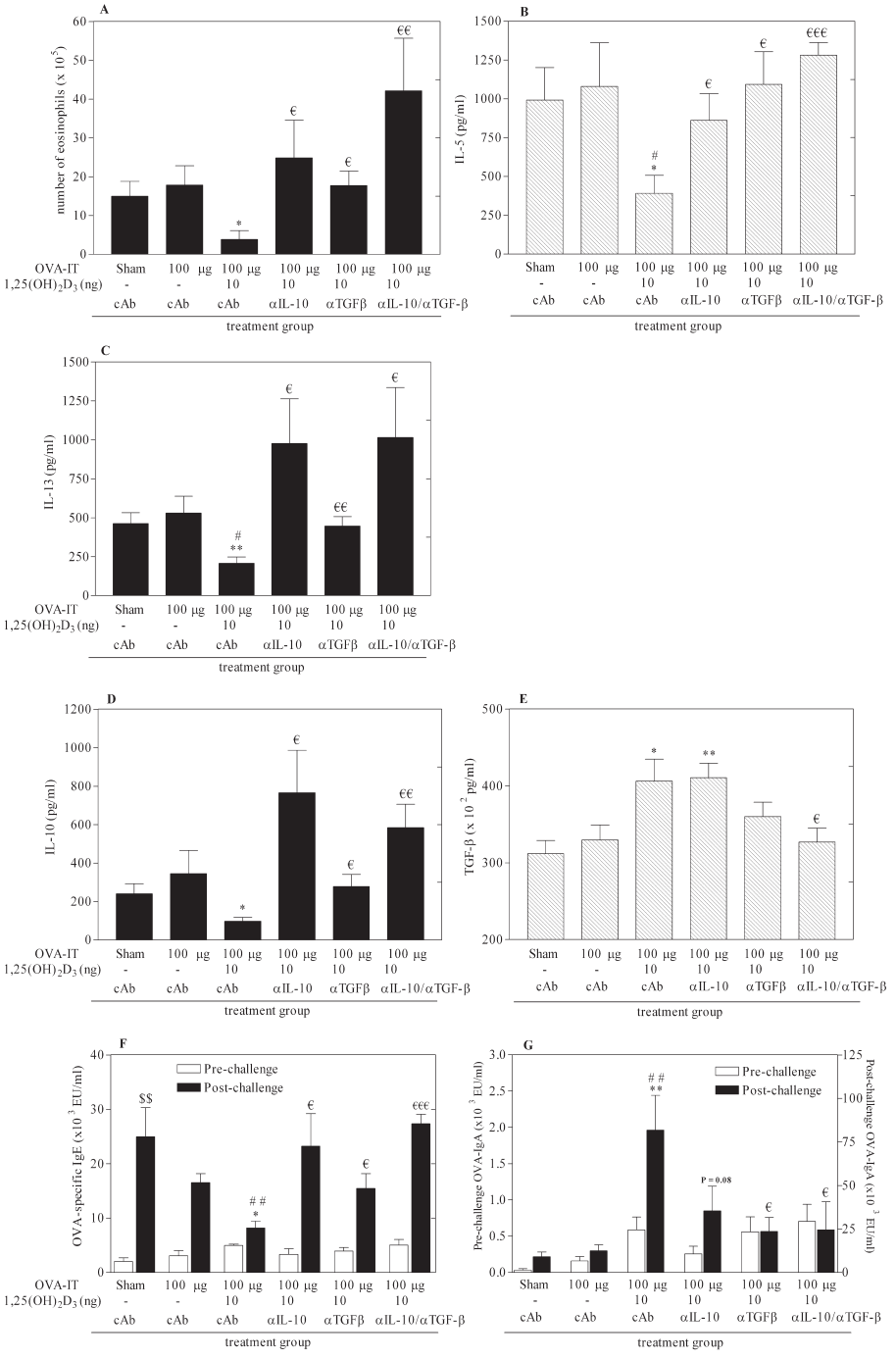

Figure 5. *Effects of anti-IL-10R, anti-TGF-β or both on the immunosuppressive effects induced by suboptimal IT combined with 1,25(OH)$_2$D$_3$. OVA-sensitized mice were treated with 100 µg OVA*

combined with 10 ng 1,25(OH)$_2$D$_3$ and received i.p. treatment with anti-IL-10R or anti-TGF-β or both just before the first OVA aerosol challenge. (A) number of eosinophils, (B) IL-5 levels, (C) IL-13 levels and (D) IL-10 levels in BAL fluid, (E) TGF-β levels in serum, (F) levels of OVA-specific IgE and (G) OVA-specific IgA in serum just before (pre-challenge) and 1 day after (post-challenge) OVA aerosol challenge. Values are expressed as mean ± SEM (n=6). $$P<0.01 compared to pre-challenge. *P<0.05, **P<0.01 compared to sham-treated mice. #P<0.05, ##P<0.01 compared to OVA 100 μg-IT treated mice. €P<0.05, €€P<0.01, €€€P<0.001 compared to mice treated with OVA 100 μg-IT combined with 10 ng 1,25(OH)$_2$D$_3$ and control antibodies (cAb= rIgG + mIgG).

Table II. *Serum OVA-specific antibody levels.*

Treatment group	Ig Isotype			
	OVA-specific IgG1 Mean ± SEM (x 10^6 EU/ml)		OVA-specific IgG2a Mean ± SEM (x 10^4 EU/ml)	
	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge
Sham + rIgG + mIgG	0.00 ± 0.00	11.30 ± 2.04 $$	0.79 ± 0.52	34.87 ± 12.94 $
OVA 100 μg-IT + rIgG + mIgG	9.42 ± 2.60	12.12 ± 2.93	1.88 ± 0.81	29.24 ± 8.05 $
OVA 100 μg-IT + 10 ng 1,25(OH)$_2$D$_3$ + rIgG + mIgG	16.51 ± 2.02	17.14 ± 1.84	11.64 ± 4.25	49.01 ± 16.46
OVA 100 μg-IT + 10 ng 1,25(OH)$_2$D$_3$ + anti-IL-10R	8.30 ± 2.74	14.82 ± 3.62	0.99 ± 0.55	28.53 ± 10.71 $
OVA 100 μg-IT + 10 ng 1,25(OH)$_2$D$_3$ + anti-TGF-β	10.91 ± 2.13	16.10 ± 1.32	5.32 ± 4.05	37.21 ± 6.90 $
OVA 100 μg-IT + 10 ng 1,25(OH)$_2$D$_3$ + anti-IL-10R + anti TGF-β	13.65 ± 3.98	17.15 ± 1.33 *	19.54 ± 12.79	31.87 ± 6.41

*OVA-sensitized mice were treated with 100 μg OVA combined with 10 ng 1,25(OH)$_2$D$_3$ and received i.p. treatment with anti-IL-10R or anti-TGF-β alone or both just before the first OVA aerosol challenge. Levels of OVA-specific IgG1 and IgG2a in serum were measured before (pre-challenge) and after (post-challenge) OVA aerosol challenge. Values are expressed as mean ± SEM (n=6). $P<0.05, $$P<0.01 compared to pre-challenge levels. *P<0.05 compared to post-challenge levels of sham-treated mice.*

Discussion

In the present study, it is demonstrated that co-administration of 1,25(OH)$_2$D$_3$ significantly augments the beneficial effects of IT on antigen-induced allergic asthma manifestations in a mouse model. Augmentation of the suppression of allergen-specific IgE levels, BAL eosinophilic airway inflammation and AHR is associated with

reduced Th2 cytokine levels in BAL fluid and reduced production of Th2-cytokines by T-cells upon antigen restimulation *in vitro*. Additionally, we demonstrated that the suppressive effects of IT combined with $1,25(OH)_2D_3$ are completely abrogated after treatment with anti-IL-10R and anti-TGF-β, indicating a crucial role of these immunoregulatory cytokines.

