Immune regulation by allergen immunotherapy

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Immune regulation by allergen immunotherapy

lessons learned from experimental approaches

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General Introduction

CHAPTER

1

Overview

Allergic diseases are the result of a complex interaction of environmental factors and genetic susceptibilities that lead to the induction of improper T helper 2 (Th2) responses and production of specific immunoglobulin E (IgE) antibodies to harmless environmental antigens [1]. The prevalence of allergic diseases has dramatically increased during the last four decades and is still increasing worldwide, affecting the quality of life of a significant proportion of the world's population [2;3]. While the reasons for this increase are largely unknown, changes in environmental factors including vast changes in life-style are thought to play an important role [4]. The following changes in environmental factors coincided with the increase in allergic diseases, and may offer explanations for their increased prevalence: (i) reduced exposure to microbial infection as stated in the hygiene hypothesis [5], (ii) increased exposure to perennial allergens [4], (iii) decline in physical exercise and changes in diet such as decreased proportion of the vitamins in the food and increased usage of iron intake by infants in affluent societies [4;6-8]. However, any causal association between the mentioned hypotheses and increased prevalence of allergic disease remains to be elucidated.

Given the high level of discomfort caused by allergic diseases, the high number of affected patients worldwide and the lack of options for disease prevention, an efficient treatment that modifies the deleterious immune reaction to allergens appears to be the best resolution for allergic diseases [9]. A wide range of pharmaceutical therapies are available for treatment of different allergic diseases [10]. Most of these therapies aim at reducing the symptoms of the disease while leaving the root immunological cause of the disease intact [10]. Currently, allergen specific immunotherapy (SIT) is the only treatment for allergic diseases that targets the root immunological base of the disease and leads to long-lasting relief of allergic symptoms [11]. However, despite the high efficacy in allergic rhinitis and venom allergies, the efficacy of SIT in allergic asthma and patients with multiple allergies is controversial and requires improvement [10-13]. Rational improvement of SIT requires a detailed knowledge of the mechanism of action of SIT in inducing allergen tolerance. This detailed mechanistic understanding of the mechanism of action of SIT is currently lacking.

In this dissertation, we will first address the role of high iron intake in exacerbating the symptoms of allergic asthma, thereby testing the hypothesis that increased iron intake by infants could contribute to the increased prevalence

of allergic diseases during the past four decades. Next, we will disclose the results of studies that we have performed to unravel the mechanisms of SIT-induced tolerance and its effector functions during allergen inhalation as well as two strategies that we used to improve the efficacy of SIT in a mouse model of ovalbumin-based SIT.

Allergic reaction: course of actions

Environmental antigens are constantly being sampled by local dendritic cells in the lungs, gastrointestinal tract and skin, and presented to T cells in the regional draining lymph nodes [14]. Allergen, at the first encounter, elicits an antigen specific Th2 response in susceptible individuals in regional lymph nodes followed by migration of the allergen specific Th2 cells to the location of allergen exposure [15]. Consequently, allergen specific Th2 cells instruct B cells to produce allergen specific IgE by secreting IL-4 and IL-13 [16]. IgE circulates in the blood and causes allergic sensitization by binding to high affinity IgE receptors (FCεRI) expressed by mast cells and basophils enabling these cells to respond to the allergen. Subsequent exposure to the allergen results in cross-linking of the cell-surface bound IgE and degranulation of mast cells and basophils, leading to the early-phase allergic reaction [15]. Early-phase allergic reaction occurs within minutes after allergen exposure due to the contents of mast cell granules such as histamine and newly synthesized lipid mediators including prostaglandin D2 and leukotrienes [15;17]. Vasodilation, increased vascular permeability, bronchoconstriction and airway mucus secretion are some of the features of early phase reaction which can last for a few hours [15;18]. Allergen presentation to Th2 memory cells by antigen presenting cells induces the late-phase allergic reaction. During the late phase reaction, which starts within a few hours of allergen exposure and lasts for several hours, cytokine contents of mast cell granules enhance activation and recruitments of Th2 cells, eosinophils, basophils to the site of allergen exposure [15;18]. Edema, skin erythema, airway narrowing and mucus hyper-production at the site of allergen are the features of the late allergic reaction [19].

Allergic asthma

Allergic asthma is a chronic allergic disease of the lungs characterized by airway inflammation, airway hyperreactivity (AHR) and excess mucus production in the airways [20]. While the prevalence of asthma is increased by approximately 50% every decade, it is estimated that more than 300 million people suffer from asthma worldwide [21;22]. Now it has become clear that the Th2 mediated allergic reaction in the respiratory system is the main underlying

cause of asthma [23]. However, pointing out only Th2 cells in the pathogenesis of asthma seems to be an oversimplification and there may be a role for other T cell subsets in the pathogenesis of asthma [24;25]. Moreover, there appear to be asthma subphenotypes including a Th2-high phenotype in approximately 50% of the patients [26-28].

Allergen specific immunotherapy

SIT is currently the only treatment for allergic diseases that aims to restore the loss of allergen tolerance by targeting the root cause of allergic diseases [11;29]. The clinical efficacy was first documented by Noon and colleagues in 1911 [30]. SIT is performed by repeated administration of high doses of the sensitizing allergen for a period of three to five years. In order to reduce the risk of side-effects such as anaphylactic reactions, the treatment starts with an initial period of low but gradually increasing doses of allergen administration [31].

Currently, SIT treatment has some disadvantages, including the need for long-term treatment, potential risk of anaphylactic reactions and variable efficacy in the treatment outcome in different patients. [32-34]. There is an urgent need for improving SIT in a way that lower doses of allergen and shorter treatment protocols can induce optimal tolerogenic efficacy in a short period of time in all treated patients. Rational improvement of SIT requires of an exact and detailed understanding of the mechanism of action of SIT. The mechanism(s) by which SIT generates or restores tolerance to the sensitizing allergen is incompletely understood to date [35].

Routes of SIT

Subcutaneous immunotherapy (SCIT)

SCIT is the classical form of SIT [36], in which the allergen is administered to the hypo-dermal layer of the skin where it can be captured by *in situ* antigen presenting cells such as skin conventional dendritic cells (DCs) and macrophages or by skin recruited plasmacytoid DCs [37]. Captured allergen is then conveyed by the APCs and presented to T cells in the draining lymph nodes [37]. SCIT is accompanied by a potential risk of anaphylactic reactions and therefore, it needs to be performed by authorized health care professionals. Requirement for repeated referral to the clinic for a period of 3-5 years in SCIT imposes practical difficulties to the patients [38].

Sublingual immunotherapy (SLIT)

Sublingual immunotherapy has been developed as a less invasive alternative for SCIT that could be performed outside the clinic [38]. In this method of SIT, allergen is given to the patients in the form of oral tablets and droplets which have to be used under the tongue at specified intervals [39;40]. Presence of patients in the clinic is not required in SLIT [39]. The type of APCs involved in SLIT has yet to be determined, however, mucosal langerhans cells in humans and oral macrophage-like cells in mouse model have been implicated [41;42]. The efficacy of SLIT in suppressing the allergic symptoms has been shown in several studies [43-46].

Other methods

Beside SLIT and SCIT other methods of SIT have been suggested. Senti and colleagues have shown that intra-lymphatic application of SIT requires lower doses of allergen and less frequent need for injection than SLIT while still being highly efficient in suppressing the symptoms of the disease [47]. The use of skin patches has also been suggested as a safe method of SIT. Applied allergen to the skin using skin patches could be captured by Langerhans cells that are highly tolerogenic [48]. The efficacy and safety of intra-lymphatic SIT and skin patches have yet to be further elucidated.

Potential mechanisms of allergen immunotherapy

Successful SIT leads to allergen tolerance as evident by suppressed Th2 as well as effector cells such as eosinophils and basophils. In addition, allergen-specific immunoglobulin responses display a shift from IgE to IgG and IgA [35]. Different hypotheses have been postulated to explain the mechanism of SIT-induced allergen tolerance. Generation of inducible regulatory T cells, blocking IgG antibodies, clonal deletion and anergy of allergen-specific Th2 cells are the major mechanisms of action that are thought to contribute to clinical efficacy of SIT [35]. The long-term efficacy of SIT indicates the presence of immunological memory, implicating either B or T cell subsets in SIT-induced allergen tolerance [49].

B cell mediated tolerance induction: Regulatory B cells and Blocking IgG

B cells can contribute to SIT-induced allergen tolerance by playing two roles. The first role is the acquisition of suppressive regulatory activities. It has been shown that B cells have the potency to become regulatory cells that are

capable of suppressing the allergic airway inflammation [50]. Interestingly, it has been shown that regulatory B cells induce their suppressive effects by producing interleukin-10 (IL-10) [51], the tolerance associated cytokine that has also been implicated in SIT-induced allergen tolerance [35;52]. However, the role of regulatory B cells in SIT-induced allergen tolerance has yet to be elucidated.

The second potential role of B cells in SIT-induced allergen tolerance is by producing blocking IgG antibodies [53]. Induction of allergen specific IgG, especially IgG4, and IgA has been observed after successful SIT in human patients [52;54;55]. SIT-induced allergen-specific IgG antibodies can contribute to the therapeutic effects of SIT by two mechanisms. These antibodies can bind to the allergen, thereby effectively competing with the specific IgE for the allergen, preventing IgE cross linking and subsequent mast cell and basophil degranulation as well as preventing IgE facilitated antigen presentation [53;56;57]. Moreover, the specific IgG antibodies can bind to FcγRIIB expressed by B cells, mast cells and basophils, which upon allergen-mediated IgG cross linking provides a negative intracellular signal and prevents cell activation, even upon co-aggregation with FcεRI [58].

T cell mediated tolerance induction: Regulatory T cells

Role of DCs in the induction of tolerance by allergen immunotherapy

To elicit an efficient T cell mediated immune response, antigen presentation is the first key step. DCs are heterogeneous population of specialized antigen presenting cells that have the unique capability of presenting antigens to naïve T cells in an immunogenic fashion [59;60]. Nevertheless, DCs can, like other subsets of APCs, also induce tolerance in naïve T cells. DCs can induce functional T cell responses by providing two signals, while determining the phenotype of the T cell response by a third signal [61;62]. The first signal is antigen specific, and delivered to $CD4^+$ T cells by presenting antigen in the context of MHC-II molecules. The second signal is given through co-stimulatory molecules such as CD80 and CD86 (B7 molecules). A third signal is provided by cytokines produced by the DC or present in the microenvironment, and determines the nature of the induced T cell phenotype, such as Th1, Th2 or Th17 [63;64]. DCs can contribute to the induction of allergen tolerance through several mechanisms. Tissue resident DCs including epidermal langerhans cells and dermal DCs show an immature profile by expressing low level of major histocompatibility complex-II (MHC-II) and the absence of co-stimulatory molecules [65;66]. Antigen presentation by immature DCs can induce T cells anergy [67] or the

induction of IL-10 producing iTreg cells [68]. DCs exposed to an antigen under non-inflammatory conditions acquire a semi-mature phenotype by expressing high levels of MHC-II and low levels of co-stimulatory molecules. Similar to immature DCs, semi-mature DCs can induce iTreg cells, while mature DCs induce effector T cells [69]. Approaches that restrain the maturation of DCs such as application of vitamin D3 facilitate the induction of tolerance and may have therapeutic implications [70].

Different sub-types of DCs including plasmacytoid and conventional DCs play distinct roles in the induction of tolerance or immunity [71]. Plasmacytoid DCs (pDCs) express the immune-regulating and tryptophan degrading enzyme indoleamine 2, 3-dioxygenase (IDO) and have been associated with tolerance induction [72]. De Heer and colleagues have shown that pDCs play an essential role in maintaining tolerance in the lungs to a harmless antigen in a mouse model [73]. In contrast, conventional DCs have been associated with the induction of Th2 and Th1 responses [74], as well as Treg cells [75]. Surprisingly, there is little known about the role of different subtypes of DCs in SIT-induced allergen tolerance [35].

Regulatory T cells

Regulatory T cells (Treg) are key elements of the immune system playing crucial roles in controlling immune responses to pathogens and eliciting immunological tolerance to self as well as to harmless environmental antigens [76]. Based on the origin and the conditions of their generation, Treg cells are classified as naturally occurring (nTreg) and inducible regulatory T cells (iTreg) [76]. nTreg cells represent a distinct lineage during T cell differentiation in the thymus, and constitutively express Forkhead Box P3 (FOXP3), Cytotoxic T-Lymphocyte Antigen 4 (CD152) and high levels of interleukin-2 receptor α-chain (CD25) [77]. nTreg cells are thought to have a specificity towards, not yet identified self antigens, facilitate the induction of iTreg cells and suppress self-reacting effector T cells [78]. iTreg cells are generated from naïve T cells in the periphery in an antigen-specific manner and these can only be induced under certain conditions during antigen presentation, and could therefore potentially play important roles in SIT-induced allergen tolerance [76]. Different subtypes of $CD4^+$ iTreg cells with suppressive capacities have been described: (i) $FOXP3^+$ iTreg cells [79], (ii) IL-10 producing Tr-1 cells that do not express FOXP3 [80], (iii) IL-10 producing $FOXP3^+$ iTreg cells [81] and (iv) TGF-β producing iTreg cells called Th3 cells [82].

There is an increasing body of evidence indicating that iTreg cells contribute to the generation and maintenance of SIT-induced peripheral allergen tolerance

[54;55;83-86]. Several studies have reported that FOXP3$^+$ [87], IL-10 producing [55], FOXP3$^+$IL-10 producing [88] or TGF-β secreting [89] T cells are increased after SIT in human subjects suggesting that SIT generates allergen-specific inducible Treg cells that contribute to the therapeutic effects of SIT in humans.

FOXP3$^+$ regulatory T cells in SIT

Several clinical studies have provided evidence for a possible role of FOXP3$^+$ Treg cells in SIT-induced generation of allergen tolerance. Kerstan and colleagues have observed that venom SIT induces activation and homing of circulating CD4$^+$CD25$^+$FOXP3$^+$ Treg cells to the secondary lymphoid organs leading to a transient decrease of these cells in the circulation one month after initiating the treatment [90]. During this time, venom-stimulated T cell proliferation and IFN-γ production was decreased suggesting a functional role for CD4$^+$CD25$^+$FOXP3$^+$ Treg cells in controlling both specific Th2 and Th1 immune responses. A different study has reported that at six months after the treatment, venom SIT increases the number of circulating CD4$^+$FOXP3$^+$ Treg cells which significantly correlates with venom-specific IgG4/IgE ratio shift, suggesting a therapeutic role for CD4$^+$FOXP3$^+$ Treg cells [87]. It seems that after venom-SIT, initially FOXP3$^+$ Treg cells home to the lymphoid organs where antigen presentation occurs, followed by their later expansion. Radulovic *and colleagues* have observed that there is an increased number of CD4$^+$CD25$^+$FOXP3$^+$ Treg cells in nasal mucosa two years after the initiation of grass pollen SIT, suggesting a putative role for these cells also in the local suppression of allergic manifestations upon allergen exposure [85]. However, the functional importance of the observed increase in FOXP3$^+$ Treg numbers for the therapeutic effects of SIT has not been clearly shown.

IL-10, an important tolerance mediator in SIT

IL-10 production has been frequently reported during and after SIT [44;89;91-93], underscoring the role of this anti-inflammatory cytokine for the induction of allergen tolerance by SIT. IL-10 can induce its anti-inflammatory effects through different mechanisms. IL-10 can directly inhibit the activation of Th2 cells. It has been shown that IL-10 inhibits CD28 costimulatory signaling, which is the second signal necessary to elicit a T cell response when the first signal is given through T cell receptor [94]. IL-10 skews the production of antigen specific IgE towards production of IgG4 antibodies [94]. Moreover, it has been shown that IL-10 can directly dampen the mast cell sensitization by inhibiting the expression of FCεRI on mast cells which is a high affinity receptor for IgE [95]. Different immune cells can produce IL-10 including B cells [51], T cells [80] and dendritic cells [96].

IL-10 producing Treg cells in SIT

Several lines of research have provided evidence for IL-10 producing Treg cells in SIT. IL-10 production by $CD4^+CD25^+$T cells has been observed in *ex vivo* stimulated PBMCs after venom [97], grass pollen [91], house dust mite [98] and birch pollen SIT [84]. It has been shown that IL-10 producing $CD4^+CD25^+$T cells suppress the allergen induced T cell proliferation in *ex vivo* stimulated PBMCs through an IL-10 dependent mechanism [97]. Induction of IL-10 producing $CD4^+CD25^+$T cells has been concurrent with alleviated clinical symptoms and skin prick test reactivity, suggesting a functional role for these cells in SIT-induced suppression of allergic symptoms [98;84]. Local production of IL-10 has also been observed in the skin at the site of SIT injections three month after venom SIT [99], and in nasal mucosa two years after grass pollen SIT [100] suggesting an involvement of a local tolerogenic mechanism through induction of IL-10. In the studies mentioned above it is not clear, however, whether the observed $CD4^+$ $CD25^+$ IL-10 producing cells are $FOXP3^-$ Tr-1 cells or $FOXP3^+$ IL-10 producing cells, since FOXP3 has not been measured in the studies. Although it has been shown that both FOXP3 positive and negative IL-10 producing subsets have similar tolerogenic capacities [80;81], the two subsets are induced by different mechanisms.

Evidence for a potential role of $IL\text{-}10^+FOXP3^+$ Treg cells in therapeutic effects of SIT comes from three recent clinical studies. In the first study, the investigators observed that house dust mite (HDM) SIT increased the number of circulating $CD4^+CD25^+FOXP3^+IL\text{-}10^+$ Treg cells one year after the SIT treatment in house dust mite sensitized asthmatic children [88]. HDM-SIT in these children was reported to also increase soluble IL-10 in plasma, which was significantly and positively correlated with the Forced Expiratory Volume in 1 second (FEV1), an index of lung function, indicating an association of IL-10 production with clinical improvement. Similarly, the second study found that 1.5 to 2 years after HDM-SIT in HDM sensitized allergic children, the number of $CD4^+CD25^+FOXP3^+IL\text{-}10^+$ Treg cells were increased in *ex vivo* HDM-stimulated peripheral blood mononuclear cells (PBMCs) in comparison to SIT-untreated asthmatic children [83]. One final study reported that SIT to Japanese cedar pollinosis induces $CD4^+CD25^+FOXP3^+IL\text{-}10^+$ Treg cells in *ex vivo* stimulated PBMCs, which was accompanied by attenuated IL-4 and IL-15 responses and improved clinical symptoms [92].

All together, clinical studies suggest a role for IL-10 producing Treg cells in SIT-induced allergen tolerance. Although these studies have observed a concurrent alleviation of clinical symptoms with the increased frequency of

IL-10 producing T cells, it has yet to be elucidated whether the observed induction of IL-10 producing Treg cells is critically important for the therapeutic effects of SIT.

TGF-β producing Th-3 regulatory T cells in SIT

Transforming Growth Factor-b plays important roles in immune regulation and homeostasis [101]. TGF-β has been implicated in SIT and can contribute to SIT-mediated tolerance induction through different mechanisms. The first mechanism of action for TGF-β is facilitating the induction of $FOXP3^+$ Treg cells. TGF-β can induce $FOXP3^+$ iTreg cells from naïve T cells upon antigen stimulation in the absence of IL-6 [102;103] or presence of all-trans retinoic acid [104]. TGF-β producing Th3 cells are a subtype of iTreg cells that were first identified during the induction of oral tolerance [105]. The second mechanism of action for TGF-β is instructing B cells to produce antigen specific IgA which can bind to allergen preventing its binding to IgE [106]. Several clinical studies have observed increased TGF-β production after different SIT treatments. The first study showed that HDM-SIT reduces the *ex vivo* allergen specific proliferation of T cells from the pool of PBMCs during the first 70 days of the treatment [89]. Neutralization of TGF-β in these cultures using soluble TGF-β receptor II increased the specific proliferation of T cells in this study, suggesting that SIT-induced suppression of T cell proliferation is mediated by TGF-β. Likewise, O'Hehir and colleagues have observed that sublingual HDM-SIT resulted in suppressed *ex vivo* allergen-specific proliferation of T cells 6 month after starting the treatment [107]. Again, neutralization of TGF-β in the cultures using TGF-βRII-Ig augmented the allergen-specific proliferation of T cells of SIT-treated patients. In a different study, it was shown that grass pollen SIT increased the expression level of mRNA of TGFb in nasal mucosa and specific IgA_2 in serum of the treated patients 2 years after the treatment [100]. The authors observed that the TGF-β expression level positively correlated with the level of specific IgA2 in serum indicating a potential contribution of TGF-β to the immunoglobulin class switching of B cells. A similar observation was reported 2 years after pollen SLIT [45]. Increased levels of TGF-β were observed in the serum of treated patients, and these correlated with the increased level of serum IgA two years after the treatment. Another study reported that HDM-SIT increased the level of TGF-β in the serum of treated patients which significantly correlated with the improved FEV1 in these patients one year after the treatment [88]. All together, data from clinical studies suggest a role for TGF-β in SIT-induced allergen tolerance. TGF-β can be produced by a variety of immune cells including dendritic cells, B

cells and T cells [75;108;109]; however, the source of production of TGF-β in SIT and its detailed mechanism of action is currently incompletely understood.

Improvement of SIT

There is an unmet medical need for improving the clinical efficacy of SIT [35]. One approach towards improving the efficacy of SIT is modifying the immune response to the administered allergen by triggering pathogen associated pattern recognition receptors (PPRs) such as toll-like receptors and NOD-like receptors on dendritic cells during SIT treatment [110]. Alum, the classical adjuvant of SIT, triggers NALP3 on dendritic cells and has shown a good safety profile while its efficacy in improving SIT is not optimal [110;111] so there is a need for a better adjuvant. It has been shown that lipopolysaccharide, polyinosinic-polycytidylic acid (polyIC), CpG-containing DNA sequences are able to inhibit Th2 cell activation and could potentially be used as adjuvant for SIT [112]. However, safety of such compounds should be addressed cautiously since ligands of PPRs are potent immunostimulators and they can lead to undesirable immune reactions [112].

Another approach to improve the efficacy of SIT is by facilitating the generation of iTreg cells for instance by inhibiting DC maturation through administration of vitamin D3 or tryptophan deprivation, blocking CD28-mediated T cell co-stimulation, and administration of TGF-β with SIT. We have previously shown that co-administration of vitamin D3, an inhibitor of nuclear factor kappa B pathway which prevents DCs maturation, enhances the efficacy of SIT in suppressing the manifestation of allergic asthma in a mouse model of SIT [113]. The efficacy of blocking CD28-mediated T cell co-stimulation and administration of TGF-β during SIT has to be yet elucidated.

Mouse model of SIT

Since the functional role of individual cell subsets and molecules involved in SIT-induced allergen tolerance could not be addressed in clinical studies we used a well-established mouse model of ovalbumin-driven SIT to unravel the mechanisms of SIT-induced allergen tolerance. In our model mice are sensitized by two intraperitoneal injections of ovalbumin (OVA) precipitated on alum. After a period of at least ten days mice receive three subcutaneous injections of either OVA-SIT (sub-optimal: 100 μg/injection, optimal: 1 mg/injection) or saline-placebo as control. Two weeks later, mice will be three times exposed to aerosolized OVA to evaluate whether develop the manifestation of allergic asthma [114].

This model closely resembles the clinical application of SIT in several ways.

First, performing SIT in the mouse model leads to a long-term suppression of three cardinal parameters of experimental allergic asthma: AHR, airway eosinophilia and specific IgE in the serum, mirroring observations in human studies on SIT [114]. Second, SIT suppresses the manifestations of experimental allergic asthma in an IL-10-dependent manner [115]. Alike clinical SIT, the level of specific serum IgE in this model is initially increased by SIT followed by a gradual decline [114]. Taken together, the provided evidence indicates that the used mouse model of SIT is a relevant model to study the mechanisms of action of allergen immunotherapy for allergic asthma.

Aim of the this thesis

The prevalence of allergic diseases has been increased during the past five decades while a casual explanation for this increase is still lacking today [2]. The hygiene hypothesis has been postulated to explain this increase but it is under debate and has not been experimentally proven yet [116]. Besides improved hygiene status, increased prevalence of allergic diseases has been associated with increased iron intake by infants in industrialized countries [117;118]. In **Chapter 2** of this thesis with the aim of finding an explanation for the increased prevalence of allergic diseases we address the effects of high iron intake on the manifestation of allergic asthma.