In humans, specific allergen IT is widely used to treat allergic rhinitis, conjunctivitis and occasionally allergic asthma. IT reduces symptoms and medication requirements and is currently the only long-term disease-modifying treatment. Although the precise mechanisms of IT are still incompletely understood, recent data suggest an important role for IL-10-producing type 1 regulatory T (Tr1) cells and TGF-β-producing Th3 type cells in IT against bee venom, house dust mite and grass pollen (8-10). In agreement herewith, we recently demonstrated that IL-10 plays a crucial role in the suppression of airway manifestation of asthma after allergen IT in a mouse model (19). Since in the clinic, improvement of the efficacy of IT is desirable, we were interested to examine whether the suppressive effects could be potentiated by $1,25(OH)_2D_3$, the active form of vitamin D_3.

The biological effects of $1,25(OH)_2D_3$ are mediated by the vitamin D receptor which acts as a transcriptional regulator by binding to vitamin D responsive elements within the promoters of target genes. The rationale to use $1,25(OH)_2D_3$ in combination with IT is based on the observations that it inhibits the NF-κB protein RelB expression in murine DCs (20). Besides skewing T-cells towards Th1 or Th2, DCs are instrumental in the generation of adaptive Treg cells like Th3- and Tr1 cells (11, 12). Immature/tolerogenic DCs induce development of Treg cells by several mechanisms, including production of IL-10 or TGF-β (13, 17). A number of studies has demonstrated that inhibition of NF-κB by $1,25(OH)_2D_3$ induces a persistent immature/tolerogenic phenotype in human and mouse DCs characterized by reduced expression of MHC-II, costimulatory molecules and IL-12 and strongly enhanced production of IL-10, but not TGF-β (25, 26, 36). DCs modulated by $1,25(OH)_2D_3$ or its analogs induce decreased T-cell proliferation and favor development of Treg cells *in vitro* and *in vivo* (27, 28, 37). Thus, combination of $1,25(OH)_2D_3$ with allergen IT may promote the generation of Treg cells that will suppress Th2-driven asthma manifestations at the time of allergen inhalation.

This is, to our best knowledge, the first study showing that $1,25(OH)_2D_3$ potentiates the suppressive effects of IT in a mouse model of allergic asthma. Complete reversal of the suppressive effects was observed after blocking the IL-10R and neutralizing TGF-β by monoclonal antibodies at the time of antigen inhalation challenge. These data strongly indicate that IL-10 and TGF-β producing Treg cells, in particular respectively Tr1 and Th3 cells, are induced during IT and become reactivated to exert their anti-asthmogenic effects during antigen inhalation challenge. However, we did not observe increased levels of the immunoregulatory cytokines IL-10 (data not shown) and TGF-β in BAL fluid after antigen challenge. In addition, IL-10 secretion upon restimulation of T-cells isolated from lung-draining lymph nodes was decreased after IT. These findings do not support the concept that induction of cytokine producing Treg cells mediates the immunosuppressive effects. Nevertheless, it remains possible that Treg cells exert their immunosuppression earlier in time or at another location. Unexpectedly, we observed increased serum levels of TGF-β after antigen challenge in mice after IT combined with $1,25(OH)_2D_3$ which supports

a critical role for this cytokine although its cellular source remains undefined.

In addition to keeping DCs in an immature/tolerogenic state, $1,25(OH)_2D_3$ may also locally increase TGF-β production. Whereas $1,25(OH)_2D_3$ has not been shown to directly induce TGF-β secretion by DCs (37), it has been demonstrated that it induces TGF-β in a.o. fibroblasts (38). Therefore, TGF-β produced in the local microenvironment may act in concert with tolerogenic DCs in the generation of TGF-β producing Th3 type cells (39). $1,25(OH)_2D_3$ has also been shown to directly affect Th cell polarization by augmenting Th2 development, independent of an effect on antigen-presenting cells (40). However, it appears unlikely that this plays a role in the induction of a memory suppressive response as observed after IT in the mouse model (19).

Yet another explanation for the augmentation of IT effects by $1,25(OH)_2D_3$ may be related to the reported induction of the chemokine receptor CCR10 during T-cell activation in the presence of $1,25(OH)_2D_3$ which enables them to migrate to mucosal tissues expressing CCR10 ligands (41). CCR10 is expressed by a subpopulation of Treg cells that can be recruited to mucosal tissues by epithelial-derived CCL28 (42, 43). In this way, $1,25(OH)_2D_3$ may increase the number of CCR10 expressing Treg cells that can be recruited to the airways at the time of allergen inhalation.

It is well-known that TGF-β plays a critical role in the isotype switch of B cells towards IgA (44, 45). Likewise, increased levels of OVA-specific IgA in serum were detected after combination of IT with $1,25(OH)_2D_3$ while administration of anti-TGF-β significantly suppressed the increased serum IgA levels. Similarly, in human studies, it has been clearly demonstrated that TGF-β secretion by allergen-specific T-cells is increased after IT with house-dust mite which is associated with increased serum Der p1-specific IgA levels (9). Interestingly, by binding to FcαRI on leukocytes, serum IgA may have potent anti-inflammatory effects mediated by intracellular recruitment of the tyrosine phosphatase SHP-1 (46). In agreement herewith, Schwarze *et al.* demonstrated that allergen-specific IgA completely prevented the development of AHR to methacholine, reduced eosinophilic airway inflammation and increased allergen-specific IgG2a in a mouse model of allergic asthma (47). Thus, the TGF-β/IgA/FcαRI pathway may by one of the effector mechanisms to suppress asthma manifestations after IT.

In summary, we demonstrate in a mouse model of allergic asthma that co-administration of $1,25(OH)_2D_3$ augments the efficacy of IT to prevent Th2-driven development of asthma manifestations. The immunoregulatory cytokines IL-10 and TGF-β play a crucial role in these beneficial effects of this combined therapy. This may be a novel strategy to improve allergen immunotherapy for the treatment of allergic diseases, asthma included.

Aknoledgment

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1α,25-dihydroxyvitamin D_3 potentiates the beneficial effects of allergen immunotherapy in a mouse model of allergic asthma: role for IL-10 and TGF-β

Supplementry material

Supplementary Method

Resistance-compliance measurement. Before measurement mice were anesthetised by i.p. injection of urethane (2 g/kg, Sigma), tracheotomized and i.v.-cannulated (Vena jugulars). Mice were then attached to a computer controlled small animal ventilator (Flexivent, SCIREQ, Canada). Spontaneous breading was stopped by i.p. administration of d-tubocurarine (Sigma), 1 mg/kg. Mice were ventilated at a breeding frequency of 280 breaths per minute and a tidal volume of 10 ml/kg. Tidal volume was pressure-limited at 300 mmH_2O. Resistance and compliance in response to metacholine (acetyl-β-methylcholine chloride, Sigma) were calculated from the pressure response to a 2 s pseudo random pressure wave. Each mouse was challenged with increasing doses of methacholine (0, 40, 80, 160, 320, 640 and 1280 μg/kg in saline) administered i.v. as described previously (48).

Supplementary Results

To investigate whether combination of IT with 10 ng $1,25(OH)_2D_3$ also reduce the development of airway resistance, we measured respiratory system resistance changes in response to methacholine 24 h after the last challenge. OVA-sensitized and challenged mice exhibited significantly higher ($P<0.001$) airway resistance to graded doses of methacholine than those of OVA-sensitized PBS-challenged mice (fig. S1). Compared to sham treatment, OVA-IT failed to reduce the development of OVA challenge -induced airway resistance to methacholine. Interestingly, co-administration of 10 ng $1,25(OH)_2D_3$ with OVA-IT significantly suppressed the airway resistance to methacholine compared to sham-treated OVA challenged mice ($P<0.05$, fig. S1). No significant changes was found in airway resistance to methacholine in sham and 10 ng $1,25(OH)_2D_3$-treated mice.

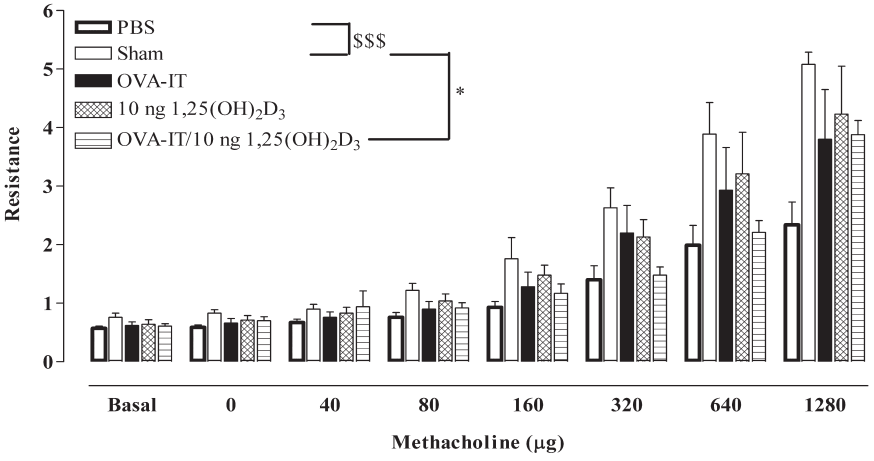

Figure S1. *Effects of co-administration of $1,25(OH)_2D_3$ on the efficacy of OVA-IT. OVA-sensitized mice received OVA-IT alone or combined with 10 ng $1,25(OH)_2D_3$. Airway resistance to methacholine 1 day after the last OVA aerosol challenge. Values are expressed as mean ± SEM. $$$P<0.001 compared to PBS challenge control mice. *P<0.05 compared to sham-treated OVA challenge mice.*

Chapter 6

Summarizing Discussion

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

1. Introduction

Allergic asthma is characterized by persistent allergen-driven airway inflammation, remodeling and airway hyperresponsiveness (AHR). $CD4^+$ T-cells, in particular Th2-type cells, play a critical role in orchestrating the disease process through the release of the cytokines IL-4, IL-5 and IL-13 (1-5). Allergen-specific immunotherapy (IT) is currently the only treatment that can have a long-term effect *via* modifying the natural course of respiratory allergy by interfering with the underlying immunological mechanisms. Allergen IT is particularly beneficial for treatment of allergic rhinitis and insect venom allergy, but it is less effective in allergic asthma where it seldom leads to complete alleviation of symptoms. The major problem of this intervention is the risk of systemic IgE-mediated reactions resulting from cross-linking by allergen. Improvement of allergen IT is needed to enhance its efficacy before widespread application in asthmatic patients can be considered. Elucidation of the precise mechanism(s) involved has potential implications for improving IT. The mechanisms underlying therapeutic efficacy of IT includes induction of blocking IgG antibodies (6), induction of T-cell anergy (7), and deviation of a Th2-type cytokine response toward a Th1 response (8, 9). In recent human studies, induction of adaptive regulatory T (aTreg) cells secreting the immunoregulatory cytokines IL-10 and TGF-β has been shown to occur after IT using bee-venom, house dust mite or pollen (10-12). However, it remains to be determined whether these cells are really responsible for the beneficial effects of IT, which is difficult to demonstrate in human studies.

Previously, we developed an allergic asthma mouse model by systemic (i.p.) sensitization and repeatedly challenge with inhalation of allergen (ovalbumin; OVA) aerosol (13). This model is characterized by allergen-specific Th2-mediated immune responses such as the presence of allergen-specific IgE in serum, airway eosinophilia and AHR to non-specific stimuli (methacholine); features comparable to that of human allergic asthma. Using this mouse asthma model we have recently shown that allergen IT induces a suppressive memory response mediated by IL-10, supporting the hypothesis that the induction of IL-10 producing type 1 regulatory T (Tr1) cells is fundamental for successful IT (14). However, we have not yet been able to identify whether this IL-10 is produced by Tr1 cells. In addition, increased IL-10 (and TGF-β) production after IT may not be limited to T-cells. B cells, monocytes and macrophages can also produce IL-10 after IT (10, 15). It is therefore possible that these cells are involved in the suppressive effects of IT, either directly or through the induction of aTreg cells. The production of these inhibitory cytokines has the potential to suppress local allergen-specific Th2 cell responses and redirect antibody class switching in favor of IgG (IL-10 isotype switch factor) and IgA (TGF-β isotype switch factor). At present little is known about the mechanisms that underlie the induction of immunological tolerance by allergen IT in allergic asthma. In this thesis, we aimed to further determine the mechanisms that contribute to the induction of tolerance by allergen IT in an OVA-induced murine model of allergic asthma with the ultimate goal to improve the treatment efficacy of allergen IT.

To this end, we addressed the following questions:

1. Do IgG Fcγ receptors (FcγR) play a role in tolerance induction by allergen IT (chapter 2)?
2. Is the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO), expressed in dendritic cells (DCs) and involved in immune suppression and tolerance induction, required for successful IT (chapter 3)?
3. Does the induction of immune tolerance by allergen IT require the suppressive activity of nTreg cells (chapter 4)?
4. Does the inhibition of NF-κB, important for DC maturation, by the active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), potentiate the suppressive effects of IT, and if so, are the immunoregulatory cytokines IL-10 and TGF-β involved in the effector phase (chapter 5)?

2. IgG Fcγ receptors are not involved in tolerance induction by allergen IT in a mouse asthma model

In 1935, Cooke *et al.* provided the first insight into the mechanism of IT by showing that the transfusion of blood from a patient who had been successfully treated with IT to an allergic recipient cured hay fever in the latter (16). A few years later, the transferable factor was identified as IgG antibody. These observations led to the so-called "blocking antibody theory". The research study described in chapter 2 was based on these and other studies demonstrating that allergen-specific IgG antibodies induced by IT contribute to a positive response both in human and animal models. IgG antibodies can compete with IgE for binding to the allergen, thereby preventing effector cell degranulation (17-19), and blocking of IgE-facilitated allergen presentation to T-cells (20, 21). In addition, antigen-IgG immune complexes may act by engaging Fc receptors on antigen presenting cells (APCs). It has been shown that antigen-IgG immune complexes can stimulate IL-10 production by murine macrophages *via* FcγRI (22). Moreover, recent findings demonstrate that signaling *via* FcγRIIB on DCs is essential to regulate antigen presentation, to down-regulate DC maturation, and for the generation of aTreg cells in a mouse model of mucosal tolerance (23).

The IgG Fcγ receptors expressed on hematopoietic cells play a key role in immune defenses by linking humoral and cellular immunity. The characterization of Fcγ receptors-deficient mice has revealed the importance of these receptors in the development or suppression of antibody-mediated responses. To elucidate the role of Fcγ receptors in tolerance induction by allergen IT, we investigated the efficacy of allergen IT in Fcγ knockout mice lacking the inhibitory receptor FcγRIIB (24), and in mice deficient in the activation receptors FcγRI and III (24). Our findings clearly demonstrate that the absence of Fcγ receptors did not disturb the efficacy of allergen IT (chapter 2). We showed that bronchoalveolar lavage (BAL) eosinophilia, Th2 cytokines, and serum OVA-specific IgE levels in Fcγ knockout mice are strongly suppressed by allergen IT, demonstrating that IgG Fcγ receptors have no major role in the beneficial effects of allergen IT in the murine model that was used. Conversely, other investigators have shown that FcγRIIB plays a critical role in mucosal tolerance to airborne and food allergy in mice treated with IT *via* nasal or oral routes (23).