SIT is the only treatment for allergic diseases that modifies the allergen-specific immunological responses underlying the disease, leading to long term relief of symptoms. However, there is an unmet medical need for further improvement of SIT to overcome drawbacks such as the requirement of application of high doses of allergen, the risk of severe side-effects including anaphylaxis, the long duration of treatment and the controversial efficacy in allergic asthma [35]. Rational improvement of SIT requires a detailed knowledge of the mechanism of its action.

The blocking IgG hypothesis has been suggested as a mechanism of action of SIT since increased level of IgG in serum of SIT-treated patients has been associated with the alleviation of allergic symptoms [52;54;55]. However, blocking IgG hypothesis has not been tested in functional studies yet. Moreover, regulatory B cells might play a role in SIT-induced allergen tolerance [50;119]. To address the role of regulatory B cells and functional importance of blocking IgG antibodies for the therapeutic effects of SIT, we compared the efficiency of SIT in FcγR and B cell deficient with wild type mice as described in **chapter 3** of this dissertation.

There is evidence indicating that plasmacytoid dendritic cells play tolerogenic roles in the lungs [73]. It has been shown that pDCs express inducible indoleamine

2,3 dioxygenase enzyme (IDO) which shows anti-inflammatory activities [120]. The contribution of IDO to the suppressive effects of SIT in our model has been shown previously [121]. In the **Chapter 4** of this thesis we address the role of pDCs in the induction of SIT-mediated allergen tolerance in our model.

There is evidence suggesting that $CD4^+FOXP3^+$ Treg cells contribute to the therapeutic effects of SIT [90], however, exact details of their contribution has yet to be elucidated. In **Chapter 5** of this thesis we address the role of $CD4^+FOXP3^+$ Treg cells in SIT-mediated suppression of the manifestation of experimental allergic asthma at two time points: 1- during the SIT treatment 2- during inhalation challenges where SIT-induced tolerance suppresses the manifestation of experimental allergic asthma.

IL-10 production has been associated with successful SIT in human patients and plays indispensible role for the suppressive effects of SIT in our model [52;115]. The source of IL-10 production, however, remains to be indentified to date. In **chapter 6** of this manuscript we address the identity of the IL-10 producing cell that is critically required for the suppressive effects of SIT.

Traditionally alum has been used as an adjuvant for SIT, however, its efficacy is not optimal [35]. Cytotoxic T Lymphocyte Antigen 4 is an anti T cell activation molecule expressed by nTreg cells and activated T cells [122]. CTLA4-Ig is a fusion molecule, consist of the extracellular domain of CTLA4 fused to heavy chain of human or mouse IgG [123;124]. CTLA4-Ig is a proven compound to be clinically used in the treatment of rheumatoid arthritis and prevention of allograft rejection. Using our mouse, we test whether CTLA4-Ig could be used as an efficient and clinically applicable adjuvant for SIT in **Chapter 7** of this thesis.

Increased levels of TGF-β have been observed in SIT-treated patients that correlate with the therapeutic effects of SIT [100]. It has been shown that TGF-β can induce iTreg cells in the presence of retinoic acid [125]. In **chapter 8** we examine whether administration of TGF-β at the time of SIT-treatment can enhance the beneficial effects of SIT presumably by induction of Treg cells.

Immune complexes can pass through the milk and induce allergen tolerance in a mouse model of allergic asthma [126]. In **chapter 9** of this manuscript we address the interesting question whether SIT-induced allergen tolerance can be transferred to the offspring through the milk. **Chapter 10** of this thesis consists of summarizing conclusions of all the previous chapters.

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CHAPTER

2

Iron administration reduces airway hyperreactivity and eosinophilia in a mouse model of allergic asthma

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Abstract

The prevalence of allergic diseases has increased dramatically during the last four decades and is paralleled by a striking increase in iron intake by infants in affluent societies. Several studies have suggested a link between increased iron intake and the marked increase in prevalence of allergic diseases. We hypothesized that the increased iron intake by infants offers an explanation for the increased prevalence of allergic disease in industrialized societies during the past four decades.

A well-established mouse model of OVA-driven allergic asthma was used to test the effects of differences in iron intake and systemic iron levels on the manifestations of allergic asthma.

Surprisingly, iron supplementation resulted in a significant decrease in airway eosinophilia, while systemic iron injections lead to a significant suppression of both allergen induced airway eosinophilia and hyperreactivity as compared to placebo. In contrast, mice fed on iron-deprived diet did not show any difference in developing experimentally induced allergic asthma when compared to those fed on iron-sufficient control diet.

In contrast to our hypothesis, airway manifestations of allergic asthma are suppressed by both increased levels of iron intake and systemic iron administrations in the mouse model.

Introduction

The prevalence of allergic diseases, such as allergic asthma, hay fever, and atopic dermatitis has increased markedly in children over the past four decades [1]. Many explanations for this increase have been postulated, of which the hygiene hypothesis [2] has gained considerable attention in the last two decades. However, an undisputed causal explanation is still lacking [3]. Associated with the increased prevalence of allergic diseases, there is a steady increase in iron intake by infants in affluent societies during the last decades [4;5]. The reasons for the increased iron intake by infants are: (i) reduced frequency and duration of exclusive breastfeeding, (ii) increased use of iron-fortified formula milk and (iii) increasing concentrations of iron in the formula milk to about 20 times the level in human breast milk [6]. In addition, efforts have been made to enhance the uptake of the iron from the formula by switching to the use of highly bioavailable forms of iron, like ferrous sulfate, and by adding vitamin C. The latter agent stimulates iron uptake by keeping it in its reduced and more bioavailable form [7]. Furthermore, since 1972 there has been a widespread introduction of iron-fortified food in the weaning diet [4;5].

We hypothesized that the marked increase in iron intake by infants offers an explanation for the increased prevalence of allergic diseases during the last decades.

Although an epidemiologic study addressing the association between increased levels of dietary iron and the increased prevalence of allergic diseases is still lacking, there is evidence suggesting a link between increased iron intake and the marked increase in prevalence of allergic diseases. The prevalence of asthma is higher in patients with disorders such as thalassemia minor and sickle cell anemia which cause elevated systemic iron levels compared to patients without hemoglobinopathies [8]. Furthermore, it has been observed that asthmatic patients show increased plasma levels of iron compared to healthy controls [9]. Moreover, data from animal models also point to a role for iron in allergic manifestations. It has been shown that administration of an iron chelator significantly decreases the serum levels of IgE in a rat model of Th2-mediated autoimmunity [10]. Additionally, lactoferrin -a natural iron chelator- plays a protective role against symptoms of experimental allergy in a mouse model [11]. Furthermore, it has been shown that iron overload strikingly increases serum levels of antigen-specific IgE in experimental candidiasis in mice [12].

Altogether, these data suggest a causal relationship between iron metabolism and the development of allergic diseases. Nevertheless the effects of dietary iron supplementation on allergic manifestations have not been experimentally tested in detail yet. In the present study, we use a well-established mouse model of allergic asthma to investigate whether increased iron intake augments the manifestations of experimental allergic asthma. We further addressed the impact of elevated level of systemic iron on the symptoms of experimental allergic asthma. Finally, we tested the effects of dietary iron deprivation on the manifestations of experimental allergic asthma. Remarkably, we found that increased serum iron levels significantly reduce allergen-induced airway hyperreactivity (AHR) to methacholine and airway eosinophilia but do not affect allergen-specific IgE levels.

Materials and Methods

Animals

Specified pathogen free (according to the Federation of European Laboratory Animal Science Associations (FELASA) guidelines) 3-week-old male BALB/c mice were purchased from Charles River laboratories (L'Arbresle, France) and were kept under SPF condition. All animal experiments were

performed in accordance with the guidelines of the institutional animal care and use committee of the University of Groningen.

Iron administration

In the first experiment, mice were maintained on drinking water containing either 250 mg Fe/L as $FeSO_4$ +1 % ascorbic acid (Sigma-aldrich, Dordrecht, The Netherlands) or only 1% ascorbic acid starting two weeks before sensitization until the end of the experiment. Since the concentration of iron in the formula milk is increased to about 20 times the level in human breast milk [6], the dose of supplemented iron in this experiment has been adjusted to 20 times the concentration of iron in mouse milk which is 12-15 mg/L [13].

For the second experiment, injectable iron dextran (Cosmofer®, Pharmacosmos A/S, Holbaek, Denmark, 60 mg/Kg for treatment group) or an equivalent amount of low density dextran (Mw ≈ 6000 for the control group, Sigma-aldrich) was injected intraperitoneally on alternate days starting two weeks prior to sensitization until the end of the experiment. The dose of injectable iron is determined based on non-toxic dose of iron previously used by Mencacci et al. [12]. In the last experiment mice were fed on casein-based semi-synthetic chow (Research Diet Services B.V. Utrecht, The Netherlands) containing either 7 or 35 ppm iron. Mice maintained on the diet from two weeks prior to sensitization until the end of the experiment.

Protocol for the induction of experimental allergic asthma

Experimental allergic asthma was induced as described elsewhere [14]. Briefly, mice received 2 intraperitoneal injections of 10 µg ovalbumin (Sigma-aldrich) + 2.25 mg alum (Pierce, Rockford, IL, USA) in 100 µL of pyrogen-free saline on days 0 and 7. Two weeks later, they were exposed to aerosolized ovalbumin solution in saline (1% w/v) for 20 minutes 3 times every third day.

Evaluation of airway responsiveness

Airway responsiveness to inhaled methacholine (Sigma-aldrich) was measured twice (before and after aerosolized ovalbumin inhalation challenges) in conscious, unrestrained mice using barometric whole-body plethysmography by recording respiratory pressure curves (Buxco; EMKA Technologies) as described in detail previously [14].

Determination of serum levels of ovalbumin-specific IgE

After measuring airway responsiveness, blood was taken, serum samples

were prepared and stored at a temperature of -80°C until further analysis. Serum levels of ovalbumin-specific IgE were determined by enzyme-linked immunosorbent assay (ELISA) as described previously and results are expressed as EU/ml [15].

Analyses of the BAL fluid

Bronchoalveolar lavage (BAL) was performed as explained previously [15]. In brief, animals were lavaged five times through the tracheal cannula with 1 ml aliquots of saline containing a cocktail of protease inhibitors (complete mini tablet (Roche Diagnostics) and 1% BSA(Sigma-aldrich)). BAL cells were counted, cytospins were made and stained using Diff-Quik (IMEB, CA, USA). Cells were identified by standard morphology.

Preparation of lung tissue for cytokine measurement

Cardiac lobe of lung was taken, homogenized and used to measure cytokine levels as described previously [16]. Concisely, lung tissue was homogenized in 20% (w/v) luminex buffer (50 mM Tris-HCl, 150 mM NaCl, 0.002% Tween 20, and protease inhibitor, pH 7.5) on ice. Subsequently, supernatants were collected for cytokine measurement after spinning the lung tissue homogenates for 10 min at 12,000 x g.

Measurement of cytokines

IL-4, IL-5, and IL-13 in the lung tissue were determined by a commercially available ELISA kit according to the manufacturer's instructions (BD Pharmingen, NJ, USA). The detection limits were 32 pg/ml for IL-5 and 15 pg/ml for IL-4 and IL-13.

Serum iron quantification

Total iron in serum was measured using colorimetric Bathophenanthroline (Sigma-aldrich) assay and according to the Ramsay method [17] with minor changes. In brief, the optical density (OD) of the reaction resulted from protein-precipitated serum with Bathophenanthroline was read in 96-well half-area plates (Greiner Bio-One B.V) using ELx808™ microplate reader. The concentration of serum iron was calculated using standard curves drawn based on measured OD values of known serial dilutions of iron(II) solution. To measure total iron-binding capacity (TIBC), serum was first saturated using iron chloride solution (Sigma-aldrich) followed by removal of unbound iron. Then the concentration of total of iron was measured using the above

mentioned steps.

Hemoglobin measurement

Hemoglobin concentration in serum was measured using a commercially available kit (QuantiChromTM Hemoglobin Assay Kit, BioAssay Systems, CA, USA) according to the manufacturers instructions.

Statistical analysis

Data are expressed as mean ± SEM. The airway resistance curves to methacholine were statistically analyzed using a general linear model of repeated measurements. All the other data were compared using student's t-test. A P-value of less than 0.05 was considered significant.

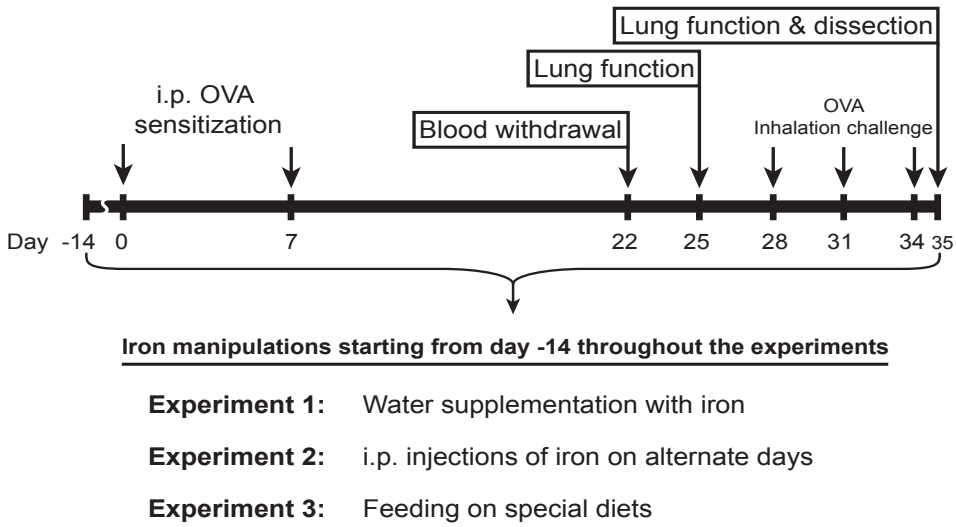

Figure 1 - Experimental layout

A mouse model of OVA-driven allergic asthma was used to test the effects of increased levels of oral or systemic iron or decreased levels of dietary iron on the manifestation of experimentally induced allergic asthma. Mice were intraperitoneally sensitized twice at day 0 and 7, followed by three inhalation provocations with aerosolized OVA solution (1% in PBS) on day 28, 31, 34 and dissected on day 35. In experiment 1, mice were maintained on drinking water containing either 250 or 35 mg/L iron as $FeSO_4$ starting two weeks before i.p ova sensitization till the end of the experiment. In experiment 2, mice received iron injections 60 mg/Kg body weight given as iron dextran on alternate days starting at day -14 before i.p. ova sensitization till the end of the experiment. In experiment 3, mice were fed on iron-deprived diet containing 7mg/Kg food or iron-sufficient diet containing 35mg/Kg food from 14 days before i.p. sensitization throughout the experiment.

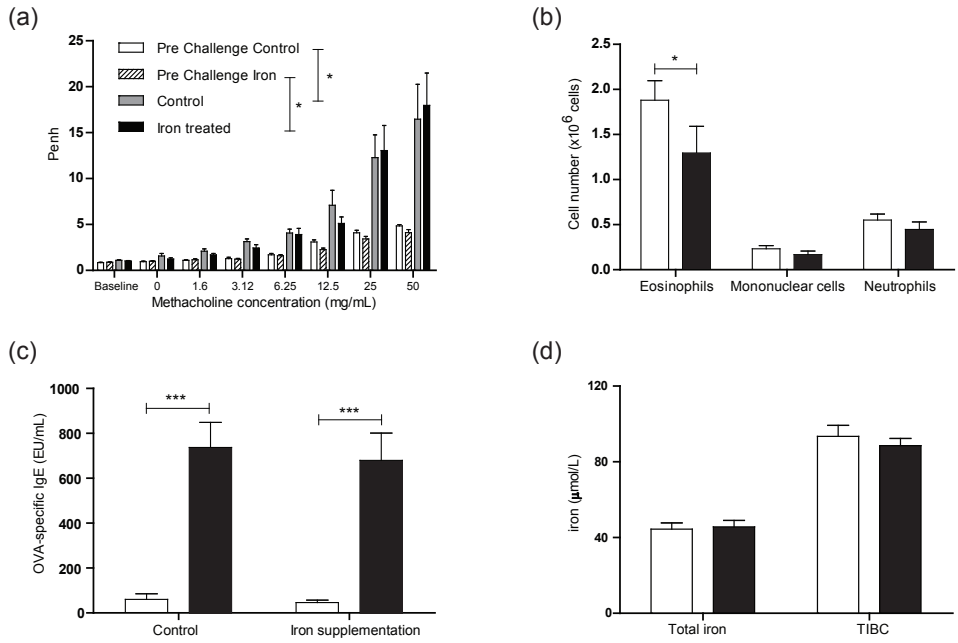

Figure 2 - Effects of iron-supplementation on the manifestation of allergic asthma

Manifestations of experimentally induced allergic asthma in mice receiving iron supplemented drinking water (black and hatched bars) or control water (open and gray bars): (a) airway responsiveness to methacholine before (open- and hatched bars) and after inhalation challenge (gray and black bars), (b) numbers of eosinophils, neutrophils and mononuclear cells in BAL, (c) levels of ova-specific serum IgE before (open bars) and after (black bars) inhalation challenge, (d) total iron and TIBC concentrations in serum. Data are shown as Mean ± SEM, N=8. (*:P<0.05 , ***,$: P<0.005).

Results

Dietary iron supplementation reduces airway eosinophilia

To address the question whether increased iron intake augments the manifestations of experimental allergic asthma, mice were maintained on drinking water supplemented with either 250 mg/L iron + 0.1% w/v ascorbic acid or 0.1% w/v ascorbic acid as control throughout the experimental procedure (Figure 1). According to the protocol, mice were first sensitized by two OVA/alum intraperitoneal (i.p) injections followed by three inhalation challenges with OVA aerosols 3 weeks later.

Airway hyperresponsiveness (AHR) to methacholine was evaluated before

and after the airway challenges. We did not observe any significant differences between experimental groups in AHR (Figure 2a).

Nevertheless, we did observe a small but significant decrease in allergen-induced airway eosinophilia in the group receiving increased concentrations of dietary iron in the drinking water as compared to the control group ($P<0.05$, $1.3x10^6 \pm 3.0x10^5$ cells in BAL in iron-supplemented compared to $1.9x10^6 \pm 2.2x10^5$, Figure 2b). Serum levels of OVA-specific IgE were not different between the two experimental groups (Figures 2c). Surprisingly, no difference between iron-supplemented and control groups was observed (Figure 2d) in total iron concentration in serum or in TIBC, which reflects transferrin levels in serum [18]. Body weight gain was constantly monitored during the experiment and no significant differences between groups were observed.

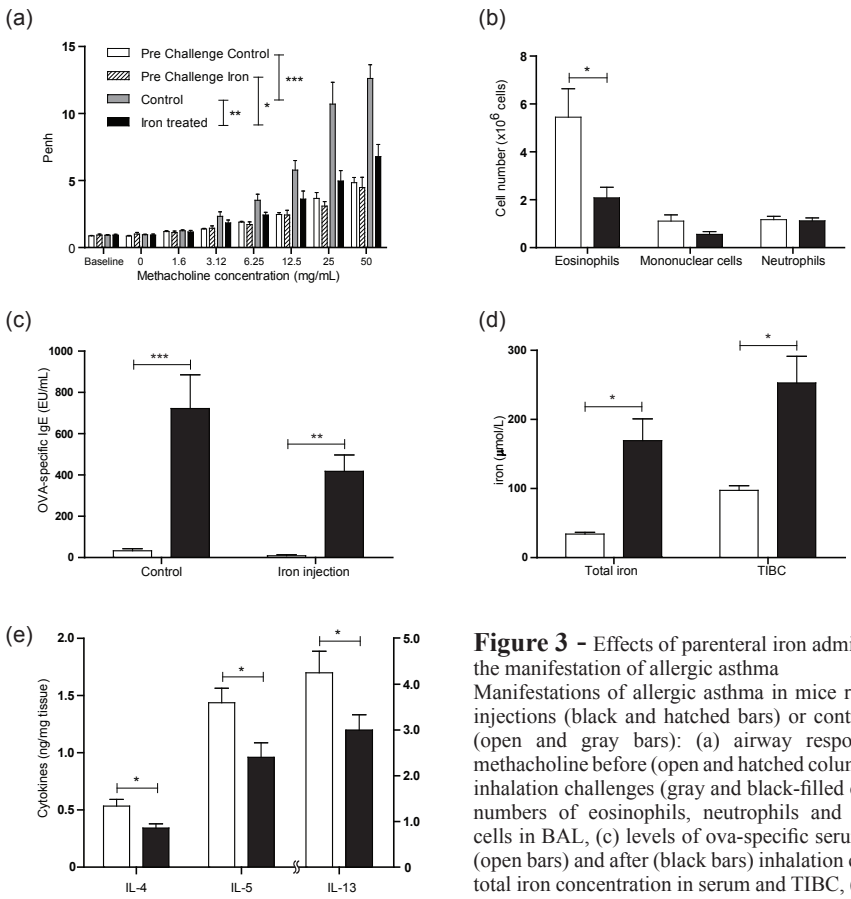

Figure 3 - Effects of parenteral iron administration on the manifestation of allergic asthma
Manifestations of allergic asthma in mice receiving iron injections (black and hatched bars) or control injections (open and gray bars): (a) airway responsiveness to methacholine before (open and hatched columns) and after inhalation challenges (gray and black-filled columns), (b) numbers of eosinophils, neutrophils and mononuclear cells in BAL, (c) levels of ova-specific serum IgE before (open bars) and after (black bars) inhalation challenge, (d) total iron concentration in serum and TIBC, (e) Cytokines levels in the lung tissue. Data are shown as Mean ± SEM, N=8. (*:P<0.05 , **:P<0.01, ***,$: P<0.005).iron and TIBC concentrations in serum. Data are shown as Mean ± SEM, N=8. (*:P<0.05 , ***,$: P<0.005).

Systemic administration of iron decreases allergen-induced airway eosinophilia and Th2 cytokine release

Since total iron concentration in serum was not increased by iron-supplemented drinking water, we used a more robust strategy to increase the systemic iron levels. To this end, mice received intraperitoneal (i.p.) injections of iron dextran (Cosmofer®; 60 mg/Kg Fe) on alternate days throughout the asthma induction protocol (Figure 1). As expected, total iron concentration in serum and TIBC were significantly increased in the iron treatment group as compared to control ($P<0.05$, 169±31 μmol/L compared to 34±2 and 252±38 μmol/L compared to 97±6, Figure 3d). Increased total iron in serum and TIBC indicate that current iron administration method is suitable for dissecting the effects of increased serum levels of iron on the manifestations of allergic asthma in our mouse model.

We measured AHR to methacholine before and after airway challenges, followed by dissecting the mice, counting airway eosinophils, and measurement of Th2 cytokines in lung and allergen-specific IgE levels in serum. As presented in figure 3b, airway eosinophilia was markedly and significantly decreased in iron-treated mice as compared to dextran-treated controls ($P<0.05$, $2.0x10^6$ cells compared to $5.4x10^6$). Surprisingly, parenteral iron treatment almost completely inhibited the allergen induced AHR as compared to dextran-treated control mice ($P<0.05$, e.g. at dose 50 mg/ml 6.8±0.9 Penh compared to 12.6±1.0 Figure 3a). However, iron treatment did not affect serum levels of OVA-specific IgE ($p=0.12$, Figure 3c). Since in this experiment total iron concentration in serum and TIBC were clearly increased and a significant suppressive effect was observed for iron administration on airway manifestation of allergic asthma, we investigated the T-cell response in the lung tissue by measuring IL-4, IL-5, IL-13, interferon-γ and IL-10 in the lung tissue. Levels of the Th2 cytokines IL-4, IL-5 and IL-13 in lung tissue were significantly reduced in iron-treated mice as compared to dextran-treated controls ($P<0.05$, 342±36 compared to 534±38, 961±126 compared to 1437±127 and 2997±335 compared to 4248±471 respectively, Figure 3e). Neither interferon-γ nor IL-10 levels in the lung tissue were affected by iron treatment (data not shown). These data show that differences in serum levels of iron do have a direct effect on manifestations of allergic asthma in the mouse model, with a marked reduction of AHR and airway eosinophilia in mice with increased levels of serum iron and TIBC.