Samsom *et al.* demonstrated that IgG-FcγRIIB signaling on DCs are essential to down-regulate DC activation, leading to mucosal regulatory T cell induction and subsequent tolerance to a systemic delayed type hypersensitivity response (23). This discrepancy could be ascribed to differences in the protocols used by these two studies (chapter 2 and (23)) or to differences between the mechanism(s) involved in immune suppression after s.c. allergen IT to that after mucosal tolerance induction. Upon mucosal tolerance induction the antigen is carried to the mucosa draining lymph nodes where mucosal regulatory T-cells are formed (25, 26). In many aspects the microenvironment in these nodes is different from that of peripheral lymph nodes (27), in particular they contain more IgG2b producing plasma cells when compared to peripheral nodes (28), the site to which s.c. IT antigen will drain. Additionally, in our model OVA IT was carried out during the time at which OVA-specific IgGs were present (after parenteral sensitization of mice), whereas in the mucosal tolerance studies mucosal OVA application was performed at a time before OVA-specific IgG induction may have occurred (prior to parenteral sensitization). Therefore, due to these micoenvironmental/time differences mucosal tolerance responses may depend on FcγRIIB whereas peripheral tolerogenic responses do not. Similarly, different mechanisms are involved in tolerance induction *via* different routes e.g. oral tolerance has been shown to be mediated by TGF-β producing Th3 cells, whereas intranasal tolerance appears to be mediated by IL-10 producing Tr1 cells (29, 30).

In human studies, no evidence has been found that signaling *via* FcγRIIB plays a role in the inhibitory effect of allergen IT using the major allergen from birch pollen *Bet v 1* (31). Ejrnaes *et al.* showed that the blocking of FcγRIIB on human basophils did not reverse the specific *Bet v 1* IT -IgG induced inhibition of human basophil histamine release (31). In addition, although various studies displayed increases in specific IgG4 (and IgA) levels which correlated with clinical improvement (11, 32, 33), in most studies, correlation of specific IgG (and IgA) with the clinical benefit of IT is poor or cannot be demonstrated, especially for IT with aeroallergens (34-37). In the case of venom allergy, the rise of IgG correlates, at least at the onset of desensitization, with protection achieved by the treatment (38). However, in some protected subjects, an increase in IgG cannot be demonstrated (39). Furthermore, increased allergen-specific IgG failed to predict the clinical response to treatment, raising the possibility that IgG may reflect high allergen exposure rather than play a causal role in successful IT (34, 38, 40). Therefore, the role allergen-specific antibodies as putative "blocking factor(s)" in IT providing protection against allergic disease appears unlikely.

3. Role of IDO in the efficacy of allergen IT

IDO has been widely investigated for the induction of immunological tolerance since its crucial role as immunosuppressive agent was demonstrated. Literature data show that the expression of IDO by DCs enables this professional APCs to inhibit T-cell proliferation and to promote tolerance (41, 42). A mechanistic link has been demonstrated between IDO and the tolerance-inducing activity of recombinant CTLA4-Ig (43). Binding of CTLA4-Ig to B7-1 (CD80) or B7-2 (CD86) receptors on

DCs was shown to activate a signaling pathway leading to the induction of IDO *in vitro*. Indeed, inhibition of IDO by using the IDO inhibitor 1-methyltryptophan (1MT) abrogated the tolerogenic effect of CTLA4-Ig in a model of islet cell transplantation (43). Furthermore, recent studies demonstrated that DCs expressing IDO contribute to peripheral tolerance *via* induction of aTreg cells (44, 45). In a more recent study, Curti *et al.* showed that IDO expression increased $CD4^+CD25^+$ Treg cells through the conversion of $CD25^-$ into $CD25^+$ cells (46).

Therefore, we investigated whether IDO plays a role in tolerance induction by allergen IT in a mouse model of allergic asthma (chapter 3). Allergen IT demonstrated partial IDO-dependent efficacy in suppression of asthma-like symptoms. We show that inhibition of IDO throughout IT abrogated the efficacy of IT on the reduction of BAL eosinophil numbers and AHR to methacholine, but no changes in serum OVA-specific IgE levels were observed. In addition, in chapter 3 it is reported that inhibition of IDO throughout the aerosol challenge period did not interfere with tolerance induction by allergen IT. These findings reveal that IDO plays a role only during induction of tolerance by allergen IT and that activity of IDO is irrelevant for the effects of IT thereafter. In addition, we did not observe an effect of IDO inhibition by 1MT during allergen inhalation challenge in control (sham)-treated mice. These observations are in line with findings reported by Hessel *et al.* (47) and Hayashi *et al.* (48). However, Hayashi and colleagues did observe a role for IDO during allergen inhalation challenge in mice treated systemically with immunostimulatory oligodeoxynucleotide sequences (ISS-ODN). This might indicate that the mechanism(s) of suppression after allergen-IT is different from that after ISS-ODN treatment.

The downstream molecular mechanism by which IDO affects immune outcomes is still a subject of active investigation. Different mechanisms have been proposed for IDO-mediated immunoregulation during pregnancy, autoimmunity and transplantation (49). Conceptual possibilities include either tryptophan depletion or downstream tryptophan metabolites (50, 51). In chapter 3, we show that formation of tryptophan metabolites, rather than tryptophan depletion, is the mechanism by which allergen IT induces tolerance to the induction of airway manifestations of asthma. Indeed, injection of tryptophan during IT did not inhibit the efficacy of optimal allergen IT, while the tryptophan metabolites, in particular kynurenine, 3-hydroxykynurenine and xanthurenic acid potentiated the efficacy of suboptimal IT. Although the other tryptophan metabolites we used (chapter 3) did not potentiate the suppressive effects of OVA IT we cannot completely exclude that they might play a role in IT considering that this may merely be a matter of different pharmacokinetic profiles *in vivo*. In addition, our data demonstrate that there are no additive or synergistic effects between the two active metabolites, 3-hydroxykynurenine and xanthurenic acid, when given together during suboptimal IT. Since 3-hydroxykynurenine is further metabolized into xanthurenic acid (through the enzyme kynurenine aminotransferase pathway (52)), this finding suggests that one of these two substances is responsible for the induction of immune-tolerance mediated by IDO. Furthermore, there is also possibility that these active metabolites (3-hydroxykynurenine and xanthurenic acid) might act *via* a similar mechanism.

The immunological mechanism(s) by which IDO-dependent tryptophan metabolites mediate the suppression of AHR and eosinophilia by allergen IT is

unknown at present but is not due to a shift from Th2 to Th1 responses as indicated by undetectable IFN-γ levels in the BAL fluid. Our present data (chapter 3) propose that induction of aTreg cells might be implicated, particularly Tr1 cells since the beneficial effects of allergen IT in our model were earlier shown to be mediated by IL-10 (14). Studies by Fallarino *et al.* (45), Belladonna *et al.* (44) and Munn and Mellor (53) demonstrated that IDO expressing DCs induce a regulatory phenotype in naïve T-cells through tryptophan starvation and tryptophan catabolites. Our data are not completely in line with the hypothesis that the combined effects of tryptophan depletion and kynurenine production are required for the generation of IL-10 and TGF-β producing regulatory T-cells. One likely explanation for this discrepancy may be that we used an *in vivo* model while others used *in vitro* T-cell activation. Interestingly, Platten *et al.* have recently found that treatment with 3-hydroxykynurenine inhibits proliferation, increases IL-10 production by murine splenocytes stimulated with a Th1- response-inducing peptide antigen, and stimulates IL-10 production *in vivo* (54). However, it remains to be elucidated how the modulation of tryptophan catabolism by IDO-expressing APCs, in particular DCs, may be implicated in the mechanism governing the generation of aTreg cells. To date, there have been no human studies of the role of IDO in asthma and/or in IT to prevent or treat this disease.