Low dietary iron does not affect the development of allergic asthma

In contrast to our initial hypothesis, we found that increased iron intake and high

systemic iron administration suppress the manifestations of allergic asthma in the mouse model. Therefore, we asked the question whether restricting dietary iron causes augmented allergic response. It is recommended that mice are maintained on diets containing at least 35 ppm iron to prevent iron deficiency. [19]. However, lower concentrations of iron have been considered sufficient and

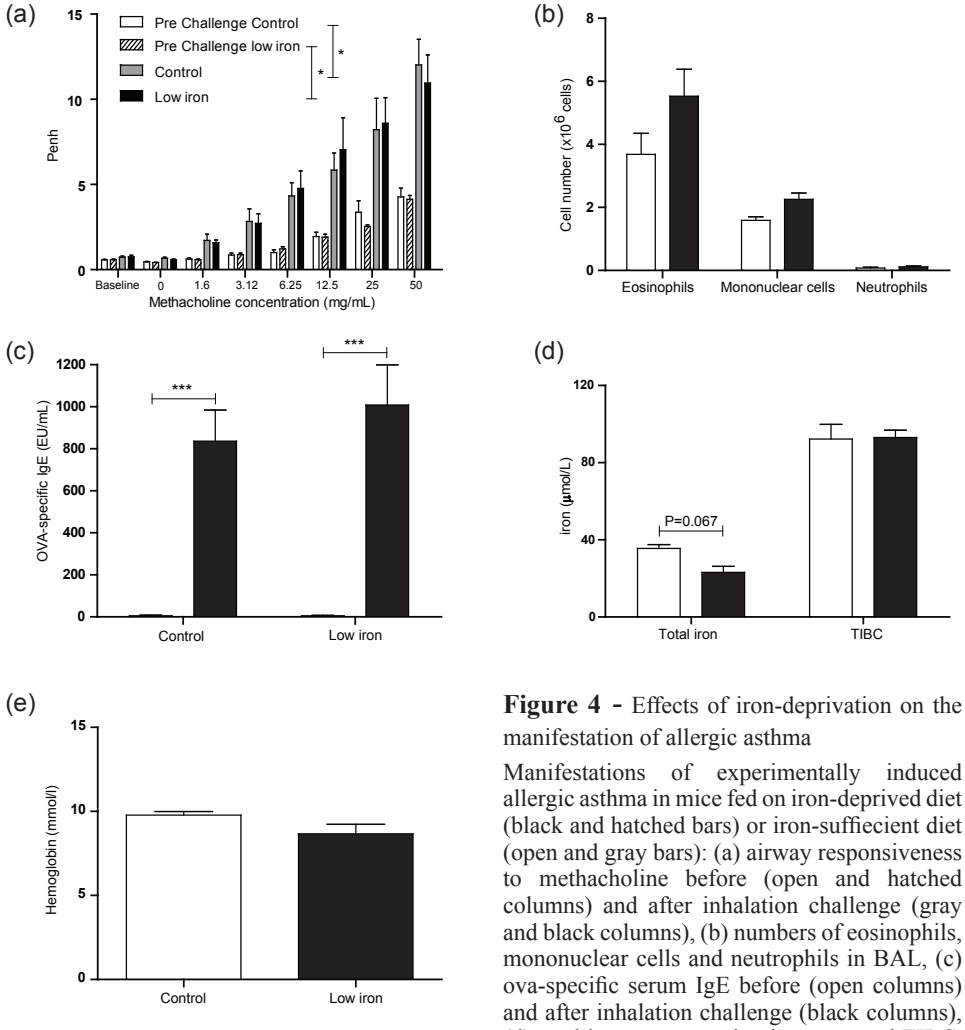

Figure 4 - Effects of iron-deprivation on the manifestation of allergic asthma

Manifestations of experimentally induced allergic asthma in mice fed on iron-deprived diet (black and hatched bars) or iron-suffiecient diet (open and gray bars): (a) airway responsiveness to methacholine before (open and hatched columns) and after inhalation challenge (gray and black columns), (b) numbers of eosinophils, mononuclear cells and neutrophils in BAL, (c) ova-specific serum IgE before (open columns) and after inhalation challenge (black columns), (d) total iron concentration in serum and TIBC, (e) Hemoglobin concentration in blood. Data are shown as Mean ± SEM, N=8. (*:P<0.05 , **,#:P<0.01, ***,$: P<0.005).

previously used in other studies without causing deficiencies [20]. Therefore, we examined the effect of a low-iron diet (7 ppm iron) as compared to iron-sufficient control diet (35 ppm iron) in our mouse model of allergic asthma. As presented in figure 1, mice were fed on diets starting two weeks prior to the first i.p sensitization and continued throughout the experiment. Total iron concentration in serum at the end of the experiment showed a trend towards a decrease ($p=0.067$, 26.8 ± 3.3 compare to 35.5 ± 2.4 Figure 4d) in the low iron diet group as compared to iron-sufficient control group. Nevertheless, the mice maintained on a low dietary iron diet did not show any difference in allergen-induced airway eosinophilia, AHR and serum levels of OVA-specific IgE as compared to mice fed on regular iron diet (Figures 4a-c). Hemoglobin concentration was measured in this experiment as an indication of iron body status and showed no difference between mice fed on low iron containing chow as compared to control treated mice (Figure 4e).

Discussion

In the present study we demonstrate that in contrast to our initial hypothesis, increased iron intake does not exacerbate the manifestations of experimental allergic asthma. Conversely, high iron intake induces a small but significant decrease in allergen induced airway eosinophilia, while serum allergen-specific IgE levels and AHR are not affected. Moreover, we show that elevated levels of systemic iron due to parenteral iron administration strongly impacts upon the symptoms of experimental allergic asthma by completely inhibiting AHR and largely suppressing airway eosinophilia as well as Th2 cytokines in lung tissue.

Remarkably, our data show that supplementation of drinking water with iron does not increase the total iron concentration in serum as compared to control mice. This is most likely due to a phenomenon known as mucosal blockade of iron absorption which leads to a rapid reduction in iron absorption from the gut subsequent to a high dose iron intake [21]. Interestingly, it has also been shown that iron supplementation does not increase iron absorption in infants under the age of 6 month while increases iron absorption in older infants [22]. Despite the absence of increased serum iron levels, we observed a significant reduction of BAL eosinophil numbers in mice receiving iron supplementation in drinking water. Reduced number of eosinophils in the BAL can be an indirect consequence of several iron-induced regulatory mechanisms. It has been shown that increased dietary iron enhances the expression of lactoferrin, L-ferritin and hepcidin, to reduce iron absorption and increase iron storage capacity [23-25]. Interestingly, Kruzel and colleagues showed that lactoferrin

decreases pollen-induced airway eosinophilia by decreasing the formation of reactive oxygen species in a murine model of asthma [11]. Therefore, we speculate that enhanced expression of lactoferrin as a result of increased iron intake may explain the reduction of BAL eosinophilia in mice receiving iron supplemented drinking water.

Since serum iron levels were not increased after iron supplementation, we studied the effect of parenteral iron administration in the mouse model of allergic asthma. Our data clearly demonstrate that elevated systemic levels of iron due to parenteral iron administration completely inhibit allergen induced AHR and largely suppress, airway eosinophilia. These suppressive effects are associated with reduced levels of Th2 cytokines in lung tissue. The observed inhibition in AHR and eosinophilia could be caused by increased expression of heme oxygenase-1 (HO-1) as a result of the elevated iron levels in serum. HO-1 is an enzyme involved in heme metabolism that has been demonstrated to be increased, particularly in lungs, by iron overload [26]. Interestingly, Xia and colleagues have demonstrated that induction of HO-1 by hemin or Sn-protoporphyrin was able to suppress allergen induced AHR, eosinophilia and antigen-specific serum IgE in a mouse model of allergic asthma [27]. However, these data are not completely in line with our observations since we did not observe an effect on serum IgE levels.

Our data also reveal that OVA-specific IgE level in serum was not influenced either by high iron intake or by parenteral iron administration. This appears to be in sharp contrast to the study of Mencacci, et al. , who showed that iron overload as a result of i.p. iron administration induces high levels of antigen specific IgE in serum [12]. However, it should be noted that they used an infection model using c. albicans which is fundamentally different from our model of allergic asthma. Candidiasis induces Th1-dominated immune responses for protection [28] while in our asthma model is dominated by a Th2 response. Moreover, Candida infection is associated with abundant production of pathogen recognition pattern molecules triggering innate and adaptive immunity through different pattern recognition receptors [29] whereas in our model, ovalbumin lacks intrinsic danger signals.

Considering the suppressive effects on airway manifestation of allergic asthma in our model after oral or parenteral iron administration, we were interested whether deprivation of dietary iron causes the opposite effect, e.g. exaggerated allergic responses. However, we demonstrate that there is no difference between mice maintained on a diet containing 7 ppm versus those maintained on a diet containing 35 ppm iron in developing the manifestation of experimental allergic asthma.

We used a well-established mouse model of allergic asthma under the situation of oral and systemic iron manipulations to find evidence supporting our hypothesis that the marked increase in iron intake by infants offers an explanation for the increased prevalence of allergic diseases during the last decades. Using inbred animals with a single genetic background we did not observe augmented manifestations of allergic diseases due to increased oral or systemic iron administrations. Genetic background may be an important determinant of the outcome of increased iron intake on allergic diseases. So, at this point it can not be excluded that increased consumption of dietary iron by infants may lead to an increased prevalence of allergic diseases, including asthma in human populations. Hence, examining the effects of high iron intake in different mouse strains would be an informative experiment to perform along with comprehensive retrospective epidemiological and genetic studies on the association between increased dietary iron and the prevalence of allergic diseases. Overall, we demonstrate that in contrast to our hypothesis antigen-induced airway manifestations of allergic asthma in the mouse can be inhibited by parenteral iron administration.

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Disclosure

The authors confirm that there are no conflicts of interest to disclose.

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CHAPTER

3

Suppression of Th2-Driven Airway Inflammation by Allergen Immunotherapy Is Independent of B Cell and Ig Responses in Mice

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Abstract

Allergen-specific immunotherapy (SIT) uniquely renders long-term relief from allergic symptoms and is associated with elevated serum levels of allergen-specific IgG and IgA. The allergen-specific IgG response induced by SIT treatment was shown to be critical for suppression of the immediate phase of the allergic response in mice, and this suppression was partially dependent on signaling through FcγRIIB. To investigate the relevance of the allergen-specific IgG responses for suppression of the Th2-driven late-phase allergic response, we performed SIT in a mouse model of allergic asthma in the absence of FcγRIIB or FcγRI/FcγRIII signaling. We found that suppression of Th2 cell activity, allergic inflammation, and allergen-specific IgE responses is independent of FcγRIIB and FcγRI/FcγRIII signaling. Moreover, we show that the SIT-induced allergen-specific systemic IgG or IgA responses and B cell function are dispensable for suppression of the late-phase allergic response by SIT treatment. Finally, we found that the secretory mucosal IgA response also is not required for suppression of the Th2-driven allergic inflammation by SIT. These data are in contrast to the suppression of the immediate phase of the allergic response, which is critically dependent on the induced allergen-specific serum IgG response. Hence, SIT-induced suppression of the immediate and late phases of the allergic response is governed by divergent and independent mechanisms. Our data show that the SIT-induced suppression of the Th2 cell-dependent late-phase allergic response is independent of the allergen-specific IgG and IgA responses that are associated with SIT treatment.

Introduction

Allergen exposure in sensitized individuals results in cross-linking of IgE bound to FcεRI on the mast cell surface and induces the immediate type I hypersensitivity response, which is driven by inflammatory mediators released from the mast cells [1]. This immediate-phase allergic response is strictly IgE dependent and is treated by pharmacotherapy aimed at blocking mast cell effector molecules, such as histamine. The late response to allergen exposure is characterized by an influx of eosinophils, neutrophils, basophils, and activated CD4+ T cells and is sensitive to immunosuppressive drugs, such as steroids [1]. Allergen-specific immunotherapy (SIT) is the only disease-modifying treatment for allergic disorders and consists of a series of s.c. injections with increasing doses of allergen, which induces long-term desensitization and relief of symptoms [2, 3]. SIT is commonly used for the treatment of allergic rhinitis and insect venom hypersensitivity, yet its efficacy

in allergic asthma is controversial. The main drawbacks of SIT are the long-term treatment protocols and the risk for eliciting anaphylactic reactions [2]. Improvement of safety and efficacy can only be achieved by characterizing the immunological mechanisms that contribute to SIT-induced suppression of allergic inflammation in more detail.

Although the critical mechanisms by which SIT suppresses allergic symptoms have not been fully identified, several potential mechanisms have been postulated to contribute to SIT, converging on the modulation of T and B cell responses to the allergen [4]. With regard to the T cell responses, SIT was postulated to divert the allergen-specific Th2 response into Th1 responses and induce regulatory T cell activity [5]. With regard to the B cell responses, SIT was shown to induce an IgG1 and IgG4-dominated Ab response to the allergen with a mild increase in IgA levels, whereas the serum levels of allergen-specific IgE showed an early increase followed by a long-term gradual decline [1]. However, the relationship between the efficacy of SIT and induction of allergen-specific IgG remains controversial, with serum concentrations of allergen-specific IgG not consistently correlating with clinical improvement[1; 6; 7].

The increase in IgG serum levels, and especially IgG4, following SIT treatment led to the formulation of the blocking Ab hypothesis, which states that the IgG Abs compete with IgE for binding to the allergen [8]. As a result, the allergen is neutralized, preventing IgE cross-linking by the allergen and degranulation of mast cells [9]. In addition, signaling through the low-affinity IgGR (FcγRIIB) was shown to directly suppress IgE cross-linking–induced mast cell activation [10]. Cross-linking of FcγRIIB receptors on memory B cells by the allergen-specific IgG4 induced by SIT was shown to inhibit IgE production [11; 12]. In the mouse, allergen–IgG complexes can also induce IL-10 production by macrophages [13;14] and induce deviation of the Th cell response, resulting in increased IgG1 serum levels [13]. Allergen-specific IgA responses in serum are also induced by SIT for grass pollen [15] and house dust mite [16], although not consistently [17]. The possible role for allergen-specific IgA responses induced by SIT is thought to encompass the induction of IL-10 expression in monocytes by FcαRI cross-linking and allergen capture at mucosal surfaces [15].

We developed a mouse model for allergen-specific SIT based on the OVA-induced mouse model for allergic asthma. In this mouse model, SIT treatment by s.c. application of gradually increasing doses of OVA inhibits allergen-induced allergic airway inflammation and hyperresponsiveness in OVA-sensitized mice and significantly suppresses the OVA-specific IgE response in serum [18]. Subcutaneous delivery of three high-dose (1 mg) OVA

injections was shown to suppress asthma manifestations as efficiently as the gradual up-dosing of OVA in the mouse model [18]. The suppressive effects on asthma manifestations were found to last for ≥5 wk after SIT treatment and to be dependent on IL-10 production [19]. Moreover, SIT treatment also induces increased serum levels of OVA-specific IgG and IgA Abs, mirroring the observations in human subjects. It is unknown whether the induction of allergen-specific IgG and IgA responses contributes to the SIT-mediated suppression of Th2-driven allergic inflammation that is responsible for the late-phase allergic response in vivo. Interestingly, very recent data from a mouse model ofFelis domesticus allergen 1 (Fel d1)-specific SIT indicate that suppression of the immediate allergic reaction is dependent on the allergen-specific IgG response [20]. Our protocol of OVA-SIT specifically allows analysis of the SIT-induced suppression of the Th2-mediated late-phase allergic response, because the allergen-induced eosinophilic airway inflammation[21;22] and airway hyperresponsiveness (AHR) [23] are independent of allergen-specific IgE responses and the immediate phase of the allergic response in the OVA/alum mouse asthma model.

In this article, we tested whether the SIT-induced suppression of the Th2 cell-driven late-phase response is also critically dependent on the induction of allergen-specific IgG and IgA responses. Surprisingly, we found that suppression of allergic inflammation by OVA-SIT was efficiently induced in the absence of FcγRIIB signaling and even in the absence of an adaptive IgG or systemic IgA response in B cell-deficient mice. Finally, we show that mucosal IgA production is dispensable for suppression of allergic inflammation by SIT. Our data show that suppression of the Th2-driven late-phase allergic response by allergen-specific SIT is independent of B cell function and the induction of allergen-specific Ig responses.

Materials and Methods

Animals

Animal housing and experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Groningen. All mice used in the experiment were 6–8-wk-old males. Mice deficient for FcγRI/III and FcγRIIB and polymeric IgR (pIgR)-deficient mice were on the BALB/c background. Wild-type BALB/c control mice were obtained from Charles River Laboratories (Maastricht, The Netherlands). B cell-deficient Igh-6tm1Cgn (μMT) and wild-type littermates (both on a C57BL/6 background) were purchased from The Jackson Laboratory (Bar

Harbor, ME). All mice were housed in Makrolon cages in a laminar flow cabinet and provided with food and water ad libitum.

Sensitization, challenge, and SIT protocol

The short-term protocol (OVA-SIT in OVA-sensitized mice, Fig. 1D) was the same as previously described [19]. Briefly, mice received two i.p. injections of 10 μg OVA (endotoxin-free, <0.5 U/ml; Seikagaku Kogyo, Tokyo, Japan) adsorbed onto 2.25 mg alum (ImjectAlum, Pierce, Rockford, IL) 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 (SIT) or 200 μl saline (sham) on alternate days. One week after the last OVA or sham treatment, mice were challenged three times every third day with OVA aerosols in saline (1% w/v) for 20 min in a Plexiglas exposure chamber coupled to a Pari LC Star nebulizer (PARI Respiratory Equipment, Richmond, VA; particle size 2.5–3.1 μm) driven by compressed air at a flow rate of 6 l/min. The long-term protocol (OVA-SIT in OVA-sensitized and -challenged mice;Fig. 1A) was identical to the short-term protocol, except for three additional OVA inhalation challenges (or PBS control treatment) given 2 wk prior to sham/SIT treatments. The effects of possible endotoxin contamination in the OVA preparations on the efficiency of SIT was previously tested in our laboratory (A.J.M. van Oosterhout and B.C.A.M. van Esch, unpublished data), and all OVA preparations used in these experiments were in the lower range of endotoxin levels that were shown to efficiently induce SIT-dependent suppression of allergic inflammation (0.5–500 endotoxin unit/ml).

Airway responsiveness to methacholine, serum levels of Igs, cellular infiltration, and Th2 cytokine levels in bronchoalveolar lavage (BAL) fluid were measured 24 h after the last OVA inhalation challenge in each mouse.

Measurement of airway responsiveness

Airway responsiveness was assessed by measuring airway resistance in response to i.v. administration of increasing doses of methacholine (acetyl-b-methylcholine chloride, Sigma-Aldrich, St. Louis, MO), as described previously [24]. Briefly, anesthetized (ketamine/domitor) mice were attached to a computer-controlled small-animal ventilator (Flexivent; SCIREQ, Montreal, Quebec, Canada). Mice were ventilated at a breathing frequency of 280 breaths/min and a tidal volume of 10 ml/kg, which was pressure limited at 300 mm H2O. Airway resistance in response to increasing doses of methacholine was calculated from the pressure response to a 2-s pseudorandom pressure

wave, as described previously [24].

Determination of serum levels of OVA-specific Igs

Blood for assessment of serum Igs was collected 7 d before the first OVA aerosol challenge (via orbital puncture) and 24 h after the last challenge (via cardiac puncture). Serum was collected, and OVA-specific IgE, IgG1, IgG2a, and IgA levels in serum were measured by ELISA, as described previously [18]. A reference standard was used with arbitrary units of each isotype of 1000 experimental unit/ml. The detection levels of the ELISAs were 0.05 experimental unit/ml for IgG2a, 0.5 experimental unit/ml for IgE and IgA, and 0.005 experimental unit/ml for IgG1.

Analysis of the cellular composition in BAL fluid

Immediately after bleeding, the lungs were lavaged through a tracheal cannula with 1 ml saline at 37°C containing 5% BSA and 2 mg/ml aprotinin (F. Hoffman-La Roche, Basel, Switzerland). Cells were pelleted, and supernatants were used for measurement of cytokines by ELISA. Subsequently, lungs were lavaged with 4 ml saline, and BAL cells were pooled and counted. For differential BAL cell counts, cytospin preparations were stained with Diff-Quick (Merz & Dade, Dudingen, Switzerland) and evaluated by one observer in a blinded fashion. Cells were differentiated into mononuclear cells, lymphocytes, neutrophils, and eosinophils by standard morphology. At least 200 cells were counted per cytospin preparation.

Measurement of cytokines

IL-5 and IL-13 in the BAL fluid were determined by ELISA, according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). The lower detection limits of the ELISAs were 32 pg/ml for IL-5 and 15 pg/ml for IL-13.

Statistical analysis

All data are expressed as mean ± SEM. The Mann–Whitney U test was used to analyze the results, and $p < 0.05$ was considered significant.

Results

OVA-specific SIT suppresses airway inflammation and hyperresponsiveness in asthmatic mice

We previously developed and used a mouse model for OVA-specific SIT in the classical OVA/alum mouse asthma model [18]. In this model, gradual up-dosing or application of three high doses of OVA by s.c. injections in OVA/alum-sensitized mice renders these mice protected against clinically relevant asthma manifestations, such as eosinophilic airway inflammation and AHR upon subsequent OVA inhalation challenge. In this model, the mice have not experienced an asthmatic response prior to the SIT treatment, which is in contrast to the routine clinical situation in which a patient is treated with allergen-specific SIT only upon presenting with clinical manifestations of an atopic disease, such as rhinitis or allergic asthma. Hence, we first tested whether OVA-specific SIT would also be protective in mice that had experienced a prior OVA-induced asthmatic response. To address this question, we designed the experimental layout depicted in Fig. 1A. Before SIT treatment, mice were given an OVA/alum sensitization followed by OVA inhalation challenges (asthmatic mice) or PBS control treatment (sensitized-only mice).

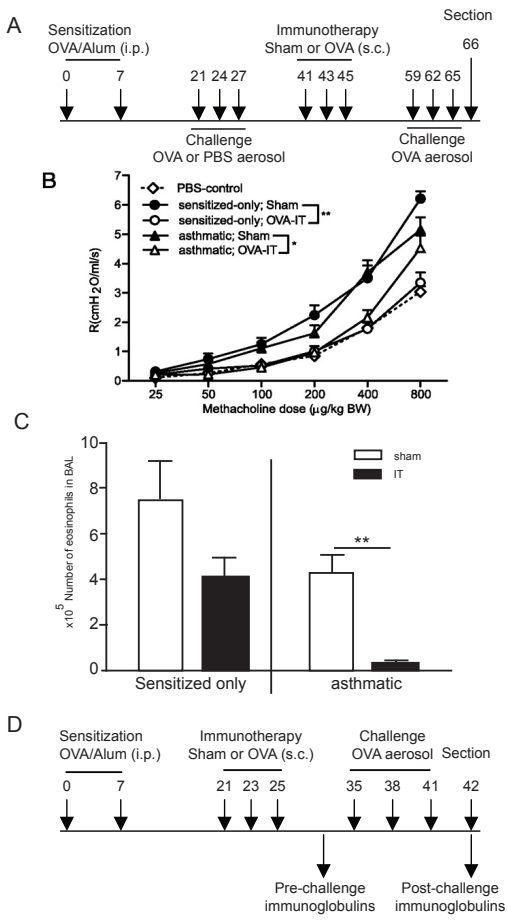

Figure 1 - Allergen-specific SIT protocol in a murine model of allergic asthma. A, Long-term protocol of OVA-SIT in the mouse model: OVA/alum i.p.-sensitized mice received a series of three PBS- (referred to as sensitized-only mice) or OVA- (referred to as asthmatic mice) inhalation challenges at week 3. Two weeks later, mice were treated with three s.c. injections of saline (sham treatment) or OVA (OVA-SIT treatment). After an additional 2 wk, all mice received a series of three OVA-inhalation challenges to induce an asthma phenotype. AHR was measured, and BAL was collected 24 h after the last challenge. B, Airway responsiveness to methacholine by sham- (solid symbols) or OVA-SIT– (open symbols) treated sensitized-only mice (● and ○) or asthmatic mice (▲ or Δ) in comparison with PBS-challenged control mice (-◊-) and eosinophil counts in BAL fluid of sham- and OVA-SIT–treated mice (C). Values are expressed as mean ± SEM of six mice per group. *$p < 0.05$; **$p < 0.01$. D, Short-term protocol of OVA-SIT in the mouse model.