4. Role of nTreg cells in the efficacy of allergen IT

A few years after the discovery of $CD4^+CD25^+Foxp3^+$ nTreg cells in mice, a similar T-cell subset has been identified in human peripheral blood, thymus, and lymph nodes (55). It is now generally accepted that nTreg cells play an important role in the induction and maintenance of peripheral self-tolerance (56, 57). Phenotypic characterization and microarray analysis of nTreg cells revealed their constitutive expression of several surface molecules including $CD45RB^{low}$/CD45RO (mouse/human), CD38, L-selectin (CD62L), CTLA-4 (CD152), CD25, and glucocorticoid-induced tumor necrosis factor receptor (GITR or TNFRSF18), as well as neuropilin-1, a molecule that is downregulated on activated conventional T-cells (58-62). It has been hypothesized that nTreg cells employ several mechanisms to suppress immune responses. These cells can both directly suppress responding T-cells and down-modulate APC function *in vitro* (63, 64). Furthermore, nTreg cells may convert $CD4^+$ T-cells into regulatory T-cells expressing IL-10 and/or TGF-β in culture systems (65, 66). Interestingly, Mekala *et al.* have recently shown that nTreg cells can promote the formation of antigen-specific IL-10 regulatory T-cells *in vivo* (67).

In chapter 4, we describe the contribution of nTreg cells to the induction of tolerance by allergen IT *via* functional inactivation of these cells by the administration of anti-GITR or anti-CTLA-4 in a mouse asthma model. GITR was shown expressed on nTreg cells but not on conventional naive T-cells (60). High expression levels of GITR on nTreg cells compared to other activated T-cells suggest an important role for GITR in their function (58, 60). Stimulation of GITR by activating antibodies (anti-GITR; DTA-1) inactivates nTreg cells *in vitro* and breaks immunological self-tolerance *in vivo* (58, 60). We (in chapter 4) show that ligation of GITR by DTA-1 mAb at the time of IT partially abrogated the suppressive effects of allergen IT on

the reduction of BAL eosinophil numbers and serum IgE, but completely reversed the suppressed AHR *in vivo*. Although these findings did not fully exclude that the observed effects of anti-GITR treatment might relate to an effect on conventional OVA-specific T-cells, we suggest that the beneficial effectiveness of IT is partially achieved *via* promoting tolerance by nTreg cells. Previously, it has been shown that the interaction of CTLA-4 with CD80 and CD86 ligands expressed on APCs, in particular DCs, can modulate immune responses. CTLA-4 is constitutively expressed by nTreg cells (68). Furthermore, engagement of CTLA-4 on nTreg cells is essential for their suppressive function (59) and for promoting induction of aTreg cells (69). This raises the possibility that treatment with anti-CTLA-4 mAb can disrupt nTreg function by preventing a CTLA-4-mediated signal through CD80/CD86 expressed on DCs. Our findings that anti-CTLA-4 treatment partially abrogated the induction of tolerance by allergen IT is in agreement with the anti-GITR experiment and strengthens a potential role for nTreg cells. Alternatively, other investigators have suggested that anti-CTLA-4 treatment can stimulate activation of effector T-cells by blocking negative signaling provided by CTLA-4:CD80/CD86 interaction (70). However, this seems unlikely because (i) neither anti-CTLA-4 nor anti-GITR treatment did abrogate the suppressive effect of allergen IT on OVA-challenge induced upregulation of the Th2-dependent IgG1 level in serum and (ii) previous observations by us (71) and others (72) show that anti-CTLA4 treatment during OVA inhalation challenge did not potentiate the asthma manifestations in OVA sensitized mice.

The mechanism(s) through which nTreg cells contribute to tolerance induction by allergen IT are yet unclear. nTreg have previously been shown to trigger the induction of IDO in DCs through CTLA-4 signaling, leading to the degradation of tryptophan into kynurenines and subsequent inhibition of T-cell proliferation (43, 73) and promoting tolerance induction by the generation of aTreg cells (45). Moreover, it has recently been demonstrated that GITR activation plays a role in this nTreg – IDO pathway (74). Grohmann *et al.* showed that GITR:GITR ligand (GITRL) interaction can also induce IDO expression and activation in a mouse plasmacytoid DCs (74). Therefore, since we demonstrated that inhibition of IDO partially abrogated the beneficial effects of allergen IT (chapter 3) it can be speculated that tolerance induction by allergen IT is in part mediated by a nTreg – (CTLA-4 and GITR) – IDO pathway.

5. 1α,25-dihydroxyvitamin D_3 potentiates the beneficial effects of allergen IT in a mouse asthma model

Since many of the observed immunological changes following allergen IT may be mediated by the induction of aTreg cells and since immature tolerogenic DCs play a critical role in Treg cell generation and peripheral tolerance, we aimed to investigate whether the treatment efficacy of allergen IT could be improved by using a compound that inhibits DC maturation. The rationale to use $1,25(OH)_2D_3$ in combination with IT is based on the observations that the expression of the transcription factor NF-κB protein RelB is inhibited by $1,25(OH)_2D_3$. The NF-κB protein RelB is essential for DC differentiation and maturation (75, 76). Professional APCs, particularly DCs, express the vitamin D receptor (VDR) and are key target of VDR agonists. Inhibition of NF-

κB by $1,25(OH)_2D_3$ induces a persistent immature tolerogenic phenotype in human and mouse DCs characterized by down-regulated expression of the costimulatory molecules CD40, CD80, and CD86, and decreased IL-12 and strongly enhanced IL-10 production. Moreover, $1,25(OH)_2D_3$ and its analogs can induce DCs with tolerogenic properties *in vivo*, as demonstrated in model of allograft rejection (77).

In the study described in chapter 5, it was investigated whether $1,25(OH)_2D_3$ could potentiate the suppressive effects of allergen IT in a murine model of allergic asthma. Interestingly, mice treated with allergen IT combined with $1,25(OH)_2D_3$ did show enhanced beneficial effectiveness of IT compared to mice treated merely with allergen IT, thus clearly demonstrating the success of this combined IT in this model. We demonstrated that co-administration of $1,25(OH)_2D_3$ effectively suppressed AHR and potentiated the reduction of serum allergen-specific IgE levels and BAL eosinophilia in the suboptimal IT regime matched by a reduction in Th2 cytokines, IL-5 and IL-13. We further show that *in vitro* antigen-induced productions of Th2-cytokines are decreased in lung-draining lymph node cells after this combination IT. These findings reveal the induction of tolerance to Th2-mediated allergic inflammatory responses. Moreover, we demonstrated that the immunoregulatory cytokines IL-10 and TGF-β are involved in tolerance to allergen-induced airway manifestation of asthma since the suppressive effects of combined IT are completely abrogated by blocking of the IL-10R and neutralizing of TGF-β by using mAbs at the time of antigen inhalation challenge. These findings demonstrate that IL-10 and TGF-β mediate the suppressive effects of IT combined with $1,25(OH)_2D_3$.

In chapter 5, we found that the increase in serum TGF-β observed after combined IT was associated with a significant increase in OVA-specific IgA in serum. Binding of IgA to Fc*α*RI on leukocytes may have an anti-inflammatory effect (78). Therefore, our findings suggest that the TGF-β/IgA/Fc*α*RI pathway may by one of the effector mechanism(s) involved in suppressing asthma manifestations by allergen IT. However, this appears rather unlikely considering the beneficial effects of allergen IT in FcγRI and III double knock-out or IIB knockout mice, which do not display increased serum OVA-IgA levels after IT (chapter 2). In humans, the role of IgA in IT is at present a matter of debate (11, 37, 79).

In addition to keeping DCs in an immature/tolerogenic state, others have recently reported that $1,25(OH)_2D_3$ signaled T cells to express chemokine receptor CCR10 during activation, which enables them to migrate to mucosal tissues expressing CCR10 ligands (80). CCR10 is expressed by a subpopulation of Treg cells that can be recruited to mucosal tissues by epithelial-derived CCL28 (81, 82). Thus, it can be concluded that augmentation of IT effects by $1,25(OH)_2D_3$ may, at least partially, be mediated by increased numbers of CCR10 expressing Treg cells by $1,25(OH)_2D_3$ that can be recruited to the airways at the time of allergen inhalation.

The present findings described in chapter 5 are of major importance for the future therapeutic approach using allergen IT. One can expect several advantages from the combination of allergen IT with the administration of compound(s) that could specifically inhibit DC maturation such as $1,25(OH)_2D_3$ or more specific NF-κB inhibitors for a clinical application in patients with allergic asthma and other allergic disorders. In this respect, a multicenter, randomized, double-blind, placebo-controlled clinical trial has been started in grass-pollen allergic rhinitis patients using grass-pollen IT with or without $1,25(OH)_2D_3$.

Functional activation of NF-κB pathway signaling as a result of CD40 ligation on DCs has been described previously (83, 84). Moreover, O'Sullivan and Thomas (85) have demonstrated that CD40 ligand (CD40L; CD154) can induce sustained high levels of RelB/p50 activation in monocyte derived DCs. Likewise, several studies have demonstrated that DC maturation occurs following ligation of CD40 on DCs with CD40L on specific $CD4^+$ T cells (86-88). Major consequences of this interaction are the upregulation of co-stimulatory molecules CD80 and CD86 on DCs and the release of inflammatory cytokines IL-12 and TNF-α (83, 89-91). Therefore, we hypothesized that if the interaction of CD40/CD40L costimulator pathway can be prevented, the maturation of DCs may be inhibited, resulting in those conditions of antigen presentation that lead to Treg cell induction. Noticeably, we found that co-injection of anti-CD40L mAb MR1 significantly improved the suppressive effects of suboptimal OVA IT on AHR and serum OVA specific IgE levels, although there was no change with respect to BAL eosinophil numbers (Taher *et al.*, unpublished observation). Unlike the effects of $1,25(OH)_2D_3$ which may affect T-cells and APCs, the potentiation of the IT effects by coadministration of anti-CD40L are most likely explained by a selective effect on APCs since they are expressing CD40 and T-cell do not. The observations that direct effects of $1,25(OH)_2D_3$ on T-cells facilitates Th2 generation (92), supports our suggestion that the potentiation of IT by this compound is also mediated by an effect on APCs.

6. Conclusions

Evaluating all data presented in this thesis, we can draw the following conclusions:

- First, allergen IT effectively suppresses asthma like-symptoms in a mouse model. However, allergen IT must be administered in an optimal dose to achieve beneficial effectiveness since IT with suboptimal dose was only partially effective in suppressing the allergic and asthmatic responses. The sensitivity for down-regulation of different asthma-like symptoms by OVA-IT follows the rank order of IgE > BAL eosinophils > AHR. Allergen IT does not induce its beneficial effects by a shift from allergen-induced Th2 responses towards Th1 responses considering the undetectable IFN-γ levels in BAL fluid after IT. Most likely, induction of aTreg cells is the mechanism by which IT induces tolerance to the induction of airway manifestations of asthma. Although we could not detect increased levels of IL-10 and TGF-β in the BAL fluid after allergen IT, it remains possible that aTreg cells execute their suppressive effects at another time-point or location. However, involvement of yet other regulatory immune cells than aTreg cells cannot be fully ruled out.
- Second, the research described in this thesis has led to potentially important new findings with regard to the mechanism(s) involved in tolerance induction by allergen IT.
    - Tryptophan downstream metabolites generated by IDO are partially required in tolerance induction by allergen IT in a mouse asthma model.
    - Natural Treg cells appear to partially contribute to the induction of

tolerance by allergen IT, probably *via* CTLA-4.

- Fcγ I, IIB and III receptors are not required by allergen IT to achieve its beneficial effectiveness in our model.
- Co-injection of allergen IT with the active form of vitamin D_3, $1,25(OH)_2D_3$, improved the efficacy treatment of allergen IT. The regulatory cytokines IL-10 and TGF-β play a crucial role in the beneficial effects of this combined IT. Though this approach is still far from being used as a common practical application in humans, we suggest that it might improve allergen IT in human allergic disorders and may hold promise for the future of allergen IT treatment strategy.

7. Implications for the development of allergen IT

Peripheral T-cell tolerance is the key immunological mechanism in healthy immune response to self- and noninfectious nonself-antigen. This phenomenon is clinically well documented in allergy, autoimmunity and transplantation. Changes in the balance between allergen-specific Treg and Th2 and/or Th1 cells are very crucial in the development and also treatment of allergic diseases. In addition to the treatment of established allergy, early administration of allergen IT may be able to reduce the inflammatory process and to modify the natural course of the disease. Induction of immune tolerance could be improved, and allergen-specific Treg cells may in turn regulate/dampen both the Th1 and Th2 cells and their cytokines, ensuring a well-balanced immune response. Overall, the data described in this thesis demonstrate that different mechanisms are involved in tolerance induction by allergen IT. For the beneficial effects of IT, we postulate that IDO expressing DCs, nTreg cells and immature/tolerogenic DCs play a crucial role in the generation of regulatory T-cells, and subsequent down-regulation of allergen-induced asthma-like symptom *via* their immunoregulatory cytokines IL-10 and TGF-β. The mechanism(s) that lead to the induction of regulatory T-cells during allergen IT are not completely defined, but some pieces of the puzzle can be put together and are schematically summarized in Fig. 1.

These findings may stimulate translation research on allergen IT in allergic patients and may be helpful to guide novel immunotherapeutic strategies to prevent and cure allergic diseases including asthma.

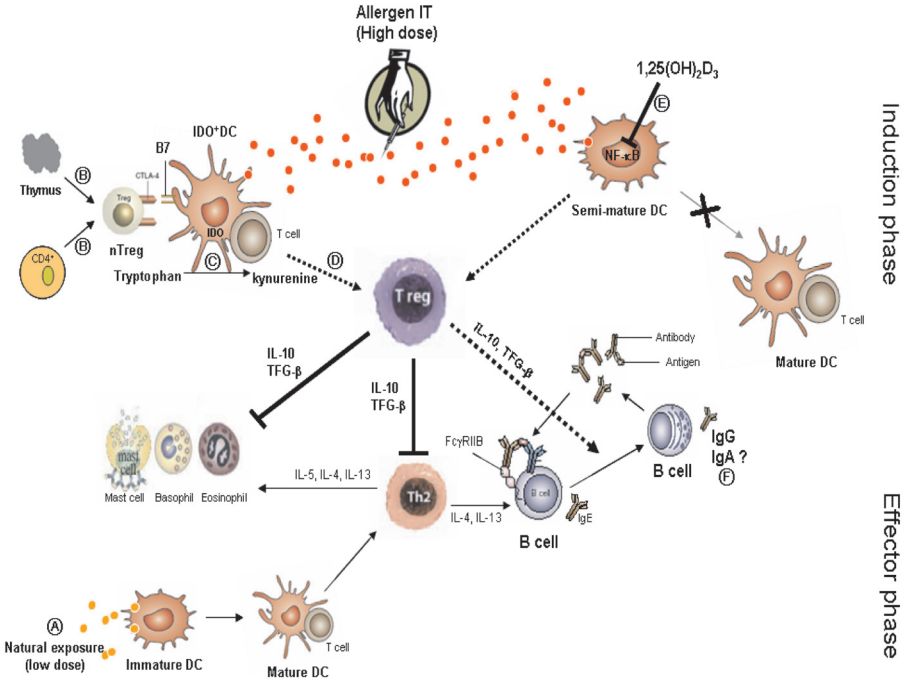

Figure 1. *Hypothetical scheme of the proposed mechanisms(s) contributing to the induction of tolerance by allergen IT. A) Natural exposure to an allergen leads to activation of Th2 cells. B) nTreg cells are generated in thymus, but can also develop from conventional CD4⁺ T-cells by specific condition or signals. nTreg cells induce IDO expression on dendritic cell (IDO⁺DC). This is partially mediated via the interaction of CTLA-4 expressed on nTreg and CD80 (B7) expressed on DC. C) IDO catalyzes the initial and rate-limiting step of tryptophan degradation. D) IDO expressing DC induces development of Treg after allergen IT by a mechanism at present not completely defined. E) 1,25(OH)$_2$D$_3$ inhibits transcription factor NF-κB thereby inhibiting maturation of DC and resulting in tolerogenic DC, which direct the induction of Treg cells. Treg cells suppressed Th2 responses and effector cells by the release of immunoregulatory cytokines IL-10 and TGF-β. F) The role of allergen-specific IgA is unclear. Dotted arrows represent immune response pathway to allergen IT; blocked lines represent inhibition.*