Two weeks after the last OVA/PBS inhalation challenge, both groups (sensitized only and asthmatic) were sham (saline) or SIT (OVA) treated. After an additional 2 wk, all groups received a series of three OVA inhalation challenges to induce the asthma phenotype, and AHR to methacholine was assessed by direct measurement of airway resistance, followed by section as described in Materials and Methods.

As shown in Fig. 1B, OVA-inhalation challenges in sham-treated mice that had not received prior OVA-inhalation challenges (sensitized only) induced a dose-dependent increase in AHR to methacholine, which was increased compared with PBS-challenged control mice. In concordance with the data from our previous studies, OVA-inhalation challenges in OVA-SIT–treated sensitized-only mice induced a significant reduction in airway responsiveness, almost to the level of PBS-challenged control mice (Fig. 1B). These data are in agreement with our previous studies [18; 24] in which we showed that OVA-specific SIT efficiently repressed the induction of an asthmatic phenotype by OVA-inhalation challenges in OVA/alum-sensitized mice.

Importantly, OVA challenges in sham-treated mice that had received prior OVA-inhalation challenges (asthmatic mice) induced a similar level of AHR compared with sham-treated sensitized-only mice, indicating that the AHR to methacholine was similar between the first and second series of OVA-inhalation challenges. Remarkably, OVA-SIT also strongly suppressed the AHR to methacholine in the asthmatic mice (Fig. 1B), supporting the notion that OVA-SIT in our mouse model suppresses AHR, irrespective of the presence of a pre-existing asthmatic phenotype in the treatment group.

Analysis of the eosinophilic airway inflammation yielded similar results (Fig. 1C). OVA-SIT treatment in sensitized-only mice reduced the BAL numbers of eosinophils compared with sham treatment, as we reported in several previous studies [18; 19; 24–26], although the effect was not statistically significantly ($p = 0.161$) in this specific experiment. Importantly, however, OVA-SIT in asthmatic mice strongly and significantly suppressed the levels of BAL eosinophils (Fig. 1C), indicating that OVA-SIT also renders protection against the induction of eosinophilic airway inflammation in mice that received prior OVA-inhalation challenges. Taken together, these data indicate that OVA-SIT in the mouse model of OVA-induced asthma efficiently suppresses the induction of clinically relevant asthma phenotypes upon OVA-inhalation challenges, irrespective of the presence or absence of a prior asthma phenotype at the time of OVA-SIT treatment. Therefore, the remaining experiments were performed using the short protocol as our model for SIT, using sensitized-only mice as the recipient for the SIT injections (Fig. 1D).

OVA-specific SIT suppresses the late-phase allergic response in FcγRIIB-deficient mice

SIT treatment in human subjects, as well as in mouse models, induces strong allergen-specific IgG responses [1; 18], which were shown to be critically required for suppression of the immediate response in a mouse model of Fel d1 SIT [20]. We hypothesized that induction of an allergen-specific IgG response by SIT is critical for suppression of the Th2-dependent late-phase allergic response in an FcγRIIB-dependent fashion. To test our hypothesis, we assessed whether SIT treatment was able to suppress Th2 cell-driven allergic inflammation in the absence of FcγRIIB signaling. To this end, we performed our experimental mouse model of allergen SIT (schematically depicted in Fig. 1D) using FcγRIIB-deficient mice.

In OVA-sensitized and sham-treated wild-type or FcγRIIB−/− mice, OVA aerosol challenges induced a significant OVA-specific IgE response (Fig. 2A, pre- versus postchallenge). In addition, the OVA challenges induced moderate IgG1, IgG2a, and IgA responses in sensitized and sham-treated wild-type and FcγRIIB−/− mice (Fig. 2B–D). OVA-SIT treatment in sensitized wild-type mice induced a strong IgG1 and IgG2a response, which was not further upregulated by subsequent OVA challenges as we showed previously [18]. In

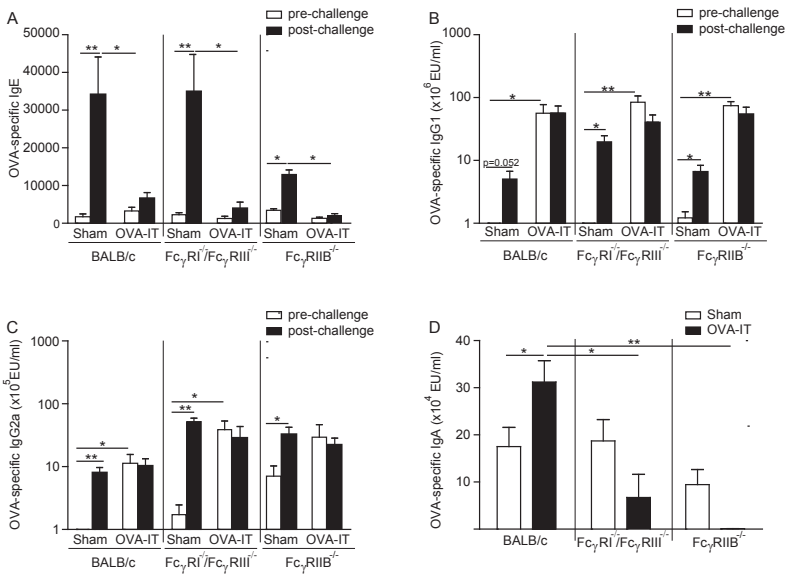

Figure 2 -OVA-specific Ig responses to SIT treatment in FcγRI/FcγRIII−/−, FcγRIIB−/−, and wild-type mice. Levels of OVA-specific IgE (A), IgG1 (B), IgG2a (C), and IgA (D) in serum were measured before (prechallenge) and after (postchallenge) OVA aerosol challenges in sensitized and SIT/sham-treated mice. Values are expressed as mean ± SEM of six mice per group. *$p < 0.05$; **$p < 0.01$.

FcγRIIB−/− mice, OVA-SIT also induced strong IgG1 and IgG2a responses (Fig. 2B, 2C), in agreement with the recent observations in the mouse model of Fel d1 SIT [20]. Finally, OVA-SIT strongly suppressed the OVA-specific IgE response in wild-type mice (Fig. 2A). Surprisingly, FcγRIIB−/− mice also displayed a strong reduction in the OVA-specific IgE response upon OVA-SIT, indicating that the SIT treatment efficiently suppressed allergen-specific IgE, independent of FcγRIIB signaling. Remarkably, the OVA-specific IgA response induced in wild-type mice by OVA-SIT was not observed in FcγRIIB mutant mice (Fig. 2D).

Because OVA-SIT efficiently suppressed the allergen-specific IgE response in mice deficient for FcγRIIB, we next analyzed Th2-driven eosinophilic airway inflammation. In line with the observed loss of the OVA-specific IgE response

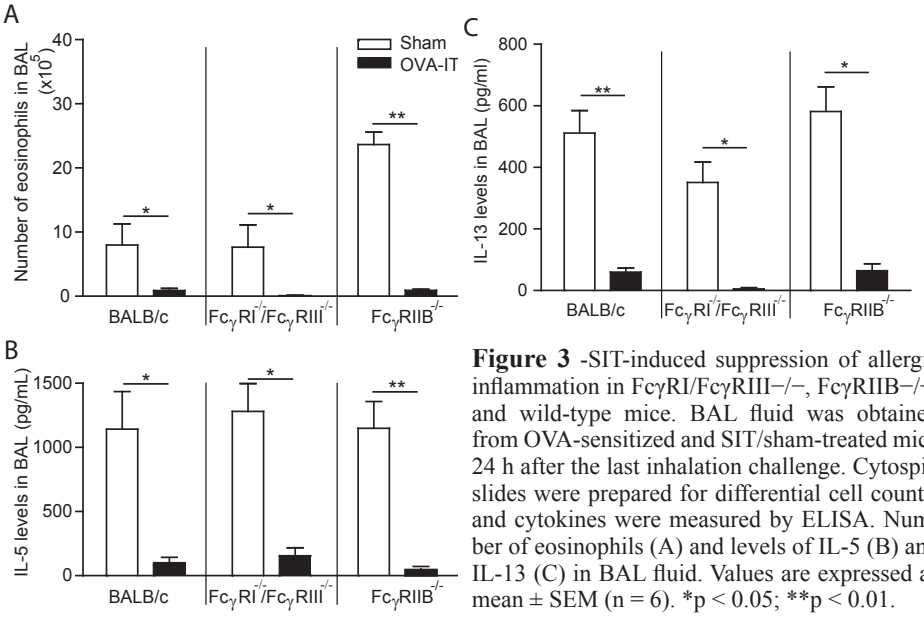

Figure 3 -SIT-induced suppression of allergic inflammation in FcγRI/FcγRIII−/−, FcγRIIB−/−, and wild-type mice. BAL fluid was obtained from OVA-sensitized and SIT/sham-treated mice 24 h after the last inhalation challenge. Cytospin slides were prepared for differential cell counts, and cytokines were measured by ELISA. Number of eosinophils (A) and levels of IL-5 (B) and IL-13 (C) in BAL fluid. Values are expressed as mean ± SEM (n = 6). *p < 0.05; **p < 0.01.

after OVA-SIT treatment, wild-type mice displayed an aborted eosinophilic airway inflammation upon OVA-SIT, accompanied by strongly decreased local Th2 cell activity, as measured by IL-5 and IL-13 levels in BAL fluid (Fig. 3). Intriguingly, FcγRIIB mutant mice showed an identical suppression of eosinophil cell counts, as well as IL-5 and IL-13 levels, in BAL fluid after OVA-SIT. Taken together, these data indicate that OVA-specific SIT is fully capable of suppressing allergen-specific IgE responses, as well as Th2-driven

eosinophilic airway inflammation, in the absence of FcγRIIB-dependent signaling.

OVA-specific SIT suppresses IgE responses and Th2-driven inflammation in FcγRI/FcγRIII−/− mice

Although the suppressive activities of FcγRIIB signaling on mast cell activation and APC function are well established [10], cross-linking of FcγR by OVA–IgG immune complexes was also shown to induce IL-10 expression in macrophages through FcγRI [14], resulting in deviation of the T cell phenotype and induction of an OVA-specific IgG response [13].

Hence, we next aimed to assess whether signaling of IgG–allergen immune complexes through FcγRI or FcγRIII contributed to the SIT-induced IgG and IgA responses or the suppression of the late-phase allergic response. To this end, we performed OVA-SIT in double-mutant FcγRI−/−/FcγRIII−/− mice. As observed in wild-type mice, the OVA-specific IgE response was strongly suppressed by OVA-SIT treatment in FcγRI/FcγRIII−/− mice and was accompanied by strongly increased OVA-specific IgG1 and IgG2a responses (Fig. 2A–C). These data indicate that neither the increased IgG responses induced by SIT nor the suppression of IgE responses are dependent on FcγRI or FcγRIII signaling. Interestingly, we found that although OVA-SIT induced significantly increased OVA-specific IgA levels in wild-type mice, decreased levels of OVA-specific IgA were detected in serum from FcγRI/FcγRIII−/− mice after OVA-SIT.

To evaluate the suppression of allergic airway inflammation by OVA-SIT in FcγRI/FcγRIII−/−mice, we also analyzed eosinophil counts and Th2 cytokine levels in BAL after sham or OVA-SIT treatment followed by OVA challenge. Similar to wild-type mice, BAL eosinophil counts in FcγRI/FcγRIII−/− mice were significantly decreased after OVA-SIT compared with sham-treated mice (Fig. 3A). In addition, OVA-SIT significantly reduced levels of IL-5 and IL-13 in BAL fluid compared with those of sham-treated FcγRI/FcγRIII−/− mice (Fig. 3B,3C). Taken together, these data indicate that FcγRI/FcγRII-mediated signaling is not required for the suppression of the late-phase allergic response by SIT. Hence, we next asked whether the allergen-specific Ig responses were required for the suppression of the Th2-driven allergic inflammation by allergen SIT.

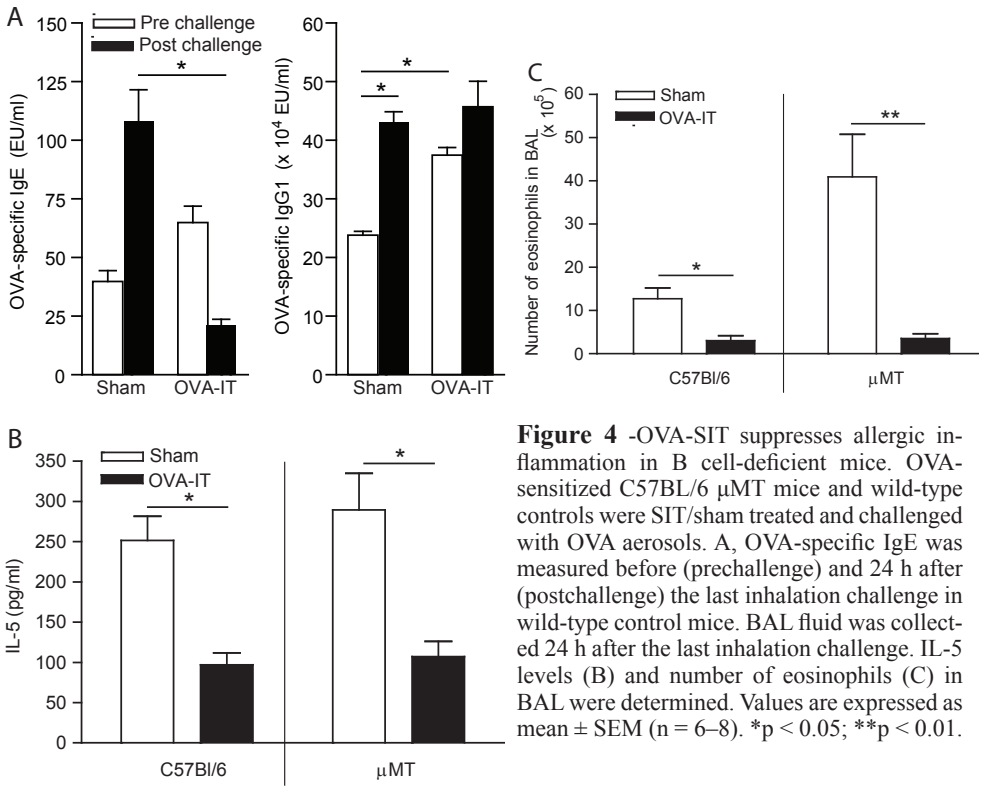

Figure 4 -OVA-SIT suppresses allergic inflammation in B cell-deficient mice. OVA-sensitized C57BL/6 µMT mice and wild-type controls were SIT/sham treated and challenged with OVA aerosols. A, OVA-specific IgE was measured before (prechallenge) and 24 h after (postchallenge) the last inhalation challenge in wild-type control mice. BAL fluid was collected 24 h after the last inhalation challenge. IL-5 levels (B) and number of eosinophils (C) in BAL were determined. Values are expressed as mean ± SEM (n = 6–8). *p < 0.05; **p < 0.01.

SIT suppresses allergic airway inflammation in B cell-deficient mice

Because we showed that SIT efficiently suppresses Th2 cell activity and eosinophilic airway inflammation in mice deficient for FcγRI and FcγRIII, as well as in mice deficient for FcγRIIB, FcγR-mediated signaling seems to be redundant for the suppression of the late-phase allergic response by SIT. Nevertheless, SIT treatment induces a strong OVA-specific IgG response in FcγRIIB−/− mice and FcγRI−/−/FcγRIII−/− mice (Fig. 2B, 2C). Therefore, we aimed to test whether the IgG serum responses contribute to SIT by a non–FcγR-dependent mechanism. Moreover, we wanted to assess the putative role of IL-10–producing B cells [27] in the SIT-dependent suppression of Th2 cell activity. Therefore, we subjected B cell-deficient µMT mice to our mouse model of SIT in allergic asthma (Fig. 1D).

As a result of the functional inactivation of the H chain of IgM in µMT mice, B cell differentiation is arrested at the pro-B cell stage, and these mice lack most mature B cells capable of producing Ig [28]. Except for some residual mucosal IgA production [29], IgM and IgG serum responses are absent in µMT

mice. Interestingly, in the OVA/alum mouse model of allergic asthma, μMT mice were shown to display Th2 cytokine production and airway eosinophilia and hyperresponsiveness, despite the absence of OVA-specific IgE responses [30]. Therefore, the μMT mouse model allows analysis of the regulation of the allergen-specific Th2 cell-driven late-phase allergic response by SIT in the absence of allergen-specific IgE and IgG and systemic IgA responses and the immediate phase of the allergic response.

To confirm that our SIT protocol was successful, we first evaluated serum levels of OVA-specific IgE in wild-type controls. As expected, OVA-SIT significantly inhibited the OVA-specific IgE response induced by OVA inhalation in sham-treated mice (Fig. 4A). Furthermore, the SIT treatment

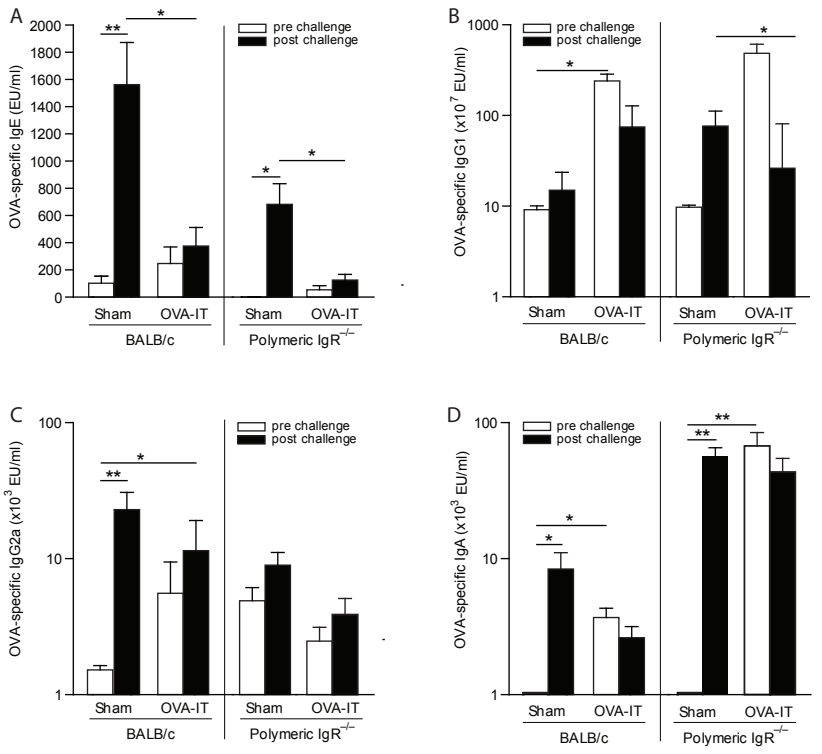

Figure 5 - OVA-specific Ig responses after SIT treatment in pIgR−/− mice. OVA-sensitized mice received SIT/sham treatments. Levels of OVA-specific IgE (A), IgG1 (B), IgG2a (C), and IgA (D) in serum were measured before (prechallenge) and 24 h after (postchallenge) the last OVA aerosol challenge. Values are expressed as mean ± SEM of six mice per group. *p < 0.05; **p < 0.01.

itself induced a strong upregulation of OVA-specific IgG1 levels, in agreement with our typical SIT-induced Ig responses.

To evaluate the suppression of Th2-driven airway inflammation after OVA-SIT in μMT mice, we measured eosinophil cell counts and Th2 cytokine levels in BAL fluid. After sham treatment, OVA challenge resulted in high IL-5 levels in BAL fluid and the infiltration of a high number of eosinophils into the airways in wild-type and μMT mice (Fig. 4B, 4C). As expected, BAL levels of IL-5 and airway eosinophilia were strongly suppressed by OVA-SIT in wild-type mice. Interestingly, the same level of suppression of BAL IL-5 and airway eosinophilia was observed in SIT-treated μMT mice (Fig. 4B, 4C), indicating that the suppression of Th2 cell activity and allergic inflammation by allergen-specific SIT is exerted independently of the allergen-specific IgG and systemic IgA responses induced by SIT.

Suppression of allergic inflammation by SIT in pIgR-deficient mice

Our data in the μMT mouse model indicate that the IgG and systemic IgA responses induced by OVA-SIT are dispensable for the suppression of Th2-driven allergic inflammation. Nevertheless, μMT mice were shown to retain a functional mucosal B cell population that is capable of producing luminal IgA [29]. Because airway delivery of allergen-specific IgA to sensitized mice was shown to suppress airway responses upon subsequent allergen challenge and to induce a systemic allergen-specific IgG2a response [31] resembling some SIT-induced phenotypes, we wanted to test whether modulation of the airway

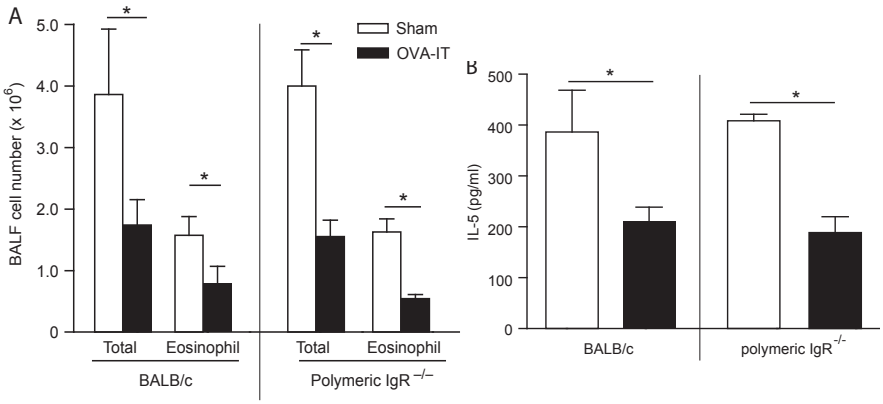

Figure 6 - SIT suppresses allergic inflammation in pIgR−/− mice. OVA-sensitized and SIT/sham-treated pIgR-deficient and wild-type mice were challenged with OVA aerosols. At 24 h after the last OVA challenge, BAL fluid was recovered and the total number of cells and eosinophils (A) and the levels of IL-5 (B) were determined. Values are expressed as mean ± SEM (n = 6). *$p < 0.05$.

luminal IgA response by SIT could contribute to the suppression of allergic inflammation. To this end, we performed our mouse model of OVA-SIT in pIgR-deficient mice, which are incapable of transporting polymeric IgA across the airway epithelium and, therefore, lack luminal IgA [32]. As shown in Fig. 5, pIgR-deficient mice displayed a strong IgG1 response upon OVA-SIT treatment (Fig. 5B). In contrast, the OVA-specific IgG2a response induced by OVA-SIT in wild-type mice was not observed in pIgR−/− mice, even though these mice have a higher basal level of OVA-specific IgG2a after sensitization (Fig. 5C). Nevertheless, OVA-specific IgE responses are suppressed by OVA-SIT in pIgR−/− and wild-type mice to a similar degree (Fig. 5A). Finally, the OVA-specific serum IgA responses were strongly increased in pIgR−/− mice, in agreement with previous observations [32; 33], but no differential effect of SIT treatment on serum IgA was seen between wild-type and pIgR−/− mice (Fig. 5D).

Surprisingly, measurement of OVA-specific IgA levels in BAL of the nontransgenic littermates indicated that, in our model, OVA inhalations in SIT-treated mice resulted in a reduced level of BAL IgA compared with OVA inhalations in sham-treated control mice, although the differences were not statistically significant (1504.5 ± 673.49 experimental unit/ml in SIT versus 5978 ± 1545.4 experimental unit/ml in sham-treated controls; $p = 0.157$).

To extend our findings to the effects of SIT on the suppression of Th2-driven airway inflammation in pIgR−/− mice, we also analyzed BAL Th2 cytokine levels and eosinophil cell counts (Fig. 6). IL-5 levels and eosinophil cell counts in BAL were equally suppressed by OVA-SIT in wild-type and pIgR−/− mice, indicating that the suppression of allergic inflammation by SIT is independent of airway luminal IgA levels.