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Nederlandse samenwatting

Samenvatting

Allergisch astma is een chronische ontstekingsziekte van de luchtwegen waarin cellen van het immuunsysteem zoals mestcellen, T- en B-lymfocyten, eosinofiele granulocyten en dendrietische cellen een belangrijke rol spelen. De ziekte kenmerkt zich door regelmatig terugkerende benauwdheid, verhoogde spiegels van allergeen-specifiek IgE, toename van de gevoeligheid van de luchtwegen voor een grote variëteit aan specifieke en niet-specifieke prikkels (hyperreactiviteit), luchtwegontsteking en veranderingen ("remodelling") van het luchtwegweefsel. Van de immuuncellen spelen in het bijzonder geactiveerde $CD4^+$ Th2-cellen een belangrijke orkestrerende rol bij het ontstaan en in stand houden van astma door hun productie van specifieke cytokinen en hun interacties met andere immuuncellen.

Naast het vermijden van contact met de allergenen die het ziektebeeld verergeren, bestaat de therapie bij astma uit het onderdrukken van de ontstekingsreactie m.b.v. corticosteroïden en/of bronchodilatatie m.b.v. β2-sympathicomimetica. Beide groepen geneesmiddelen werken echter alleen op de symptomen van astma maar veranderen niets aan de onderliggende oorzaak van dit ziektebeeld. Allergeen immunotherapie (hyposensibilisatie), voor het eerst toegepast in 1911, is een manier om de reactie op een allergeen voor lange duur tegen te kunnen gaan. Hierbij wordt de allergische patiënt, volgens een langzaam oplopende dosering, onderhuids ingespoten met een extract van het allergeen waar deze patiënt allergisch voor is. Deze therapie wordt voornamelijk toegepast voor de preventie van een allergische reactie op bijen- of wespensteken en het verminderen van allergische rinitis veroorzaakt door pollen-, huisstofmijt- of kattenantigenen. Allergeen immunotherapie kan ook gebruikt worden ter behandeling van allergisch astma. Deze therapie is echter minder effectief bij astma en wordt zelden toegepast.

Over het werkingsmechanisme van allergeen immunotherapie in het lichaam bestaat nog veel onduidelijkheid. In de loop van de 20^e eeuw zijn de rol van blokkerende IgG antilichamen, de inductie van T-cel anergie, een verschuiving van een Th2-cytokine respons naar een Th1 respons en de inductie van bepaalde regulerende T-cellen nader onderzocht. Het is echter nog steeds noodzakelijk om meer te weten te komen over het precieze werkingsmechanisme van allergeen immunotherapie om deze behandeling ook effectief en veilig toe te kunnen gaan passen bij allergische astmapatiënten.

Hiervoor is enkele jaren geleden in ons laboratorium een muismodel voor allergisch astma ontwikkeld waarin de verschillende astmatische verschijnselen door allergeen immunotherapie onderdrukt konden worden. Muizen kunnen astmatisch worden gemaakt door deze dieren te sensibiliseren middels intraperitoneale injecties met het model allergeen ovalbumine en een paar weken later de luchtwegen herhaaldelijk bloot te stellen aan een nevel van hetzelfde allergeen ("provocatie"). Bij deze dieren wordt een door Th2-cellen gereguleerde afweerreactie waargenomen met een toename van allergeen-specifiek IgE in het serum, een verhoogde hoeveelheid eosinofielen in de longen en de long lavage vloeistof en hyperreactiviteit voor diverse luchtwegvernauwende stimuli. Door het toepassen van allergeen immunotherapie na de sensibilisatie maar voor de inhalatieprovocatie, is het mogelijk de astmatische verschijnselen te onderdrukken. In dit proefschrift zijn de resultaten beschreven van experimenten die als doel hadden meer te weten te komen over

het werkingsmechanisme van allergeen immunotherapie met als uiteindelijk doel te komen tot een verbeterde immunotherapie voor de behandeling van allergische patiënten, inclusief astma.

Is er een rol voor IgG Fcγ-receptoren bij de inductie van tolerantie tijdens allergeen immunotherapie? (hoofdstuk 2)

Een van de eerste verklaringen voor de therapeutische werking van allergeen immunotherapie was de zogenaamde "blokkerende antilichamen" theorie. Door immunotherapie neemt de spiegel van allergeen-specifieke IgG antilichamen in het bloed sterk toe. Deze IgG antilichamen zouden de binding van het allergeen aan IgE kunnen blokkeren en daardoor allergische reacties kunnen afremmen. Hoewel dit niet meer als mogelijke verklaring wordt gezien, kunnen deze IgG antilichamen mogelijk wel *via* Fcγ-receptoren allergische reacties onderdrukken. Fcγ-receptoren komen tot expressie op verschillende soorten immuuncellen en spelen een belangrijke rol in humorale en cellulaire immuunreacties. Er zijn drie typen Fcγ-receptoren: I, II en III, die elk een rol zouden kunnen spelen in de beschermende werking van immunotherapie. Om de rol van IgG Fcγ-receptoren bij de inductie van tolerantie door allergeen immunotherapie te onderzoeken is gebruik gemaakt van speciale muizen waar de genetische informatie van Fcγ-receptoren ontbrak. Echter bij zowel muizen waar de Fcγ-receptor IIB, als bij muizen waar de Fcγ-receptoren I en III niet tot expressie komen, waren de astmatische verschijnselen (verhoogde hoeveelheden eosinofielen, Th2 cytokinen en IgE) middels allergeen immunotherapie even goed te onderdrukken als in controle (wild-type) muizen. Uit deze resultaten kan geconcludeerd worden dat de typen I, IIB en III Fcγ-receptoren geen rol spelen bij allergeen immunotherapie in dit muizenmodel voor astma.

Is het tryptofaan-metaboliserende enzym indoleamine 2,3-dioxygenase (IDO) betrokken bij allergeen immunotherapie? (hoofdstuk 3)

Uit diverse studies is het duidelijk geworden dat IDO wat o.a. tot expressie komt in dendrietische cellen (DCs), betrokken is bij immuunsuppressie en de inductie van immunologische tolerantie. Of dit enzym ook belangrijk is bij allergeen immunotherapie is bestudeerd door tijdens de therapie muizen gelijktijdig te behandelen met een remmer van het IDO, namelijk het 1-methyltryptofaan. Door toediening van deze stof werd een deel van de effecten van allergeen immunotherapie te niet gedaan, namelijk de afname van de infiltratie van eosinofielen in de luchtwegen en het verminderen van de luchtweghyperreactiviteit voor methacholine. Het verlagen van de IgE spiegels door allergeen immunotherapie werd niet beïnvloed door 1-methyltryptofaan. Het immuunregulerende enzym IDO speelt dus een differentiële rol in allergeen immunotherapie. Er zijn twee mechanismen beschreven waarmee IDO dit soort immuunsuppressie c.q. tolerantie kan bewerkstelligen. Enerzijds is dat lokale depletie van het IDO substraat tryptofaan, wat essentieel is voor proliferatie van T-lymfocyten. Anderzijds is het bekend dat bepaalde tryptofaanmetabolieten immuunregulerende eigenschappen hebben. Uit het vervolg van dit onderzoek bleek dat het mechanisme waardoor IDO effect had op de inductie van tolerantie bij allergeen immunotherapie niet het gevolg was van een verlaging van de hoeveelheid

tryptofaan, maar door de productie van een aantal metabolieten gevormd door IDO uit tryptofaan. Het toedienen van tryptofaan tijdens allergeen immunotherapie kon het effect van deze behandeling namelijk niet opheffen, terwijl toediening van bepaalde metabolieten, namelijk kynurenine, 3-hydroxy-kynurenine en xanthurinezuur, de effectiviteit van de therapie deden toenemen. Bepaalde metabolieten gevormd uit tryptofaan door het enzym IDO spelen dus een belangrijke rol bij de inductie van tolerantie door allergeen immunotherapie.