Discussion

We present a comprehensive set of data assessing the relevance of allergen SIT-induced Ig responses for the suppression of the Th2-driven allergic inflammation that underlies the late-phase allergic response. We show evidence that cardinal features of the late-phase allergic response in the OVA-driven mouse model of allergic asthma are suppressed by SIT via a mechanism that does not depend on the induction of IgG or IgA responses, FcγRIIB signaling, or B cell function. The use of s.c. injections of high-dose OVA in the classical OVA-driven mouse model of asthma was previously established as a mouse model that phenocopies the human response to SIT treatment in considerable detail [18; 19]. Nevertheless, OVA as a model allergen lacks several aspects of natural allergens and consequently requires i.p. immunization adsorbed to

alum to achieve sensitization. Moreover, repeated airway administrations with OVA were shown to induce a gradual loss of the asthmatic phenotype in sensitized mice [34–36], although this effect is not Ag specific. We observed a limited reduction in eosinophilic airway inflammation in mice receiving two series of three OVA inhalations compared with mice that received only a single series of three OVA-inhalation challenges (Fig. 1C; compare sham-treated asthmatic mice with sham-treated sensitized-only mice, which might be attributed to the repeated OVA inhalations in this extended SIT protocol. This observation underscores the notion that the data from these mouse studies can only be extrapolated to the human situation with great care. Nevertheless, the suppressive effect of repeated airway administrations with OVA was not observed on the AHR to methacholine, and, importantly, OVA-SIT still suppressed AHR and airway eosinophilia in the asthmatic group very efficiently, indicating that despite the side effects of repeated OVA exposure, SIT is still fully operational. Importantly, the three high doses of OVA used in our model were previously shown to be as efficacious as a gradual up-dosing of OVA in the mouse model [18], and suppression of clinically relevant parameters of the asthmatic phenotype, such as AHR and airway eosinophilia, are as efficiently suppressed by the SIT treatment in mice that were only sensitized to OVA as in mice that had experienced a prior asthmatic phenotype at the time of SIT treatment, the latter reflecting the clinical practice more closely.

In human allergic individuals, allergen SIT was shown to efficiently suppress the late phase of the allergic response [37–39], associated with reduced numbers of infiltrating T cells, basophils, neutrophils, and eosinophils. Intriguingly, the effects of SIT on the immediate phase of the allergic response are modest, although a reduction in the magnitude of the immediate-phase allergic response was reported [39; 40]. The most prominent response to allergen SIT treatment in allergic individuals is the induction of allergen-specific IgG1 and IgG4 responses, yet the relevance of the SIT-induced IgG Ab response has remained controversial [1]. Although some studies reported observations that indicate a contribution of blocking Abs to SIT [41], several groups failed to show a correlation between the presence of neutralizing IgG Abs and improved clinical outcome[6; 42]. The allergen-specific IgG Abs might contribute to SIT by blocking access of the allergen to IgE on mast cells or basophils or by preventing FcεRI-dependent mast cell activation by binding to FcγRIIB (reviewed in Ref. 1). Upon cross-linking of the FcεRI–IgE complex on the mast cell surface with the FcγRIIB–IgG complex by the same multivalent ligand, FcγRIIB ITIM phosphorylation by FcεRI-associated kinases can directly suppress FcεRI signaling and mast cell activation [43; 44]. In the mouse model of Fel d1-specific SIT, both mechanisms have been postulated to contribute to the suppression

of the immediate phase of the allergic response [20]. Although suppression of systemic anaphylaxis by SIT was critically dependent on FcγRIIB signaling, suppression of tissue mast cell degranulation was maintained in FcγRIIB-deficient mice. Remarkably, the suppression of the systemic and the local immediate-phase allergic response could be passively transferred by serum or the serum IgG fraction, indicating the requirement for allergen-specific IgG [20]. The allergen-induced Th2 cell-driven airway inflammation [21; 22] and AHR [23] in the OVA/alum mouse model of experimental allergic asthma were previously shown to be independent of allergen-specific IgE responses and the immediate-phase allergic response induced by cross-linking of IgE on mast cells and basophils. This is in marked contrast to the human situation, in which allergic asthma is characterized by a clear immediate and late-phase allergic response, depending on allergen-specific IgE and Th2 cells, respectively [45]. Our mouse model of OVA-SIT exclusively allows the analysis of the mechanism by which the treatment suppresses the late-phase allergic response. We found that the allergen-specific IgG response induced by SIT is not critically required for suppression of the Th2 cell-dependent late-phase allergic response or the allergen-specific IgE responses.

The FcγRIIB-independent suppression of allergen-specific IgE responses was also observed in the Fel d1 virus-like particle vaccination study [20]. Although the investigators hypothesized a contribution of the ssRNA within the virus-like particles to suppression of the IgE response through TLR3/7 signaling, our data replicate their findings on the FcγRIIB-independent suppression of the IgE response by SIT, even in the absence of TLR signaling. Therefore, the mechanism by which allergen SIT represses the IgE response seems to be independent of direct suppression of IgE-producing B cells via FcγRIIB [46;47]. In fact, the SIT-induced suppression of the Th2-driven allergic inflammation and the allergen-specific IgE responses seem to correlate, indicating that inhibition of Th2 cell activity might be critical for the reduced IgE levels after SIT.

In serum, OVA-SIT induces a specific IgA response in our mouse model (Fig. 2D), in agreement with human studies [15; 16]. Nevertheless, the use of a reference serum containing an arbitrary level of OVA-specific IgA as a standard in these assays precludes the absolute quantification of the OVA-specific IgA levels induced by OVA-SIT or a direct comparison with the magnitude of the OVA-specific IgG response. Consequently, the biological relevance of this response remains to be determined. Moreover, the secreted form, rather than the serum levels, of IgA is critical for mucosal protective activity of this Ig isotype. For

instance, direct nasal installation of allergen-specific IgA was shown to have systemic effects, reflected in an altered allergen-specific CD4+ T cell response and increased serum IgG2a levels [31], indicating that increased luminal IgA levels can contribute to suppression of allergic manifestations. We did not directly measure OVA-specific IgA levels in BAL after OVA-SIT but prior to inhalation challenge. However, after OVA challenge, specific IgA levels were not significantly reduced in the serum (Fig. 5D) and BAL fluid of OVA-SIT–treated mice compared with sham-treated mice. These data seem to indicate that in our mouse model, the luminal IgA levels might not be critical for the suppression of the late-phase response by SIT treatment. Our data in the pIgR−/− mice lend further support to the notion that mucosal IgA responses are dispensable in SIT-induced suppression of local allergic inflammation. In addition, our data from the μMT mice strongly indicate that the systemic IgA responses induced by SIT are also dispensable for suppression of the late phase of the allergic response.

Remarkably, we found that pIgR deficiency selectively abrogates the SIT-induced OVA-specific IgG2a response, whereas other Ig responses and suppression of allergic inflammation remain unaffected, in line with the observations of Schwarze et al. [31]. These data seem to indicate the presence of a feedback mechanism by which luminal IgA levels regulate the systemic Ag-specific IgG2a response. Given the Ag specificity of this process, we would assume the involvement of a Th1 cell population, as was previously postulated [31]. Nevertheless, we find that this mechanism does not contribute to the SIT-induced suppression of the late-phase allergic response or allergen-specific IgE.

Our experiments in μMT mice also indicated that allergen-specific SIT can efficiently suppress Th2-driven eosinophilic airway inflammation in sensitized mice in the absence of B cells. This is in contrast to the critical role for B cells in tolerance induction in naive mice by mucosal application of allergen [48; 49], but is in agreement with earlier observations that T cells can efficiently be tolerized in vivo in the absence of B cells [50]. Because FcγRIIB-dependent signaling was shown to be required for the induction of mucosal tolerance in naive mice by intranasal installation of OVA [51], oral-tolerance induction in naive mice seems to be at least partially dependent on the Ig response. Because we found that suppression of Th2 cell activity can be induced independently of B cells, it remains of interest to determine which APC is required for the observed suppression of Th2 cell activity by SIT. We recently showed that SIT treatment is partially dependent on IDO [24], the rate-limiting enzyme in tryptophan metabolism that contributes to the induction of adaptive regulatory

T cells by plasmacytoid dendritic cells [52]. Because we and other investigators showed a dependency of the suppression of allergic inflammation by SIT on IL-10 [19; 53], it is tempting to speculate that IDO-competent dendritic cells induce adaptive regulatory T cells during SIT treatments that then suppress Th2 activity upon allergen exposure. Alternatively, the high-dose OVA treatment might induce Th2 cell anergy, although the IL-10 dependence of the SIT-induced suppression of allergic inflammation would not seem to support anergy induction as a major mechanism for SIT in our mouse model. In this article, we show that, irrespective of the involvement of regulatory or anergic T cell populations, the SIT-induced suppression of the late-phase allergic response is induced independently of allergen-specific IgG and IgA responses and FcγR signaling.

In summary, to our knowledge, we demonstrate for the first time that the allergen-specific IgG and IgA responses induced by allergen-specific SIT in a sensitized host are not critically required for the suppression of allergen-specific IgE responses and the Th2 cell-driven late-phase allergic response upon allergen provocation. This is in marked contrast to recent data showing that allergic desensitization by an Fel d1 vaccination strategy caused suppression of the immediate phase of the allergic response that could be passively transferred by serum or the serum IgG fraction and which was, in part, dependent on FcγRIIB signaling [20]. We postulate that allergen-specific SIT suppresses the immediate and late-phase allergic response by divergent and independent mechanisms. Other mechanisms that could contribute to the SIT-induced suppression of allergic inflammation, such as local IL-10 production, induction of regulatory T cells, or immune deviation to Th1 or Tr-1 cells, need to be addressed in future studies.

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Plasmacytoid dendritic cells are dispensable for tolerance induction during SIT in a mouse model of allergic asthma

CHAPTER 4

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Abstract

Allergen-specific immunotherapy (SIT) is the only treatment for allergic diseases that leads to long-lasting alleviation of the allergic symptoms by targeting the root cause of the disease. There is an urgent requirement for improving the efficacy of SIT which needs a detailed understanding of the mechanism of action of SIT. Antigen presentation during SIT is a less-known key step of the mechanism of action of SIT. Plasmacytoid dendritic cells (pDCs) have been shown to play tolerogenic effects by expressing indoleamine 2,3 dioxygenase (IDO), however, it remains to be determined whether pDCs play a critical tolerogenic role during SIT treatment. In this study, we examined whether pDCs contribute to antigen presentation during SIT and whether they are critically important for SIT-induced tolerance in our model.

Performing subcutaneous SIT using fluorochrome-labeled ovalbumin (OVA) revealed that most of the APCs that captured OVA are located in head and forelimb draining lymph nodes. Proliferation of CFSE-labeled OVA-specific T cells at these locations demonstrates that the captured OVA is presented to T cells. Detailed analysis of cell surface markers revealed that pDCs constitute a high proportion of APCs that capture OVA during SIT in our model. To assess whether pDCs are required for the induction of SIT-induced tolerance, we performed SIT in pDC depleted mice. Remarkably, we found that depletion of pDCs during SIT does not abrogate the therapeutic effects of SIT. We conclude that although pDCs significantly contribute to antigen presentation during SIT, these cells are dispensable for SIT-mediated tolerance induction to the model allergen OVA.

Introduction

Allergen specific immunotherapy (SIT) is the only treatment for allergic diseases that results in long-lasting relief of the symptoms by targeting the root cause of the disease [1]. Currently, SIT has been effectively used to cure allergic rhinitis and venom allergy in which the efficacy of the treatment varies between patients [1;2]. Moreover, the efficacy of SIT in allergic asthma and patients with multiple allergies is controversial [3]. Improving the efficacy of SIT in a way that it can be used in a wider range of allergic diseases and patients is desirable, however, this requires a detailed knowledge of the mechanism of SIT-induced allergen tolerance. Classically, allergen is applied to the hypodermis layer of the skin where it can be captured by antigen presenting cells (APCs) and presented to $CD4^+$ T cells in secondary lymphoid organs [4]. To the best of our knowledge it has not been yet addressed, which

type of APCs contributes to allergen presentation during SIT and what their contribution is to SIT-induced allergen tolerance.

Dendritic cells (DCs) are the most professional antigen presenting cells, playing critical roles in the induction of T cell mediated immune response and peripheral tolerance [5]. DCs can be divided into two main subsets, conventional DCs (cDC) and plasmacytoid DCs (pDC) with distinct roles in immune regulations [6]. Conventional DCs express high level of CD11c and varying levels of CD11b. These cells are involved in the induction of Th2 and Th1 responses in the lungs [7].

pDCs can induce tolerance by expression of indoleamine 2,3 dioxygenase (IDO) and inducible costimulatory ligand (ICOSL) and through the induction of $CD4^+FOXP3^+$ and $CD4^+$IL-10 producing regulatory T cells (Tregs) [8-10]. Clinical data have shown that SIT increases mRNA level of IDO in peripheral blood mono nuclear cells at very early stages of venom SIT [11]. Furthermore, we previously showed that SIT-induced suppression of the manifestation of experimental asthma is partially mediated by IDO [12]. These data suggest that pDCs may play an important role in SIT-induced allergen tolerance.

Therefore, we were interested in evaluating the role of pDCs in the generation of SIT-induced allergen tolerance using our mouse model of ovalbumin (OVA)-driven asthma and SIT [13]. To this aim we tested which draining lymph nodes are the major sites of antigen presentation after s.c. OVA-SIT treatment and if pDCs contribute to antigen presentation in these lymph nodes. Furthermore, using antibody-mediated depletion, we determined if pDCs are critically required for the generation of SIT-induced allergen tolerance. We show that pDCs contribute to antigen presentation during SIT by capturing and presenting OVA to T cells, however, they are dispensable for SIT-induced allergen tolerance.

Materials and Methods

Mice

We purchased 6-8 week specific pathogen free BALB/cByJ mice from Charles River laboratories (L'Arbresle, France) and kept them under SPF conditions in individually ventilated cages. OVA-TCR transgenic mice (DO11.10) on a BALB/c background were bred at University Medical Center Ghent (Ghent, Belgium). The guidelines of the institutional animal care and use committee of the University of Groningen were followed when performing animal experiments.

Induction of allergic asthma and SIT

The protocol of the induction of experimental allergic asthma is described in detail elsewhere [14]. In brief, mice were sensitized by intraperitoneal (i.p.) injection of 10 µg LPS-free OVA (Seikagaku Kogyo, Tokyo, Japan, LPS<5 EU/mg) and 2.25 mg alum (Pierce, IL, USA) in 100 µl of pyrogen-free saline. SIT consisted of three subcutaneous (s.c.) injections of 100 µg OVA / 200 µl sterile saline on alternate days two weeks later. Saline was injected as placebo. Fourteen days after the last s.c. injection mice were challenged by aerosolized OVA 1% in phosphate-buffered-saline (PBS) 3 times every third day. As described in detail below, airway responsiveness to increasing doses of methacholine was measured 24 h after the last challenge, thereafter mice were dissected, bronchoalveolar lavage was performed and blood and lung samples were taken.

OVA-tracking and cell transfer

To test whether pDCs contribute to antigen uptake, 100 µg/mouse Alexa Fluor® 555 ovalbumin (4›,6-diamidino-2-phenylindole, dilactate; invitrogen, Merelbeke, Belgium) was s.c. injected to previously OVA/alum sensitized mice. 24 h after the last injection mice were dissected and mandibular, accessory mandibular and superficial parotid lymph nodes were pooled and referred to as 'head draining' LNs; Forelimb draining (proper axillary and accessory axillary) and thoracic (bronchial and mediastinal lymph nodes) and spleen were collected and analyzed for traces of AF555-labeled OVA by flow cytometry. To evaluate whether pDCs present OVA to T cells, $10x10^6$ splenocytes of DO11.10 mice were isolated, stained with Carboxyfluorescein succinimidyl ester (CFSE; invitrogen, Merelbeke, Belgium) as described elsewhere [15] and were injected intravenously to OVA/alum sensitized mice. 24 h after cell transfer, 100 µg/mouse OVA was s.c. injected. 48h after OVA injection mice were dissected and the above mentioned lymph nodes were collected and analyzed for T cells proliferation by flow cytometry.

pDC depletion protocol

Two days before SIT, 120G8 (750 µg/injection) or rat IgG (750 µg/injection) was s.c. injected two times on consecutive days. This treatment resulted in 99 ± 0.1% depletion of pDCs (MHC-II^{int}CD11c^{int}mPDCA-1^{+}) in the head and forelimb draining lymph nodes in 120G8 receiving as compared to rat IgG receiving mice (Figure 7). SIT was performed one day after the second antibody injection. Antibodies were injected again (500 µg/injection) before 2nd and 3rd SIT injections.

Measurement of airway reactivity

The resistance of airways in response to increasing doses of methacholine was directly measured using invasive measurements as previously described [12]. Briefly, mice were anesthetized by i.p. injection of ketamine (100 mg/kg; Pfizer, NY, USA) and medetomidine (1 mg/kg; Pfizer) then tracheotomized (20-gauge intravenous: i.v. cannula; Becton Dickinson, Alphen a/d Rijn, The Netherlands) and attached to a computer-controlled small-animal ventilator (Flexivent; SCIREQ, Quebec, Canada). After paralyzing the mice with i.v. injection of Pancuronium bromide (Pavulon, 50 µg/kg Merck Sharp & Dohme, NJ, USA), ventilation was adjusted at a breeding frequency of 300 breaths/minutes and a tidal volume of 10 ml/kg. Tidal volume was pressure limited at 300 mm H2O. An i.v. cannula was inserted through the jugular vein for the administration of methacholine. Methacholine (acetyl-b-methylcholine chloride, Sigma-Aldrich, Dordrecht, the Netherlands) was administered intravenously. Airway resistance was calculated from the pressure response to a 2-second pseudorandom pressure wave.

Measurement of the levels of specific IgE in serum

Serum levels of OVA-specific IgE were determined by sandwich enzyme-linked immunosorbent assay (ELISA) as described previously [16]. In brief, 96-well microplates (Greiner Bio-One, Hannover, Germany) were coated with 2 µg/ml anti-mouse IgE antibody (BD bioscience, Breda, the Netherlands) over night. Then, plates were washed using wash buffer consist of 0.005% Tween20^{Ò} (Sigma-Aldrich) in PBS. After blocking the plates with 3% bovine serum albumin (Sigma-Aldrich) in PBS for 2 h, serial dilutions of reference serum as well as sample serums were added and incubated for 1.5 h at room temperature (RT). Then, plates were washed again and digoxigenin-labeled (Roche Diagnostics, Almere, the Netherlands) OVA was added and incubated for 1 h at RT. Anti-digoxigenin-POD (Roche Diagnostics) was added after washing the plate and incubated for 1 h at RT. O-Phenylenediamine (Thermo-scientific, IL, USA) was used for chromogenic reaction. Optical density of individual wells was read at 490 nm using ELx808™ microplate reader (BioTek, Friedrichshall, Germany). Concentrations were calculated according to the standard curve drawn based on serial dilutions of reference serum and results are expressed as experimental units (EU)/ml.

Performing BAL

We lavaged the mice five times through the tracheal cannula with 1-ml aliquots of saline. The cells of bronchoalveolar lavage were pooled, counted, and cells

types were indentified using flow cytometry as described elsewhere [17].

Flow cytometry

The following were used: PerCP-Cy5.5-anti-CD11b, FITC-anti-I-A/I-E and PE-Cy™7-CD11c (BD Pharmingen™, Erembodegem, Belgium), APC-mPDCA-1 (eBioscience, Vienna, Austria). For flow cytometry, single cells were washed with FACS buffer (PBS + 1% BSA + 0.1% NaN_3) then incubated with antibody mixtures for 30 minutes on ice. Thereafter, cells were washed 3 times with FACS buffer. Flow cytometry data were analyzed using FlowJo (Treestar, RO, USA). Flow cytometry was performed using LSR-II and FACSAria-II (BD bioscience, Erembodegem, Belgium).

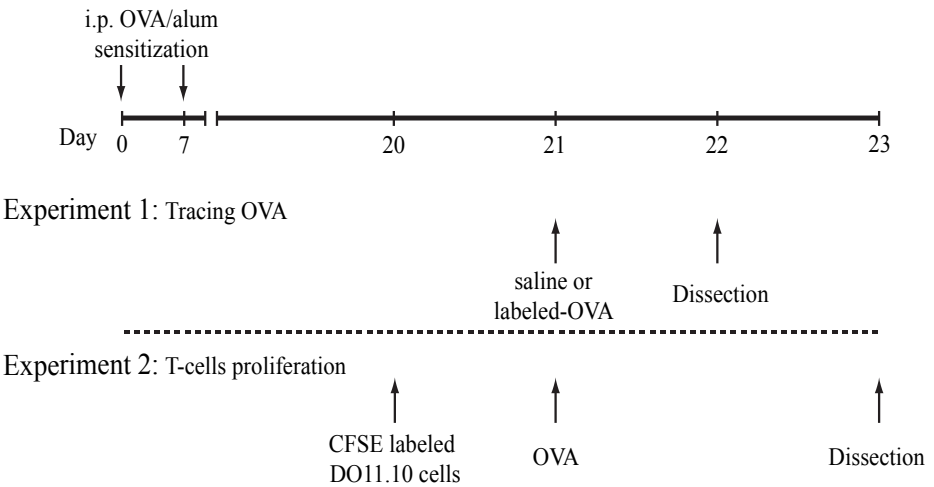


Figure 1 - Time line of experiments addressing antigen presentation during SIT. Experiment 1, mice were sensitized by OVA / alum on day 0 and 7. On day 21 fluorochrome-labeled OVA (100 µg/ 200 µl) was injected s.c. On day 21 mice were dissected and lymph nodes and spleen were checked for OVA+ cells by flow cytometry. In experiment 2, CFSE-labeled DO11.10 cells were injected i.v. to OVA/alum sensitized mice on day 20 followed by OVA-SIT 24 h later. Mice were dissected on day 23 and lymph nodes and spleen were checked for cell divisions using flow cytometry.

Statistical analysis

Data are expressed as mean ± SEM. The airway resistance curves to methacholine were statistically analyzed using a general linear model of repeated measurements. All the other data were compared using Mann–Whitney U test corrected for multiple comparisons. A P-value of less than 0.05 was considered significant.

Results

pDCs highly contribute to antigen presentation during experimental SIT

First, we aimed to test whether pDCs take up OVA during SIT. To this aim we injected fluorochrome-labeled OVA s.c. to OVA/alum sensitized mice (Figure 1). Draining lymph nodes of the head, forelimb, and thoracic cavity as well as the spleen were analyzed for cells that captured the labeled OVA using flow cytometry, 24 h after the injection. Head draining lymph nodes consist

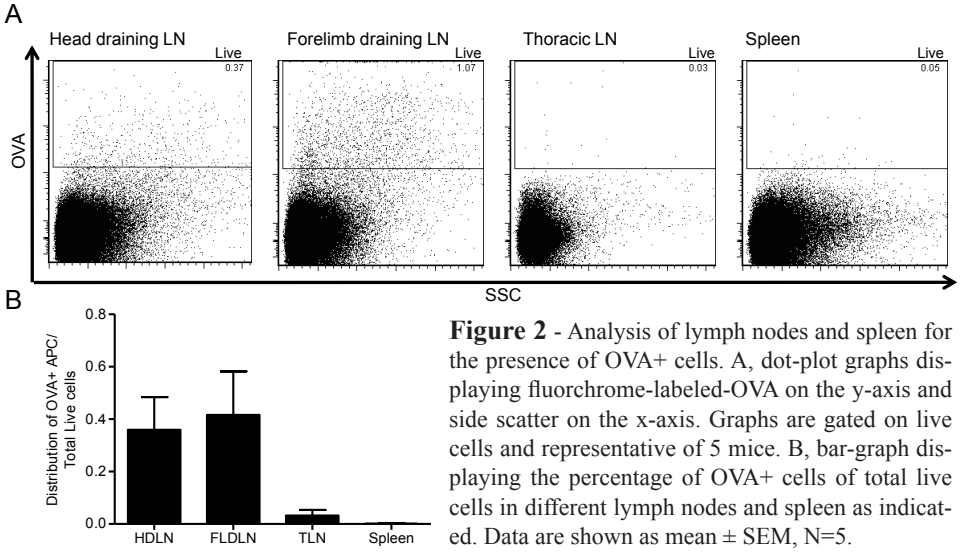
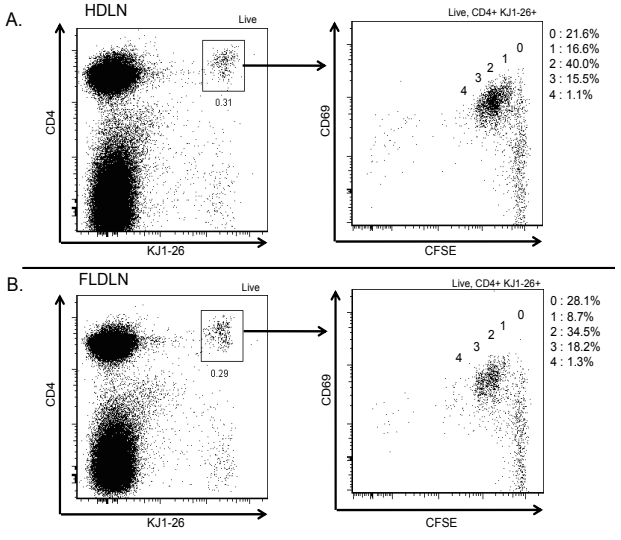


Figure 2 - Analysis of lymph nodes and spleen for the presence of OVA+ cells. A, dot-plot graphs displaying fluorchrome-labeled-OVA on the y-axis and side scatter on the x-axis. Graphs are gated on live cells and representative of 5 mice. B, bar-graph displaying the percentage of OVA+ cells of total live cells in different lymph nodes and spleen as indicated. Data are shown as mean ± SEM, N=5.

Figure 3 - Proliferation of DO11.10 CD4+ T cells in A, head and B, forelimb draining lymph nodes 48 h after OVA injection in vivo. Left panels, dot-plot graphs displaying live DO11.10 CD4+ T cells gated based on the expression of CD4 on the y-axis and DO11.10 T cell receptor on the x-axis. Right panels, dot-plot graphs displaying CD69 on the y-axis and CFSE on the x-axis. Graphs are representative of 5 mice.



of mandibular, accessory mandibular and superficial parotid lymph nodes. Forelimb draining lymph nodes include proper axillary and accessory axillary lymph nodes. Bronchial and mediastinal lymph nodes were pooled together as thoracic lymph nodes. Analysis of cells obtained from head draining lymph nodes shows that 0.36 ± 0.12% (n=5) of total live cells were positive for OVA (Figure 2A-B). In forelimb draining lymph nodes 0.42 ± 0.17% (n=5) of total live cells were positive for OVA while OVA^+ cells were absent in thoracic lymph nodes or spleen (Figure 2A-B). These data demonstrate that s.c. injected OVA has been up taken up by DCs that have migrated to head and forelimb draining lymph nodes.

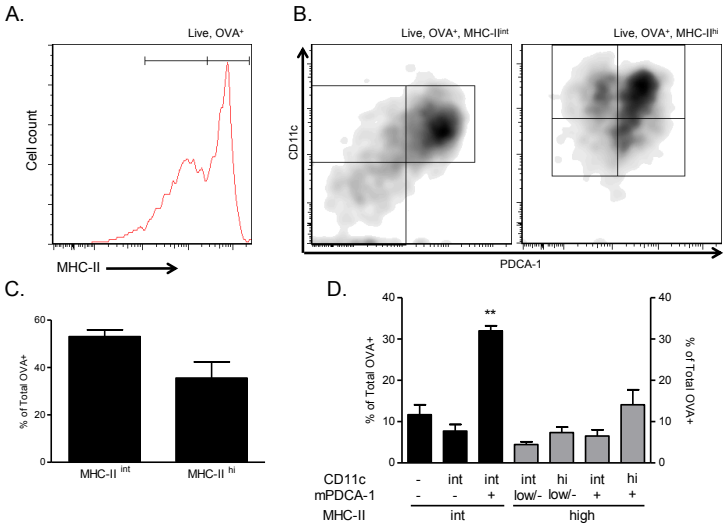


Figure 4 - Analysis of sub-types of OVA+ cells in head draining lymph nodes based on the expression of MHC-II, CD11c and mPDCA-1. A, representative histogram of 5 mice of live OVA+ cells divided into MHC-II intermediate and high cells. B, expression of CD11c and mPDCA-1 of left panel, live MHC-II intermediate, and right panel, MHC-II high cells. Representative of 5 mice C, the percentage of MHC-II intermediate and high of total live OVA+ cells. D, the percentage of different sub-types of OVA+ cells based/ total OVA+ cells on the expression of MHC-II, CD11c and mPDCA-1. Data are shown as mean ± SEM, N=5.

To determine if pDCs contribute to the APCs in head and forelimb draining lymph nodes during SIT treatment, OVA^+ cells were further analyzed for the expression of MHC-II, CD11c and PDCA-1. Live OVA^+ cells were first divided into MHC-II intermediate and MHC-II high populations followed by analyzing the levels of CD11c and mPDCA-1expression for each. As presented in figure 4A,C in head draining lymph nodes 53.0 ± 2.8% (n=5) of live OVA^+ cells express intermediate MHC-II and 35.5 ± 6.7% (n=5) express high MHC-II. In forelimb draining lymph nodes 42.9± 8.4% (n=5) are MHC-II^{int} and 50.5 ± 9.4% (n=5) are MHC-II^{hi} cells (Figure 5A,C).

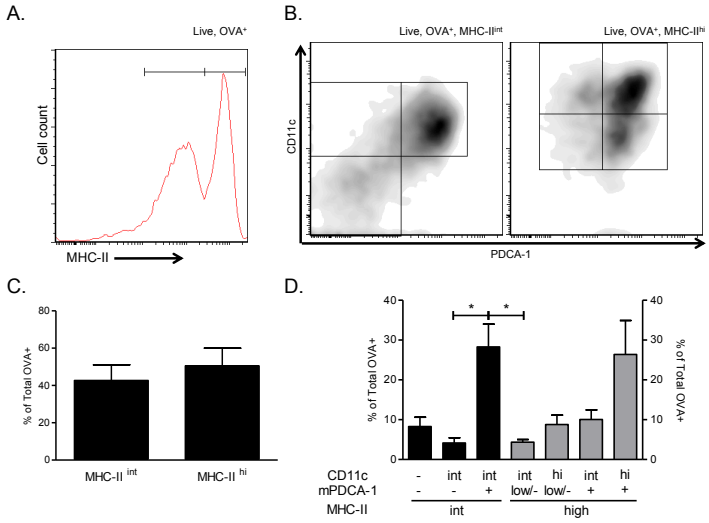


Figure 5 - Analysis of sub-types of OVA+ cells in forelimb draining lymph nodes based on the expression of MHC-II, CD11c and mPDCA-1. A, representative histogram of 5 mice of live OVA+ cells divided into MHC-II intermediate and high cells. B, expression of CD11c and mPDCA-1 of left panel, live MHC-II intermediate, and right panel, MHC-II high cells. Representative of 5 mice C, the percentage of MHC-II intermediate and high of total live OVA+ cells. D, the percentage of different sub-types of OVA+ cells based/ total OVA+ cells on the expression of MHC-II, CD11c and mPDCA-1. Data are shown as mean ± SEM, N=5.

A significant sub-population of live OVA^{+} cells in head (32.0 ± 1.2%, Figure 4B,D) and forelimb (28.3 ± 5.7%, Figure 5B,D) draining lymph nodes show the profile of pDCs by expressing intermediate level of MHC-II, CD11c and high level of mPDCA-1. Besides pDCs, the other main APC population that captures OVA is, MHC-II^{hi} CD11c^{hi} mPDCA-1^{+} cDC population, in head (14.0 ± 3.6%, Figure 4B,D) and forelimb (26.4 ± 6.5%, Figure 5B,D) draining lymph nodes. These data indicate that pDCs are major APCs during SIT treatment in our mouse model.

To determine whether the captured OVA by APCs was presented to T cells in the head and forelimb draining lymph nodes CFSE-labeled splenocytes from DO11.10 mice were intravenously injected to OVA/alum sensitized mice followed by s.c. OVA-SIT treatment 24 h later. After 48 h, the lymph nodes were taken and intensity of CFSE and expression of CD69 (a lymphocyte activation marker) were analyzed in live CD4^{+}DO11.10 T cells by flow

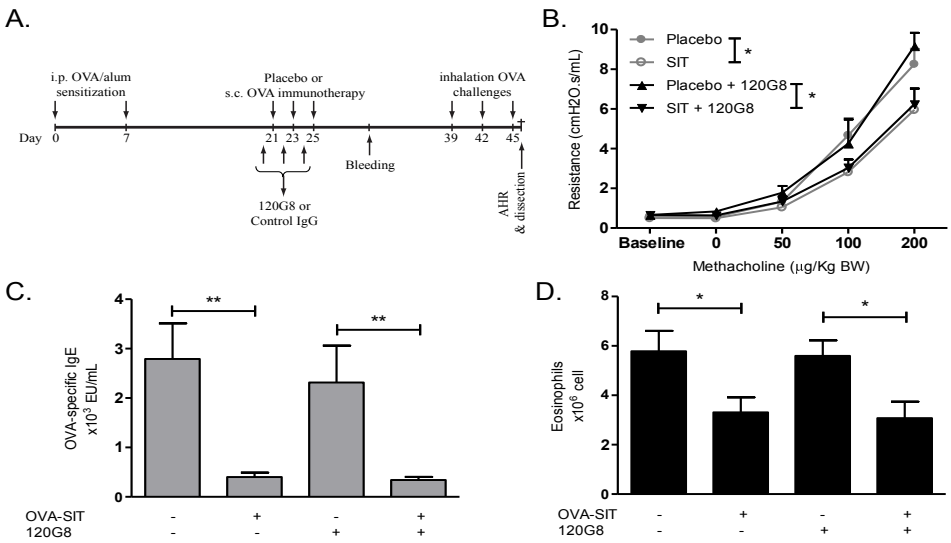


Figure 6 - The efficacy of SIT in pDC depleted versus control mice. A, timeline of experiment. Mice were sensitized using OVA absorbed to alum on day 0 and 7. pDC depleting antibody, 120G8 or rat IgG were injected on day 19 and 20 (750 µg/injection) and on day 22 and 24 (500 µg/injection). OVA-SIT was performed on day 21,23 and 25 followed by inhalation challenges on day 39, 42 and 45. Mice were dissected on day 46. B, airway resistance measured in response to injection of saline and increasing doses of methacholine. C, the levels of OVA-specific IgE in the serum 24 h after the last inhalation challenge. D, total number of eosinophils in the BAL fluid 24 h after the last inhalation challenge. Data are shown as mean ± SEM, N=5.

cytometry. As shown in figure 3A-B, OVA-specific T cells in head and forelimb draining lymph nodes, but not in other secondary lymphoid organs, showed multiple cell divisions within 48 h after s.c. OVA injection. OVA-specific T cells in placebo-treated mice show no divisions (Figure 8). T/ hese data show that the captured OVA by APCs after migration to head and forelimb draining lymph nodes is readily presented to T cells in a stimulatory fashion. Expression of CD69 is decreasing by each cell division indicating that the activation state of T cells declines progressively during cell division.

pDCs are dispensable for therapeutic effects of experimental SIT

Next, we depleted pDCs during SIT treatment to investigate whether antigen presentation by pDCs is critically required for the therapeutic effects of SIT. As demonstrated in figure 6A, mice were sensitized by OVA/alum followed by pDC depletion and SIT. Thereafter mice were challenged and dissected. SIT treatment in control IgG receiving mice significantly suppressed AHR ($P<0.05$, 27.8 ± 12.9%, Figure 6B), OVA-specific IgE in serum ($P<0.01$, 85.6 ± 3.2, Figure 6C) and airway eosinophilia

(P<0.05, 42.7 ± 10.4, Figure 6D) compared to placebo treatment. The 120G8-mediated pDC depletion resulted in 99 ±0.1% depletion of pDCs in head and forelimb draining lymph nodes (Figure 7). Performing SIT in pDC depleted mice leads to a significant suppression of AHR (P<0.05, 31.9 ± 8.5%, Figure 6B), OVA-specific IgE in serum (P<0.01, 85.2 ± 2.7, Figure 6C) and airway eosinophilia (P<0.05, 44.9 ± 11.8, Figure 6D) as compared with placebo treated 120G8 receiving mice. There was no significant difference in the level of SIT-induced suppression of AHR, airway eosinophilia and specific-IgE between pDC-depleted and pDC competent groups.

These results demonstrate that the role of pDCs in antigen presentation during SIT is dispensable for SIT-induced suppression of the manifestations of experimental allergic asthma.

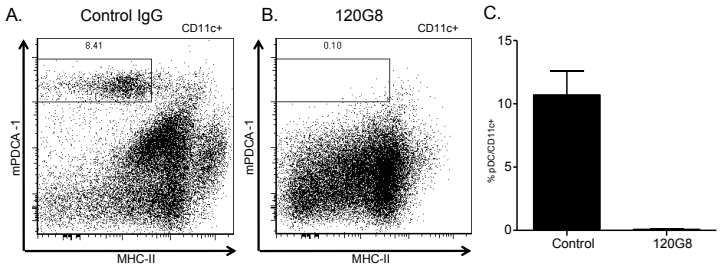


Figure 7 - The percentage of pDCs of total live CD11c cells in forelimb draining lymph nodes of mice receiving two injection of 750 µg/injection A, rat-IgG, B, pDC depleting 120G8 antibody on two consecutive days. C, Analysis of percentage of pDCs of total live CD11c cells forelimb draining lymph nodes of rat-IgG receiving mice versus 120G8 treated mice. Data are shown as mean ± SEM, N=3.

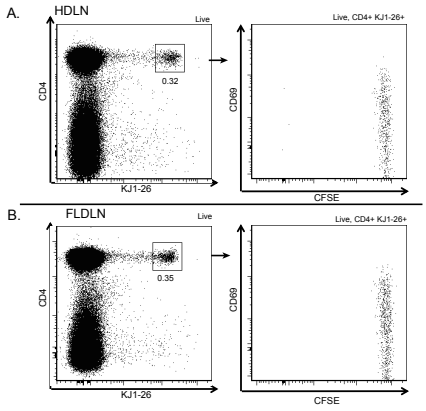


Figure 8 - Proliferation of DO11.10 CD4+ T cells in A, head draining and B, forelimb draining lymph nodes. Left panels, selection of DO11.10 CD4+ T cells out of total live cells. Right panels, dot-plot graphs displaying the expression of CD69 y-axis and CFSE x-axis. Graphs are representative of 5 mice.

Discussion

In this study we found that head and forelimb draining lymph nodes are the major sites of antigen presentation after s.c. IT as evident by the presence of OVA^+ APCs and proliferation of OVA-specific $CD4^+$ T cells in our mouse model of allergic asthma. We show that pDCs significantly but not exclusively contribute to antigen presentation during SIT in this model. However, despite their significant contribution to antigen presentation, pDCs are dispensable for the generation of SIT-induced suppression of AHR, airway eosinophilia and OVA-specific IgE in serum.

Several APCs are present in the normal skin including, langerhans cells, dermal DCs (dDCs), skin macrophages and pDCs that can be rapidly recruited from the blood upon stimulation [18;19]. Here, we show that a high proportion of OVA^+ APC in the draining lymph nodes express mPDCA-1 and intermediate levels of CD11c and MHC-II, a typical profile for pDCs [20].

pDCs play crucial roles in the induction of tolerance particularly because of their unique capacity to express large amounts of inducible IDO upon stimulation in addition to its constitutive expression [21]. These cells are found in large numbers in sentinel lymph nodes of melanoma patients [22] and it has been shown that activity of IDO is critically important for pDC-induced tolerance to tumor cells in a mouse model [23]. More importantly, in the lungs, it has been shown that pDCs play a crucial role in the induction of tolerance to harmless airborne antigen [24]. Interestingly, an early induction of IDO has been observed after venom SIT in human patients indicating an important role for IDO in human SIT [11]. We previously showed that pharmacological inhibition of IDO partially abrogated SIT-induced suppression of airway eosinophilia in our model, also pointing to a role for IDO in tolerance induction [12].

Mechanistically, pDCs contribute to tolerance induction by inducing $CD4^+CD25^+FOXP3^+$ as well as IL-10 producing Treg cells through expression of IDO [10]. Increased numbers of IL-10 producing $CD4^+$ T cells in peripheral blood have been reported after human SIT [25;26]. We have also shown that SIT-induced suppression of the manifestation of experimental allergic asthma is critically dependent of IL-10 in our model [27]. Moreover, we recently found that SIT results in a transient increase in the percentage of $CD4^+CD25^+FOXP3^+$ Treg cells in the blood and spleen (unpublished data). Taken together, these data suggest that pDCs may play an important tolerogenic role in SIT-induced allergen tolerance. However, here we show that efficient depletion of pDCs during SIT does not abrogate SIT-induced suppression of AHR, specific IgE in serum and airway eosinophilia, indicating that pDCs play a dispensable role in this model. Thus, it appears likely that other APCs play a critical

tolerogenic role in SIT-induced tolerance.

Dermal DCs are located in the dermis layer of the skin where they can efficiently take up the subcutaneously administered antigens and present it to T cells. Here we found that besides pDCs the second major population that captures OVA after SIT shows a profile similar to dermal migratory DCs by expressing high levels of CD11c and MHC-II [28]. It has been shown that dDCs have high migratory capacity *in vivo* [29] and have the capacity to generate inducible Tregs *in vitro* [30] and *in vivo* [31]. It remains to be elucidated whether dDCs play a role in tolerance induction by SIT.

Langerhans cells have also been suggested to be involved in tolerance induction by sublingual immunotherapy [32] and could potentially be involved in tolerance induction in our model. However, langerhans cells mainly reside in the epidermis layer of the skin [33] while we administer OVA-SIT to hypodermis layer of skin where langerhans cell are rare. Thus, although we cannot completely exclude a role for LCs in our SIT model, it is rather unlikely that they play a critical role.

Macrophages are also present in the skin and can play tolerogenic roles during antigen presentation [18]. We have previously shown that OVA-pulsed macrophages injected as cell-based immunotherapy induces tolerance to subsequent allergen challenges in a mouse model of asthma [34]. Furthermore, it has recently been shown that macrophage-like cells play a critical role in antigen presentation and tolerance induction during sublingual SIT in a mouse model [35]. However, the route of antigen administration and potentially the mechanism of tolerance induction in sublingual SIT are different from subcutaneous SIT. The importance of skin macrophages for SIT-induced allergen tolerance has yet to be elucidated.

We show that a high proportion of the cells that capture the s.c. injected fluorochrome-labeled OVA are located in head and forelimb draining lymph nodes where OVA-specific T cells show multiple cell-divisions after OVA-SIT. Similarly, we have previously shown that s.c. injection of OVA to naïve mice after active transfer of DO11.10 OVA-specific T cells resulted in vigorous proliferation of the transferred cells in brachial lymph nodes [36]. Here we found that the expression of CD69, an early lymphocyte activation marker is decreased by each proliferation step of T cell cells demonstrating that CD69 is rapidly induced upon stimulation followed by its gradually decline. It has been shown that induction of oral tolerance is accompanied with initial activation of $CD4^+$ T cells and rapid up-regulation of CD69 followed by subsequent down-regulation of CD69 and hypo-responsiveness of T cells underscoring the importance of T cell activation for tolerance induction [37;38].

In summary, we demonstrate that the major sites of antigen presentation during SIT in our mouse model of allergic asthma are head and forelimb draining lymph nodes. We show that pDCs highly contribute to OVA capture and antigen presentation in our model; however, these cells are not critically required for SIT-driven tolerance induction in this model. Further studies are needed to unravel the details of antigen presentation during SIT treatment and cellular mechanism of SIT-induced allergen tolerance.

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CHAPTER

5

Contribution of FOXP3+ regulatory T cells to alleviation of experimental allergic asthma after specific immunotherapy

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Abstract

Background: Allergen specific immunotherapy (SIT) has been used since 1911, yet its mechanism of action remains to be elucidated. There is evidence indicating that $CD4^+FOXP3^+$ regulatory T cells (Tregs) are induced during SIT in allergic patients. However, the contribution of these cells to SIT has not been evaluated *in vivo*.

Objective: To evaluate the *in vivo* contribution of (i) naturally occurring $FOXP3^+$ Tregs during SIT and of (ii) SIT-generated inducible $FOXP3^+$ Tregs during allergen exposure to SIT-mediated suppression of asthmatic manifestations.

Methods: We used a mouse model of SIT based on the classical OVA-driven experimental asthma. Tregs were quantified by flowcytometry 24 and 96 hours post SIT treatment. $CD4^+FOXP3^+$ Treg were depleted either during SIT treatment or prior to allergen challenges to study their contribution to the suppression of allergic manifestations by SIT treatment.

Results: Our data show that depletion of Tregs at the time of SIT treatment reverses the suppression of AHR but not of airway eosinophilia and specific IgE levels in serum. Interestingly, the number of $CD4^+CD25^+FOXP3^+$ T cells is transiently increased after SIT in the spleen and blood, suggesting the generation of inducible and presumably allergen-specific Tregs during treatment. Depletion of $CD4^+FOXP3^+$ Tregs after SIT treatment partially reverses the SIT-induced suppression of airway eosinophilia but not of AHR and serum levels of specific IgE.

Conclusion and clinical relevance: We conclude that SIT-mediated tolerance induction towards AHR requires $CD4^+CD25^+FOXP3^+$ T cells at the time of allergen injections. In addition, SIT generates $CD4^+CD25^+FOXP3^+$ T cells that contribute to the suppression of airway eosinophilia upon allergen challenges. Therefore, enhancing $FOXP3^+$ T cell number or their activity during and after SIT could be of clinical relevance to improve the therapeutic effects of SIT.

Introduction

Allergen specific immunotherapy (SIT) is a unique treatment for IgE mediated allergic diseases. SIT fundamentally alters the adaptive immune response to the allergen and results in long-lasting relief of the disease symptoms and induction of immune tolerance to the allergen [1]. The clinical application of SIT was first documented by Noon and Freeman in 1911 [2]. Although SIT is efficacious in allergic rhinitis, allergic conjunctivitis, venom allergy, and in monosensitized patients [1;3], its efficacy in patients with allergic asthma or with

multiple sensitizations is controversial and symptomatic pharmacotherapeutic strategies are often preferred in these patients [4]. Currently, SIT is accompanied by several major drawbacks including the need for long-term treatment, high risk of anaphylactic reactions and variable efficacy in the treatment outcome. Improving SIT by overcoming these disadvantages is of particular importance to encourage children to participate in SIT, since it can prevent the progression of respiratory allergies to allergic asthma as well as reducing the chance of developing multiple allergies [5]. Rational improvement of SIT is dependent on an exact and detailed understanding of the yet incompletely characterized mechanisms by which SIT induces tolerance to the allergen.

There is ample evidence suggesting that $CD4^+FOXP3^+$ Tregs may play a role in the therapeutic effects of SIT. In clinical studies it has been observed that the number of $CD4^+FOXP3^+$ Tregs is increased locally in nasal mucosa after grass pollen SIT [6] and in blood during venom SIT [7]. Using MHC-II tetramers, Aslam and colleagues have shown that there is a sustained induction of circulating FOXP3+ antigen specific T cells within 3-5 weeks of venom immunotherapy[8]. Similarly, in asthmatic children, house dust mite immunotherapy increases the number of circulating $CD4^+CD25^+FOXP3^+$ Tregs 1.5-2 years after the treatment [9]. All together this evidence suggests that inducible Tregs may contribute to the therapeutic effects of SIT.

Regulatory T cells are indispensible components of the immune system and are required for the prevention of exaggerated immune response against pathogens and immunological tolerance to self-antigens as well as environmental allergens [10]. Natural regulatory T cells (nTregs) arise as a distinct lineage in the thymus and express the transcription factor FOXP3 and high levels of interleukin-2 receptor α-chain (CD25) [11]. Tregs can also be derived from naïve T cells in the periphery by the acquisition of regulatory properties, inducing the outgrowth of so-called inducible Tregs (iTregs) under certain conditions [11]. Both naturally occurring and inducible $FOXP3^+$Tregs are capable of suppressing allergic asthma manifestations in the mouse model [12]. Interestingly, nTregs can facilitate the conversion of naïve $CD4^+$ T cells to different types of iTregs, in experimental conditions *in vitro* [13;14] and may therefore play a role in the induction of tolerance by SIT. However, the functional role for either nTregs in maintaining a tolerogenic microenvironment during SIT injections, or for iTregs in actively suppressing the allergic manifestations upon allergen challenge after SIT treatment remain unknown and are difficult to address in human studies.

We hypothesize that Tregs are critically required for the suppression of allergic manifestations by SIT. Here, we aim to test the role of $FOXP3^+$

Tregs at two time points: (i) during SIT injections where FOXP3$^+$ nTregs could facilitate the generation of allergen-specific tolerance, and at the time of allergen challenges after the completion of SIT treatment where indicible FOXP3$^+$ Tregs could contribute to suppression of the manifestations of asthma. We show that depletion of nTregs during SIT partially reverses the SIT-associated suppression of AHR but does not affect the suppression of airway eosinophilia and OVA-specific IgE in serum. We demonstrate that the number of FOXP3$^+$ Tregs is transiently increased in spleen and blood but not in the regional draining lymph nodes upon SIT. Importantly, depletion of CD4$^+$FOXP3$^+$ T cells prior to inhalation challenges partially abrogated SIT-associated suppression of airway eosinophilia.