Zijn nTreg cellen betrokken bij de inductie van immuuntolerantie door allergeen immunotherapie? (hoofdstuk 4)

Natuurlijke regulerende T-cellen (nTreg) spelen een belangrijke rol bij het remmen van de activatie van conventionele T-cellen en er zijn bovendien sterke aanwijzingen dat ze betrokken zijn bij de inductie en het in stand houden van perifere tolerantie. Deze cellen worden gekenmerkt door de expressie van een aantal oppervlaktemoleculen waaronder het "cytotoxic T-lymphocyte antigen 4" (CTLA-4) en de "glucocorticoid-induced tumor necrosis factor receptor"(GITR). Daarnaast zijn CTLA-4 en GITR ook actief betrokken bij de immuunsuppressieve functie van nTreg cellen. Door het toedienen van antilichamen gericht tegen deze oppervlaktemoleculen is de rol nTreg cellen in het muizenmodel voor allergeen immunotherapie onderzocht. Activerende antilichamen gericht tegen GITR zorgen ervoor dat nTreg cellen geïnactiveerd worden. Toediening van dit antilichaam tijdens de allergeen immunotherapie in het diermodel resulteerde in het volledig verdwijnen van het remmende effect van immunotherapie op de luchtweghyperreactiviteit en een vermindering van de remmende effecten op de infiltratie van eosinofielen in de luchtwegen en de afname van IgE in het serum. Ook blokkade van de functie van nTreg cellen middels antilichamen gericht tegen CTLA-4 resulteerde in een verminderd effect van allergeen immunotherapie op de astmatische verschijnselen in het diermodel. Deze resultaten duiden er op dat de nTeg cellen waarschijnlijk betrokken zijn bij de inductie van tolerantie middels allergeen immunotherapie.

Kan de actieve vorm van vitamine D_3 de remmende effecten van allergeen immunotherapie versterken en zijn IL-10 en TGF-β daarbij betrokken? (hoofdstuk 5)

De expressie van de transcriptiefactor van NF-κB is belangrijk voor de uitrijping van DCs en de aanmaak van deze transcriptiefactor kan geremd worden door de actieve vorm van vitamine D_3, het $1,25(OH)_2D_3$. Onrijpe DCs spelen een belangrijke rol in de aanmaak van adaptieve Treg cellen en daarmee het ontstaan van perifere tolerantie. Door nu de uitrijping van DCs te blokkeren middels remming van NF-κB door $1,25(OH)_2D_3$ was het mogelijk de remmende effecten van allergeen immunotherapie op de astmatische kenmerken in het diermodel te versterken. Om te onderzoeken of hierbij de immuunregulerende cytokinen IL-10 en TGF-β, mogelijk geproduceerd door adaptieve Treg cellen, betrokken zijn, werden antilichamen die de werking van deze cytokinen remmen, toegediend tijdens de inhalatie provocatie. Toediening van zowel antilichamen tegen de IL-10 receptor als tegen het TGF-β resulteerde in het gedeeltelijk opheffen van de effecten van de combinatietherapie

van $1,25(OH)_2D_3$ met het allergeen. Toediening van beide antilichamen gaf een complete opheffing van de combinatietherapie te zien. Hieruit kan geconcludeerd worden dat $1,25(OH)_2D_3$ de werking van allergeen immunotherapie sterk bevordert en dat dit wordt veroorzaakt door de immuunsuppressieve cytokinen IL-10 en TGF-β, die waarschijnlijk geproduceerd worden door allergeen-specifieke Treg cellen. Het positief versterkende effect van $1,25(OH)_2D_3$ op de allergeen immunotherapie zou wellicht ook bij patiënten met allergisch astma goed toepasbaar kunnen zijn, hetgeen momenteel in een klinische studie wordt onderzocht.

De resultaten beschreven in dit proefschrift tonen aan dat nTreg cellen en DCs waarin IDO tot expressie komt, betrokken zijn bij de aanmaak van adaptieve Treg cellen die middels IL-10 de astmatische verschijnselen tijdens allergeen immunotherapie kunnen verminderen. Combinatie van allergeen met $1,25(OH)_2D_3$, waardoor mogelijk DCs geremd worden in hun rijping en hiermee de aanmaak van Treg cellen versterken, bevorderd de onderdrukking van kenmerken van astma.

Abbreviation

$1,25(OH)_2D_3$	1α,25-dihydroxyvitamin D_3
1MT	1-methyltryptophan
3-OH-AA	3-hydroxyanthranilic acid
3-OH-KYN	3-hydroxykynurenine
AHR	airway hyperresponsiveness
APCs	antigen presenting cells
aTreg	adaptive regulatory T cell
BAL	bronchoalveolar lavage
CD40L (CD154)	CD40 ligand
CTLA-4 (CD152)	cytotoxic T-lymphocyte antigen 4
DCs	dendritic cells
FcγR	Fc gamma receptor
FcεRI	Fc epsilon receptor
GITR (TNFRSF18)	glucocorticoid-induced tumour necrosis factor receptor
IDO	indoleamine 2,3-dioxygenase
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IT	allergen immunotherapy
KA	kynurenic acid
KYN	kynurenine
mAbs	monoclonal antibodies
NF-κB	nuclear factor-kappa B
nTreg	natural regulatory $CD4^+CD25^+Foxp3^+$ T cell
OVA	ovalbumin
QUINA	quinolinic acid
TGF-β	transforming growth factor-beta
Th	T helper
TLN	thoracic lymph nodes
Tr1	type 1 regulatory T cells
TRP	tryptophan
XA	xanthurenic acid

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Yousef
2007

Curriculum Vitae

The author of this thesis was born in Bany Walid, Libya, on the 1st of November 1965. Tripoli, the capital of Libya was the place where he finished his studies from primary to higher school. In the year 1983, he was admitted to AL-Fateh Medical University to do 5-years studies in Pharmaceutical Sciences and he was successfully obtained B. Pharm. After graduation he worked, for four years, as a pharmacist. In the year 1993, he changed his position and worked as a demonstrator at the department of Pharmacology and Therapeutics, Faculty of Pharmacy, AL-Fateh Medical University. During the period 1996-1998, he transferred to Malaysia where he continued his studies and obtained MSc. in molecular pharmacology at Universiti Sains Malaysia. In 1991, he employed as an academic staff member of AL-Fateh Medical University and worked as assistant lecturer/researcher. Four years later, he was awarded a scholarship from the Ministry of Higher Education of Libya to carry out a PhD program in Pharmacology. In May 12, 2003, he joined the department of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, where he started his PhD program. His research project focused on the investigation of the mechanisms underlying the beneficial effects of allergen immunotherapy with the ultimate goal to improve its therapeutic efficacy for allergic asthma, using a mouse model. The results he obtained are published in this thesis. The work leading to this thesis was carried out under the supervision of Prof. Dr. Antoon van Oosterhout and Dr. Paul Henricks. During his PhD period he attended advanced courses in the field of immunology and infectious diseases at the Eijkman Graduate School.

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If you cannot run, walk
If you cannot walk, crawl
but by all means make sure you get to the end