Methods

Animals

6-8-week-old female BALB/c mice were purchased from Charles River laboratories (L'Arbresle, France) and were kept under SPF condition. All animal experiments were performed in accordance with the guidelines of the institutional animal care and use committee of the University of Groningen. *De*pletion of *reg*ulatory T cells (DEREG) mice on Balb/c and C57BL/6 background were used to specifically deplete FOXP3$^+$ Tregs *in vivo* [15]. Non-transgenic littermates of DEREG mice were used as controls.

Experimental allergic asthma, SIT

The protocol to induce experimental allergic asthma and to perform SIT is described elsewhere [16]. In brief, as shown in figure 1, mice received 2 intraperitoneal (i.p.) injections of 10 µg ovalbumin (OVA; Seikagaku Kogyo, Tokyo, Japan, endotoxin levels 5> U/mg) and 2.25 mg alum (Pierce, Rockford, IL, USA) in 100 µL of pyrogen-free saline on days 0 and 7. Two weeks after the last i.p. injection, they received three subcutaneous (s.c.) injections at the base of the neck of either 1 mg OVA in 200 µl pyrogen free saline (SIT-treated groups) or 200 µl pyrogen free saline (placebo groups) on alternate days. At least ten days after the 3rd s.c. injection mice were exposed to 3 times OVA inhalation challenges. Airway responsiveness to increasing doses of methacholine was measured 24 hours after the last challenge, thereafter mice were dissected, bronchoalveolar lavage was performed and blood and lung samples were taken.

Treg depletion

As shown in figure 1, in experiment A, nTregs were depleted before SIT using anti-CD25 (500 µg/mouse) [17] administered i.p. one day before every s.c. OVA SIT injections. Control mice received rat IgG (500 µg/mouse).

In experiment B, $CD4^+FOXP3^+$ Tregs were depleted by administration of unnicked diphtheria toxin (DT, 1 µg/mouse, Merck, Darmstadt, Germany) to transgenic DEREG mice and to their wild type littermates (as control for DT treatment) on two consecutive days, two days prior to the first OVA inhalation challenge. This approach leads to removal of more than 90% of $CD4^+FOXP3^+$ Tregs for at least 3 days [15].

Evaluation of airway responsiveness

Airway responsiveness to inhaled methacholine (Sigma-aldrich, Zwijndrecht, Netherlands) was measured twice (before and after ovalbumin inhalation challenges) in conscious, unrestrained mice using barometric whole-body plethysmography by recording respiratory pressure curves (Buxco; EMKA Technologies, Paris, France) in experiment A as described in detail previously[18].

In the experiment B airway responsiveness to increasing doses of methacholine was evaluated by directly measuring airway resistance by invasive methods as explained in detail elsewhere [19]. For details see supplementary methods in repository materials.

Determination of serum levels of ovalbumin-specific IgE

After measuring airway responsiveness, blood was drawn; sera were collected and stored at -80°C until further analysis. Serum levels of ovalbumin-specific IgE were determined by enzyme-linked immunosorbent assay (ELISA) as described previously [16] and results are expressed as EU/ml.

Analyses of the BAL fluid

Bronchoalveolar lavage (BAL) was performed as explained previously [19]. In brief, animals were lavaged five times through the tracheal cannula with 1-ml aliquots of saline. The first ml of saline contained a cocktail of protease inhibitors (complete mini tablet (Roche Diagnostics, Woerden, the Netherlands) and 1% bovine serum albumin (BSA: Sigma-aldrich, Zwijndrecht, Netherlands). BAL cells from all 5 ml were pooled, counted, and cells types were indentified using flow cytometry as described elsewhere[20]. Flow cytometry data were analyzed using FlowJo (Treestar, RO, USA) and

$CD3e^-$,$CD19^-$,$CD11c^-$,$CCR3^+$ cells were considered as eosinophils.

Preparation of lung tissue for cytokine measurement

Cardiac lobe of lung was taken, homogenized and used to measure cytokine levels as described previously [19]. Concisely, lung tissue was homogenized in 20% (w/v) luminex buffer (50 mM Tris-HCl, 150 mM NaCl, 0.002% Tween 20, and protease inhibitor, pH 7.5) on ice. Subsequently, supernatants were collected for cytokine measurement after spinning the lung tissue homogenates for 10 min at 12,000 x g and stored at -80°C until later analysis by ELISA.

Measurement of cytokines

IL-4, IL-5, and IL-13 in the lung tissue were determined by a commercially available ELISA kit according to the manufacturer's instructions (BD Pharmingen, NJ, USA). The detection limits were 32 pg/ml for IL-5 and 15 pg/ml for IL-4 and IL-13.

Isolation of LNs

To analyze the expression of $FOXP3^+$ regulatory T cells after SIT, lymph nodes were isolated after dissecting the mice. Head draining (mandibular, accessory mandibular and superficial parotid) lymph nodes and forelimb draining (proper axillary and accessory axillary) lymph nodes were collected and pooled for analysis. A sample size of 6-8 animals was used in each group. For details about preparation of single cell suspension see supplementary methods in repository materials.

Flow cytometry and antibodies

PerCp labeled anti-CD4 (clone RM4-5) was purchased from BD Bioscience (NJ, USA). PE-labeled anti-mouse FOXP3 (clone FJK-16s), eFluor450-labeled anti mouse CD25 (clone eBio3C7) were purchased from eBioscience (CA, USA). For flow cytometry see supplementary methods in repository materials.

Statistical analysis

Data are expressed as mean ± SEM. The airway resistance curves to methacholine were statistically analyzed using a general linear model of repeated measurements. All the other data were compared using student's *t*-test. A P-value of less than 0.05 was considered significant.

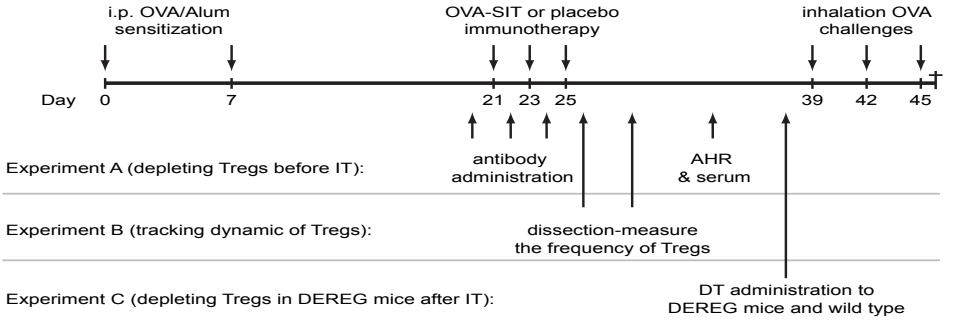

Figure 1 - Experimental setup in evaluating the role of naturally occurring and inducible Tregs in the mouse model of SIT. Mice were sensitized by OVA (10 μg) combined with alum (2.25 mg) on day 0 and 7. SIT was performed by three s.c. injections of 1 mg OVA in SIT-treated or saline in placebo-treated mice on alternate days two weeks after the last sensitization. Airway hyperreactivity, airway eosinophilia and increased OVA-specific IgE in serum, the basic manifestations of allergic asthma were induced by three inhalation challenges with aerosolized OVA every third day. One day after the last challenge lung function was measured, and then mice were dissected. In experiment A, nTregs were depleted using anti-CD25 (PC61) antibody i.p. injected one day before every SIT injection. In experiment B, a group of SIT-treated and placebo-treated mice were dissected one day after the last SIT injection and another group of SIT-treated and placebo-treated mice were dissected four days after the last SIT injection. In experiment C, all CD4+FOXP3+Tregs were selectively depleted in DEREG transgenic mice by administration of 1 μg/mouse diphtheria toxin on two consecutive days, two days prior to inhalation challenges.

Results

Depleting nTregs during SIT treatment partially reverses SIT-mediated suppression of AHR

Naturally occurring Tregs might contribute to SIT treatment by facilitating a tolerogenic microenvironment at the time of allergen injections in the sensitized host. To examine whether nTregs are required for the induction of a suppressive allergen-specific immune response during the induction phase of SIT, mice received nTreg-depleting anti-CD25 (PC61) or rat IgG control antibody, 24 hours before each SIT/placebo injection (Figure 1: Experiment A). Fourteen days after the completion of SIT treatment, mice were challenged by the relevant allergen to evaluate the suppression of allergic manifestations by SIT. In placebo-treated control antibody receiving mice, OVA inhalation challenges increased airway hyperresponsiveness (AHR) (Figure 2A). Inhalation challenges in these mice also elevated the level of OVA-specific IgE in serum (Figure 2B), and recruited eosinophils to the airways (Figure 2C). As expected, SIT-treatment in the rat IgG control-treated group significantly suppressed AHR to methacholine (63 ± 2.4% at the peak response, $P<0.005$, Figure 2A), OVA-specific IgE in serum (79 ±

5.6%, P<0.005, Figure 2B) and airway eosinophilia (83 ± 3.4%, P<0.001, Figure 2C). These results demonstrate that allergen-specific IgE and AHR were elevated and eosinophils were efficiently recruited to the airways in our mouse model of experimental allergic asthma. Importantly, all three mentioned basic manifestation of allergic asthma were efficiently suppressed by SIT treatment in this model.

To evaluate whether during SIT treatment $CD4^+FOXP3^+$ Tregs contributed to the induction of allergen tolerance, we depleted these cells using CD25-specific antibodies at the time of SIT treatment [17]. Administration of anti-CD25 antibody efficiently depleted more than 90% of $CD4^+FOXP3^+CD25^+$ Tregs in the spleen and blood of mice 24 hours after antibody injection and

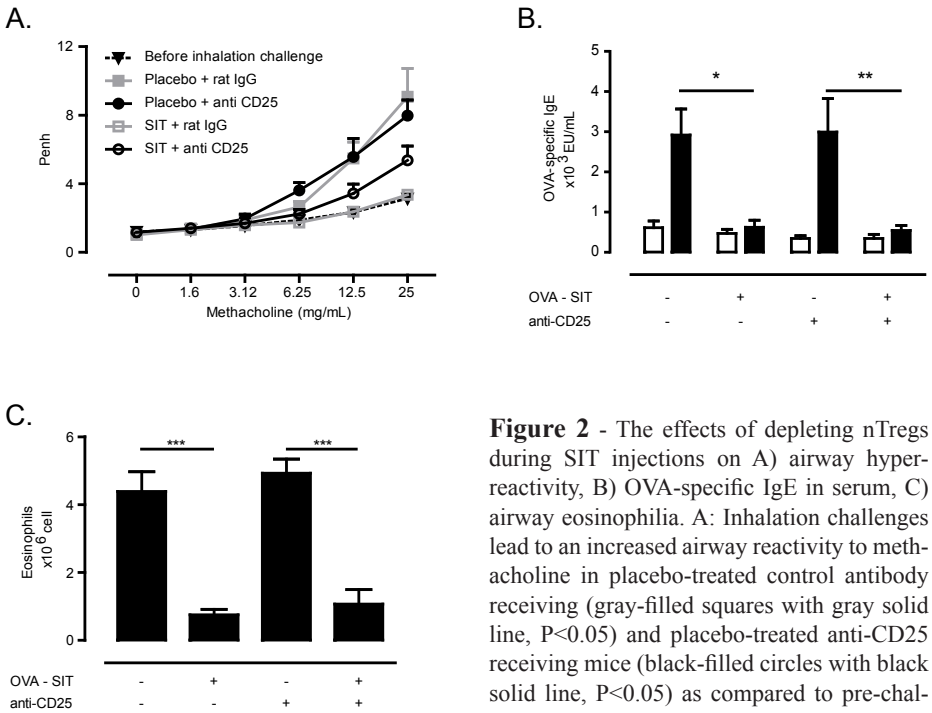

Figure 2 - The effects of depleting nTregs during SIT injections on A) airway hyper-reactivity, B) OVA-specific IgE in serum, C) airway eosinophilia. A: Inhalation challenges lead to an increased airway reactivity to methacholine in placebo-treated control antibody receiving (gray-filled squares with gray solid line, P<0.05) and placebo-treated anti-CD25 receiving mice (black-filled circles with black solid line, P<0.05) as compared to pre-challenge values (triangles with black dotted line). SIT treatment suppressed allergen-induced AHR in control antibody receiving mice (unfilled squares with gray solid line, P<0.05 compared to placebo-treated control antibody receiving mice). Depletion of nTregs partially abrogated SIT-mediated suppression on AHR (unfilled circles with black solid line, P<0.05 as compared to SIT-treated control antibody receiving mice). B: Inhalation challenges significantly (P<0.005) increased OVA-specific IgE in serum in placebo-treated mice (filled bars) as compared to pre-challenge values (open bars). SIT treatment in both control antibody receiving and anti-CD25 antibody receiving mice significantly (P<0.05) suppressed OVA-specific IgE in serum. C: Eosinophils were recruited to the airways after inhalation challenges. SIT treatment in both control antibody receiving and anti-CD25 antibody receiving mice significantly (P<0.005) suppressed airway eosinophilia

that effect lasted for at least 48 hours (Figure S1). Compared to control-antibody treated mice, anti-CD25 treatment in the placebo-treated group did not affect antigen-induced manifestation of allergic asthma (Figures 2A-2C). In the SIT-treated group, however, we observed a significantly reduced suppression of AHR by SIT treatment as compared to the suppression in control rat IgG receiving SIT-treated mice (63 ± 2.4% at the peak response). Notwithstanding, SIT still induced a significant 33% suppression of AHR in the anti-CD25-treated group (Figure 2A). Anti-CD25 treatment did not affect the suppression of airway eosinophilia (79 versus 83% in control) and OVA-specific IgE levels in serum (82 versus 79% in control) compared to control antibody receiving SIT-treated mice (Figures 2B-C). Thus, depletion of Tregs cells by CD25 antibody treatment prior to SIT injections partially reverses suppression of AHR but not airway eosinophilia and OVA-specific IgE levels in serum in this model.

$CD4^+CD25^+FOXP3^+$ regulatory T cells are transiently increased after SIT

Next, we addressed whether SIT treatment induces an increased frequency of

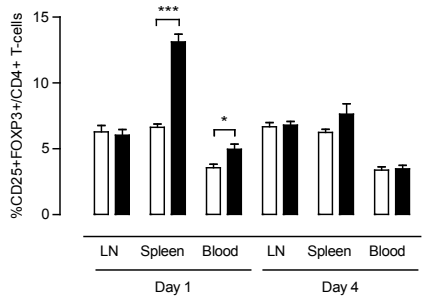

Figure 3 - Frequency of CD4+CD25+FOXP3+ Tregs in head and forelimb draining lymph nodes, spleen and blood 24 and 96 hours after the last SIT injection. The number of CD4+CD25+FOXP3+ Tregs in the spleen and blood were significantly increased in SIT-treated mice as compared to placebo-treated group ($P<0.005$ and $P<0.05$ respectively).

$CD4^+CD25^+FOXP3^+$ Tregs as has been observed in human studies [6]. To this aim, the frequency of $CD4^+CD25^+FOXP3^+$ Tregs was measured in spleen, blood and pooled head and forelimb draining lymph nodes one and four days after the last SIT injection. Interestingly, we found that SIT significantly increases the number of $CD4^+CD25^+FOXP3^+$ Tregs in the spleen by 98% and in the blood by 39% of placebo the first day after the last SIT injection (Figure 3). This difference in the number of $CD4^+CD25^+FOXP3^+$ Tregs between SIT-treated and placebo-treated control mice was no longer evident 4 days after the last SIT injection. Analyzing the number of $CD4^+CD25^+FOXP3^+$

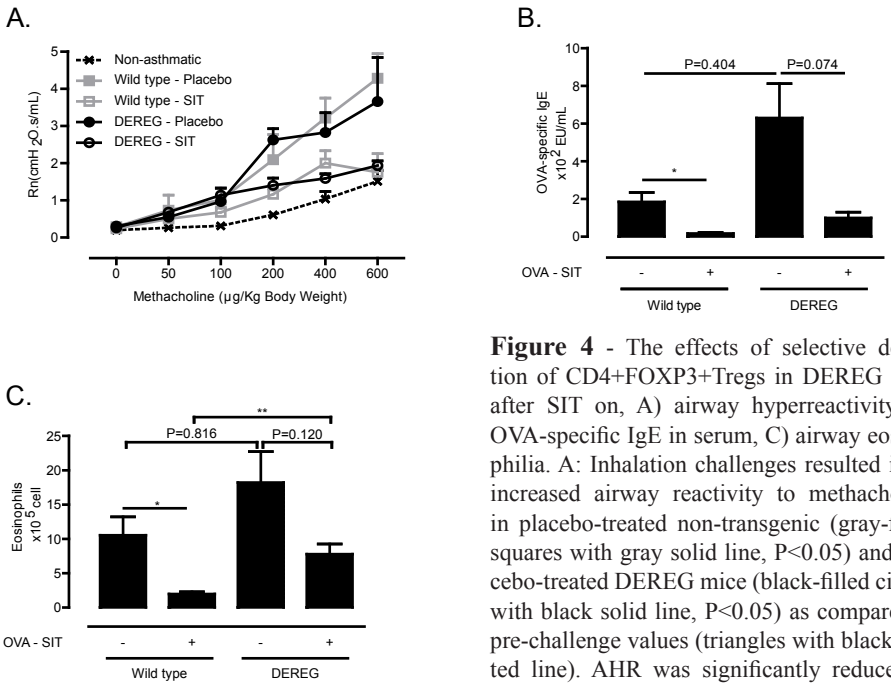

Figure 4 - The effects of selective depletion of CD4+FOXP3+Tregs in DEREG mice after SIT on, A) airway hyperreactivity, B) OVA-specific IgE in serum, C) airway eosinophilia. A: Inhalation challenges resulted in an increased airway reactivity to methacholine in placebo-treated non-transgenic (gray-filled squares with gray solid line, P<0.05) and placebo-treated DEREG mice (black-filled circles with black solid line, P<0.05) as compared to pre-challenge values (triangles with black dotted line). AHR was significantly reduced in SIT-treated non-transgenic as well as DEREG mice (unfilled squares with gray solid line and unfilled circles with black solid line, P<0.05 compared to placebo-treated control antibody receiving mice). B: Inhalation challenges significantly (P<0.005) increased OVA-specific IgE in serum in placebo-treated mice (filled bars) as compared to pre-challenge values (open bars). SIT treatment in non-transgenic mice significantly (P<0.05) reduced OVA-specific IgE in serum. SIT treatment in CD4+FOXP3+Treg depleted DEREG mice did not significantly reduce OVA-specific IgE in serum. C: Number of bronchoalveolar eosinophils was significantly reduced in SIT treated non-transgenic mice as compared to placebo treated non-transgenic mice (P<0.05). Bronchoalveolar eosinophilia in CD4+FOXP3+Treg depleted DEREG mice was not suppressed by SIT as compared to placebo-treated mice and it is significantly higher as compared to SIT-treated non-transgenic mice (P<0.01).

Tregs reveals no difference at either time point between SIT-treated and placebo in the lymph nodes draining the injection site of the allergen during treatment. These data indicate that SIT treatment induced a transient increase in peripheral FOXP3$^+$ T cells shortly after the allergen injections, indicating a potential induction of inducible allergen-specific CD4$^+$CD25$^+$ Tregs by the treatment.

Selective depletion of FOXP3$^+$ regulatory T cells partially reverses the effects of SIT

To test the hypothesis that SIT induces CD4$^+$FOXP3$^+$ inducible Tregs that contribute to the suppressive effects of SIT, DT was administered to

DEREG mice to selectively deplete $CD4^+FOXP3^+$ Tregs 24 hours prior to allergen challenges in SIT- or control- treated mice (Figure 1: experiment C). DT administration in transgenic DEREG mice resulted in more than 90% depletion of $CD4^+FOXP3^+$ regulatory T cells as compared to their wild type littermates (Figure S2). As expected, OVA inhalation challenges induced the manifestations of experimental asthma in OVA sensitized placebo-treated mice by induction of AHR, recruitment of eosinophils to the airways and increasing the level of OVA-specific serum IgE in both DEREG transgenic and their wild type littermates (Figure 4A-C).

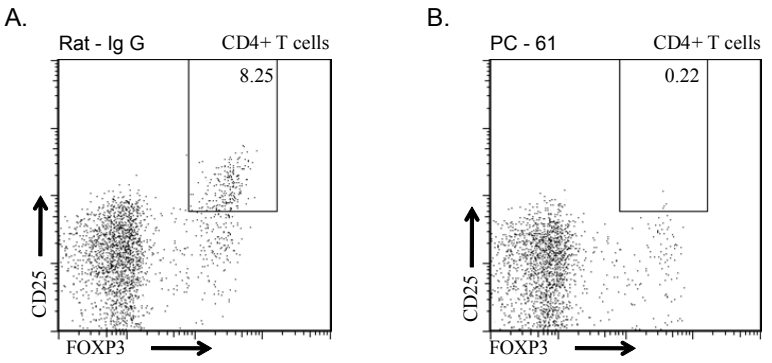

Figure S1 - Depletion efficacy of anti-CD25 antibody in wild type mice. Dot plot figures show the frequency of CD4+CD25+FOXP3+ T cells in the spleen, A) control antibody receiving mice, B) anti-CD25 receiving mice.

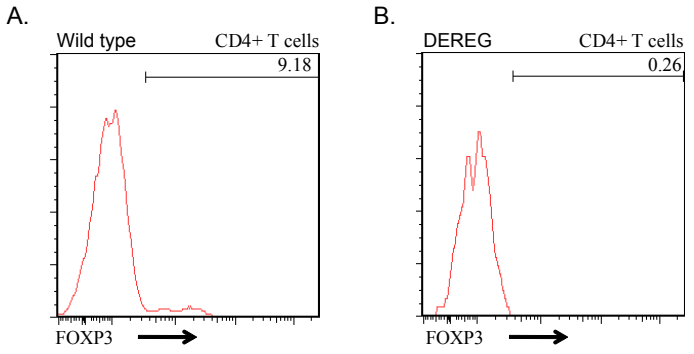

Figure S2 - Depletion efficacy by diphtheria toxin administration to DEREG mice, histogram figures show the frequency of CD4+FOXP3+ T cells in the spleen of DT treated, A) wild type mice, versus B) DEREG mice.

SIT resulted in a significant level of suppression of AHR that was similar between wild type (59%) and DEREG mice (48%) (Figure 4A, $P<0.05$). Interestingly, depletion of $CD4^+FOXP3^+$ Treg cells in DEREG mice resulted in slightly increased levels of OVA-specific IgE in serum compared to wild-type mice, while the level of suppression achieved by SIT treatment was similar between the two groups (Figure 4A and 4B). In contrast, SIT caused only a mild reduction of airway eosinophilia in DEREG mice (57.23%, $P=0.12$), whereas the suppression in wild-type mice was much more pronounced (81%, $P<0.05$) (Figure 4C). Moreover, the number of airway eosinophilia in DEREG mice was significantly higher compared to their wild-type littermates ($7.8x10^5$ versus $1.9x10^5$, $P<0.01$). Thus, specific depletion of $CD4^+FOXP3^+$ Tregs partially abrogates SIT-associated suppression of airway eosinophilia but not of AHR and specific IgE level in serum.

Discussion

This study addresses the functional relevance of nTregs for generating allergen-specific tolerance during SIT injections as well as the role of SIT-generated $CD4^+FOXP3^+$ inducible Tregs in the suppression of allergic manifestations upon allergen exposure. Using a mouse model of allergic asthma, we show that during SIT injections $CD4^+CD25^+$ Tregs are partially required for developing the SIT-induced suppression of AHR. Furthermore, we show that SIT transiently increases $CD4^+CD25^+FOXP3^+$ Tregs in spleen and blood, indicating that SIT generates inducible allergen-specific Tregs. Finally, we demonstrate that $CD4^+FOXP3^+$ Tregs partially mediate SIT-induced suppression of airway eosinophilia but not of AHR and specific IgE upon allergen provocation.

Considering that nTregs can facilitate the generation of iTregs [21;22], we evaluated the role of nTregs in tolerance induction during SIT injections. Tolerance induction in our model was evaluated based on the suppression of AHR, airway eosinophilia and specific IgE in serum [23]. Interestingly, when nTregs are depleted during SIT injections, SIT-induced suppression of AHR was partially reversed while suppression of specific IgE and airway eosinophilia remained unchanged. Confirming our previous findings [16], these data clearly demonstrate that suppression of different manifestations of allergic asthma in mice including AHR, eosinophilia and specific IgE are differentially regulated by SIT. Nevertheless, the mechanism by which nTregs specifically induce tolerance towards antigen-induced AHR remains to be elucidated. In contrast to our findings that nTreg cells are not critical for SIT-induced suppression of airway eosinophilia, Boudousquie and colleagues [24] have observed that $CD4^+CD25^+$ T cells are critical for suppression of eosinophilic airway inflammation in a model of intranasal tolerance induction. The differences might be the result of the divergent pathways of tolerance induction: Boudousqui *et al*, administered the antigen intranasally to induce tolerance, whereas SIT in our model was performed by s.c. injections. Different mechanisms are involved in tolerance induction through different routes of antigen administration [25;26]. For instance, while plasmacytoid DCs are essential for tolerance induction through inhalation [25], conventional DCs play a major role in oral tolerance [26]. Moreover, Boudousquie and colleagues injected anti-CD25 antibody at three different time points, before and after intranasal antigen application, and at the time of inhalation challenge, while we only used the anti-CD25 only during SIT injections to specifically dissect the functional importance of nTregs during SIT-induced tolerance.

Although it has been shown that SIT increases the number of $CD4^+FOXP3^+$

Tregs systemically [6] and locally [7] in SIT-treated allergic patients, a functional role for these cells *in vivo* has not been shown. We hypothesized that $CD4^+FOXP3^+$iTregs are induced by SIT and mediate SIT-induced suppression of allergic manifestations during allergen provocation in our mouse model. Here, we show that similar to human SIT studies [6;7], SIT transiently induces $CD4^+CD25^+$ $FOXP3^+$Tregs in the spleen and blood in our mouse model. Our results show that in SIT-treated mice the frequency of $CD4^+CD25^+$ $FOXP3^+$Tregs is increased in the spleen and blood 24 hours after SIT injections followed by a subsequent return to the level of placebo-treated mice 96 hours after the last SIT injection. In line with this hypothesis, Kerstan *et al*, have shown that venom immunotherapy in human subjects results in the activation and migration of blood $CD4^+CD25^+FOXP3^+$ Tregs to regional lymph nodes[27].

Using DEREG mice we show that selective depletion of $CD4^+FOXP3^+$Tregs after SIT partially abrogates the suppressive effects of SIT on airway eosinophilia, suggesting that induction of $CD4^+FOXP3^+$ iTregs is important but not the only mechanism by which SIT induces tolerance to OVA in this model. Moreover, our results show that AHR and allergen-specific IgE are not influenced by depletion of $CD4^+FOXP3^+$Tregs again confirming that the three different cardinal features of allergic asthma namely, AHR, eosinophilia and specific IgE are differentially regulated by SIT.

Besides $CD4^+FOXP3^+$ Tregs, TGF-β producing Th3 cells and IL-10 producing Tr1 cells have been also implicated in SIT [28;29]. Jutel and colleagues have shown that venom SIT induces TGF-β producing as well as IL-10 producing venom specific peripheral $CD4^+$ T cells in human patients [30]. They found that neutralization of these cytokines hampers SIT-induced suppression of proliferation of *ex vivo* venom stimulated T cells. Furthermore, Hansen and colleagues showed that CD4+ T cells engineered to express TGF-β effectively suppress AHR and allergic inflammation in a mouse model of allergic asthma [31] demonstrating that TGF-β producing CD4+ T-cells are capable to suppress asthma manifestations *in vivo*. Moreover, TGF-β instructs B-cells to produce allergen-specific IgA which competes with IgE to bind to the allergen leading to neutralizing the allergen and preventing mast cell degranulation [28]. Although OVA-IgA is increased after IT in our mouse model, we have previously shown that IgA production is dispensable for SIT-induced suppression of experimental asthma features [16]. It remains to be determined if, in addition to FOXP3 Tregs, TGF-β producing Tregs are involved in our mouse model of SIT.

Production of IL-10 has been frequently observed during and after SIT in clinical studies [29;32-37], suggesting that IL-10 plays important roles in SIT in

human patients. The induction of IL-10 producing Tregs was first shown in peripheral blood mononuclear cells which were stimulated *ex vivo* 28 days after venom SIT [30;32]. Similar results were found after grass pollen SIT in patients with systemic anaphylaxis [38] and house dust mite SIT in asthmatic children [9]. In line with clinical findings, SIT in our mouse model of allergic asthma induces a long-term IL-10 dependent tolerance that lasts for at least 20 weeks ([39] and unpublished data). The key role of IL-10 for tolerance induction in this model might be an indication for the involvement of IL-10 secreting iTregs, presumably Tr-1 cells. Kearly *et al*, have shown that transfer of OVA-specific $CD4^+CD25^+$ T cells to OVA-sensitized mice suppress OVA-induced airway inflammation and AHR in an IL-10-dependent fashion [40]. However, IL-10 secretion from the transferred Treg cells was not required for the observed effects, suggesting that $CD4^+CD25^+$ T cells instruct a different cell type to secrete the asthma suppressing IL-10. In the present study we show that $CD4^+FOXP3^+$ iTregs are only partially involved in SIT-induced tolerance induction, indicating that another, as yet unknown sub-type of $CD4^+$ $FOXP3^-$ Tregs is implicated. This unidentified regulatory T cell may be more critical for suppression of AHR and IgE than for airway eosinophilia, explaining their differential regulation by SIT.

In summary we show that nTregs are partly required for establishing tolerance during SIT injections. Furthermore, we show that SIT transiently increases the number of $CD4^+FOXP3^+$Tregs in the spleen and blood and provide evidence that $CD4^+FOXP3^+$Tregs are partially responsible for the beneficial effects of SIT. It is suggested that generation of a subtype of FOXP3 negative Tregs is a complementary mechanism underlying the induction of full-tolerance by SIT.

Acknowledgments

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CHAPTER

6

IL-10 production by dendritic cells is required for allergen-specific immunotherapy in mice

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Abstract

Background: Allergen-specific immunotherapy (SIT) is a disease-modifying treatment for allergic diseases, and is associated with desensitization of mast cells and basophils, induction of neutralizing antibody responses and suppression of Th2 cell activity. A hallmark of SIT is the expression of IL-10 by T cell subsets, and IL-10 signaling is critical for suppression of allergic inflammation in mouse models of SIT. Here, we dissect the mechanism of IL-10 induction for the suppression of allergic inflammation.

Methods: A mouse model for OVA-specific SIT based on the classical experimental asthma mouse model was used to assess IL-10 production by flowcytometric secretion assays. $CD8^+$ T cells were depleted using antibodies. OVA-pulsed dendritic cells (DCs) were adoptively transferred to sensitized and SIT-treated mice to assess the relevance of DC derived IL-10 for suppression of inflammation by SIT.

Results: SIT treatment induces IL-10 producing $CD4^+$ and $CD8^+$ T cells. $CD8^+$ T cells are not required for suppression of eosinophilic airway inflammation. Allergen challenge of SIT-treated mice does not induce IL-10 producing T cell subsets in lung tissue. In contrast, we identify a subset of DCs as the major IL-10 producing cell type in SIT-treated mice after allergen challenge. Adoptive transfer of IL-10-deficient or wild-type OVA-pulsed bone-marrow derived DCs to sensitized and SIT- or sham-treated acceptor mice demonstrates that the IL-10 production from the allergen-presenting DCs is critically required for suppression of Th2-driven eosinophilic airway inflammation.

Conclusions: We conclude that IL-10 production by allergen-presenting DCs is required for suppression of the allergic inflammation by allergen-specific IT.

Introduction

Allergic individuals respond to allergen exposure with an IgE dependent immediate type I hypersensitivity response that can be transiently suppressed by blocking mast cell effector molecules such as histamine, followed by a Th2 dependent late allergic exposure that can be suppressed by immunosuppressive drugs[1]. In contrast, allergen-specific immunotherapy (SIT) is a disease-modifying treatment for allergic disorders, inducing long-term relief of symptoms[2]. SIT treatment entails subcutaneous or sublingual applications of allergen with increasing doses. The response to SIT is characterized by early

inhibition of mast cell and basophil activity, increase of allergen-specific IgG4 titers and suppression of allergen-specific Th2 cell activity[1,3,4]. Induction of allergen-specific regulatory T cell responses is thought to be critical for the altered allergen-specific B and T cell responses, and is associated with the *ex vivo* expression of IL-10 and TGFβ in PBMC cultures[1,5,6]. IL-10 production in T cells after allergen-specific stimulation of PBMCs isolated from SIT-treated patients was found to be induced already a few hours after the first allergen injection in wasp venom immunotherapy[7] and has been observed after SIT treatment for a range of allergens, including house dust mite (HDM), birch pollen and grass pollen[8-11]. IL-10 production has also been observed *in vivo* upon allergen challenge in SIT-treated patients[10,12]. Interestingly, IL-10 secretion is not limited to $CD4^+$ T cells. For instance, IL-10 was also produced by monocytes in the original bee venom SIT study[6], and in the nasal mucosa after grass pollen SIT[10]. Finally, HDM-specific SIT was shown to induce IL-10-producing $FOXP3^+$ $CD8^+$ cells[13]. Hence, whereas the induction of IL-10 expression by SIT-treatment is well established, the cellular source of IL-10 varies between studies, and the mechanism underlying IL-10 induction has not been characterized. Therefore, it remains to be established which cell is critical for the IL-10 production required for suppression of allergic symptoms upon allergen challenge, and thereby for therapeutic efficacy in an SIT-treated individual.

In order to dissect the mechanisms underlying the therapeutic effects, we employ a mouse model for allergen-specific SIT based on the ovalbumin (OVA) induced mouse model for allergic asthma. Experimental SIT treatment renders a long-lasting protection from allergic airway inflammation and is dependent on IL-10 signaling *in vivo*[16]. Here, we aim to characterize the mechanism by which SIT treatment suppresses the allergic manifestations upon allergen challenge. We observe a strong induction of IL-10 production by $CD8^+$ T cells after SIT injections and by inflammatory DCs in the lung tissue after allergen challenge. Whilst $CD8^+$ T cells are irrelevant for suppression of allergic manifestations, we find that IL-10 expression by DCs is required for suppression of airway eosinophilia upon allergen provocation in SIT-treated mice.

Methods

Mice

Male wild-type or IL-10 deficient BALB/c mice 6-8 week old were purchased from Charles River (Maastricht, The Netherlands). Animal housing and

experiments were performed in accordance with the guidelines of and after ethical review by the Institutional Animal Care and Use Committee (IACUC/DEC) at the University of Groningen.

Experimental SIT protocol

The protocol used for OVA (endotoxin-free (<0.5 U/ml) Seikagaku; Tokyo, Japan) sensitization, SIT and inhalation challenge was previously described[15].

Ovalbumin-specific immunoglobulin in serum and cell counts and cytokine ELISAs in BAL

Serum was collected 3-5 days before the first OVA challenge and 24hrs after the last challenge. OVA-specific IgE and IgG1 levels were measured by enzyme-linked immunosorbent assays (ELISA)[15]. Lungs were lavaged with 5mL of saline, and cytokines were measured in cell-free supernatants of the first 1mL. Cellular composition of the BAL was analyzed with flowcytometry[17]. IL-5 and IL-13 in the BAL were determined by ELISA (Becton Dickinson, Franklin Lakes, NJ, USA). The detection limits were 32 pg/ml (IL-5) and 15 pg/ml (IL-13).

Isolation of lung, thoracic lymph nodes (TLNs) and spleen for flowcytometry

Single-cell suspensions were obtained from lung after enzymatic digestion and from spleen and lymph nodes by mechanical disruption[18]. IL-10 was quantified using the IL-10-secretion assay (Miltenyi Biotec, Leiden, the Netherlands), after erythrocyte (RBC) lysis and culture ($2x10^5$) in 96-well round bottom plates with 10µg/ml OVA for 18hrs using a FACSCalibur or LSRII (Becton Dickinson) for measurement and FlowJo software (Tree star inc, OR, USA) for analysis.

Depletion of $CD8^+$ T cells

$CD8^+$ T cells were depleted using two i.p. injections with YTS169 (500µg/mouse), 24hrs before the first OVA inhalation challenge and after 5 days.

Generation of bone marrow derived DC (BM-DC)

BM-DC were generated as described[19]. At day 9 of culture, BM-DCs were pulsed with 100µg/ml OVA for 24hrs, and $1x10^6$ mature DCs were injected intratracheally in a volume of 80µl PBS in anesthetized mice.

Statistical analysis

All data are expressed as mean ± SEM. Mann-Whitney U test for non-parametric data (SPSS software version 16.0) was applied to analyze the results and a $p<0.05$ was considered significant. Significance is indicated in the figures with * for $p<0.05$ and ** for $p<0.01$.

Results

SIT does not suppress airway inflammation in IL-10 knockout mice

To test whether compensatory pathways for the IL-10 mediated suppression of allergic inflammation induced by SIT exist *in vivo*, we first performed OVA-SIT in *IL-10* deficient mice. It is well-established that IL-10-deficient mice have an altered asthma phenotype[21,22]. Therefore, we evaluated the efficacy of SIT by comparing control (Sham) to OVA-SIT-treatment within IL-$10^{-/-}$ mice only (figure 1). OVA inhalation challenges induced strong OVA-specific IgE responses in both Sham- and SIT-treated IL-$10^{-/-}$ mice (figure 1B). OVA-SIT also induced strong OVA-specific IgG1 responses that were not further increased by OVA inhalation challenges in IL-$10^{-/-}$ mice. These results are very similar to the effects of OVA-SIT in wild-type mice[14-16].

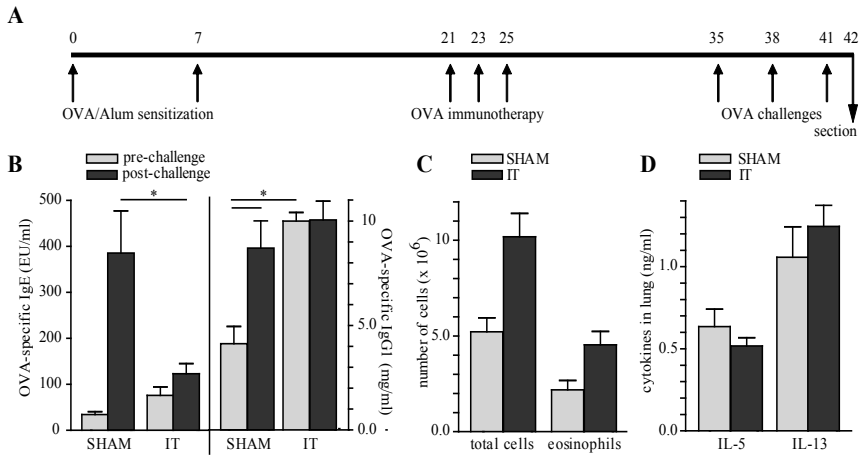

Figure 1 -(A) The experimental protocol for OVA-IT in IL-10 knockout mice. (B) OVA-specific immunoglobulin responses induced by OVA-IT or sham treatment (pre-challenge) and subsequent OVA inhalation challenges (post-challenge) in IL-10 deficient mice. (C) Total cell counts and numbers of eosinophils in bronchoalveolar lavage (BAL) of OVA-IT (black bars) or sham (grey bars) treated and OVA-challenged IL-10 deficient mice. (D) Th2 cytokine levels in lung tissue of OVA-IT (black bars) or sham (grey bars) treated and OVA-challenged IL-10 deficient mice.

In sham treated IL-10$^{-/-}$ mice, OVA inhalation challenge induced infiltration of eosinophils and other inflammatory cells into the airways, which was even more pronounced in SIT-treated IL-10$^{-/-}$ mice (figure 1C). In addition, Th2 cytokines in lung tissue were not reduced by SIT treatment (figure 1D). These results are in contrast to the SIT-induced suppression of airway eosinophilia and Th2 cytokine production observed in wild-type mice[14-16], demonstrating that IL-10 is critically required for suppression of the Th2 mediated eosinophilic airway inflammation by OVA-SIT.

IL-10 production is induced in T cells by OVA-SIT treatment.

Next, we aimed to identify the cellular source of IL-10 in SIT. IL-10 can in fact be produced by a plethora of different cell types, including regulatory B and T-cells, macrophages and dendritic cells[23]. We first analyzed the IL-10 producing cells 24 hrs after the last SIT injection (figure 2).

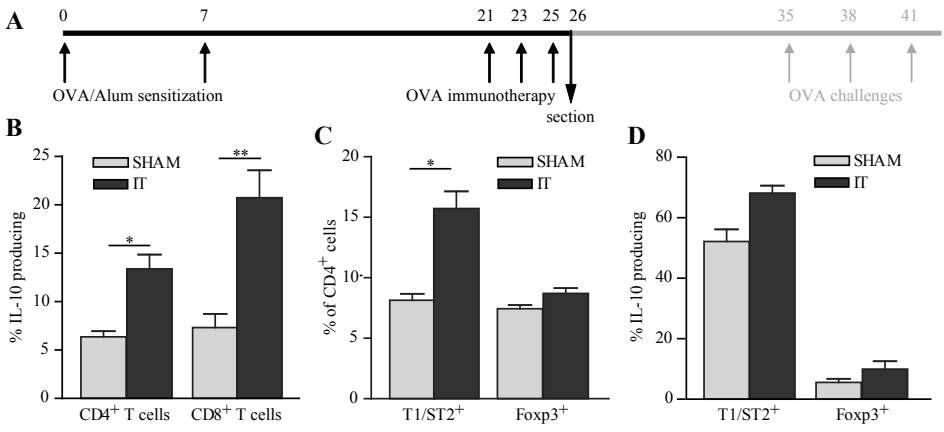

Figure 2 -(A) Schematic representation of the experimental protocol for detection of IL-10-secreting cells after IT treatment. (B) Fraction of IL-10 secreting cells within the CD4+ and CD8+ T cell population in sham (grey bars) or OVA-IT (black bars) treated mice. (C) Fraction of T1/ST2 or FoxP3-expressing cells within the CD4+ T cell population in sham (grey bars) or OVA-IT (black bars) treated mice. (D) Fraction of IL-10 secreting cells within the T1/ST2 or FoxP3-expressing CD4+ T cell subpopulation in sham (grey bars) or OVA-IT (black bars) treated mice.

In spleen, percentages of CD4$^+$, CD8$^+$ T-cells or FOXP3$^+$ CD4$^+$ regulatory T cell subsets were not changed by SIT treatment, while the T1/ST2$^+$ fraction of CD4$^+$ T-cells was increased only after OVA-SIT, indicating a rapid expansion of Th2 cells (figure 2C and data not shown). In the SIT treated group, an increased fraction of IL-10$^+$ cells was observed within CD4$^+$ and CD8$^+$ T-cells compared to Sham treated controls (figure 2B). Interestingly, the fraction of

IL-10 producing cells present in $CD8^+$ T-cells was much higher than in $CD4^+$ T-cells. We did not observe increased numbers (figure 2C) or IL-10 production of $FOXP3^+$ $CD4^+$ regulatory T-cells (figure 2D).

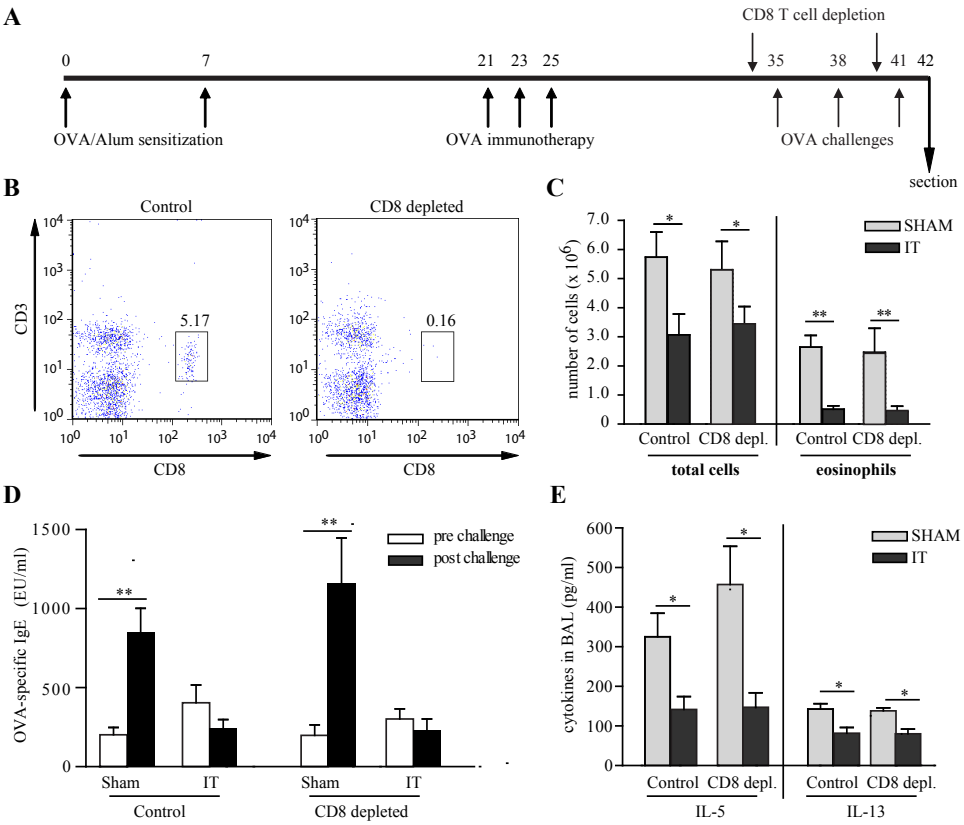

Figure 3 -(A) The experimental protocol for CD8+ T cell depletion during OVA inhalation challenges in OVA-IT or sham-treated mice. (B) Efficiency of CD8+ T cell depletion in spleen. (C) Total cell counts and numbers of eosinophils in BAL of OVA-IT (black bars) or sham (grey bars) treated and OVA-challenged mice treated with control antibody or CD8-depleting antibody as indicated. (D) OVA-specific immunoglobulin responses induced by OVA-IT or sham treatment (pre-challenge) and subsequent OVA inhalation challenges (post-challenge) in mice treated with control antibody or CD8-depleting antibody as indicated. (E) Th2 cytokine levels in BAL of OVA-IT (black bars) or sham (grey bars) treated and OVA-challenged mice treated with control antibody or CD8-depleting antibody as indicated.

CD8$^+$ T cells do not contribute to suppression of eosinophilic airway inflammation.

The presence of IL-10-producing CD8$^+$ T cells after SIT treatment might mark the induction of regulatory CD8$^+$ T cells, similar to those reported in mouse models of low-zone tolerance[24]. Therefore, we asked whether CD8$^+$ T cells contributed to SIT-induced suppression of Th2 driven eosinophilic airway inflammation, by performing antibody-mediated depletion of CD8$^+$ T cells during allergen challenges (figure 3A), resulting in a depletion of more than 95% of CD8$^+$ T cells (figure 3B). Loss of CD8$^+$ T cells, however, did not affect the suppression of eosinophilic airway inflammation (figure 3C), of OVA-specific IgE in serum (figure 3D) and of Th2 cytokines in BAL (figure 3E). These data do indicate that CD8$^+$ T cells do not make a substantial contribution to the suppression of allergic airway inflammation by SIT.

Lung CD11c+MHC-IIint cells produce IL-10 in lung tissue of SIT treated mice after OVA-inhalation challenge

In the mouse model of OVA-SIT, suppression of Th2 driven eosinophilic airway inflammation is observed after OVA challenges. The studies using IL-10R neutralizing antibodies[16,20] or using IL-10 deficient mice (figure 1) do not inform whether IL-10 is critically required during SIT treatment or during allergen challenges. We therefore also evaluated the presence of IL-10 producing cells in spleen, lymph nodes and lung tissue after OVA inhalation challenges in SIT or Sham-treated mice. We did not observe any changes in the fraction of IL-10 producing cells within the major T cell subsets in spleen, mediastinal lymph nodes or lung tissue between Sham and SIT-treated groups (figure 4B and data not shown). Remarkably, the percentage of T1/ST2$^+$ CD4$^+$ cells was strongly decreased in lung tissue after SIT, confirming

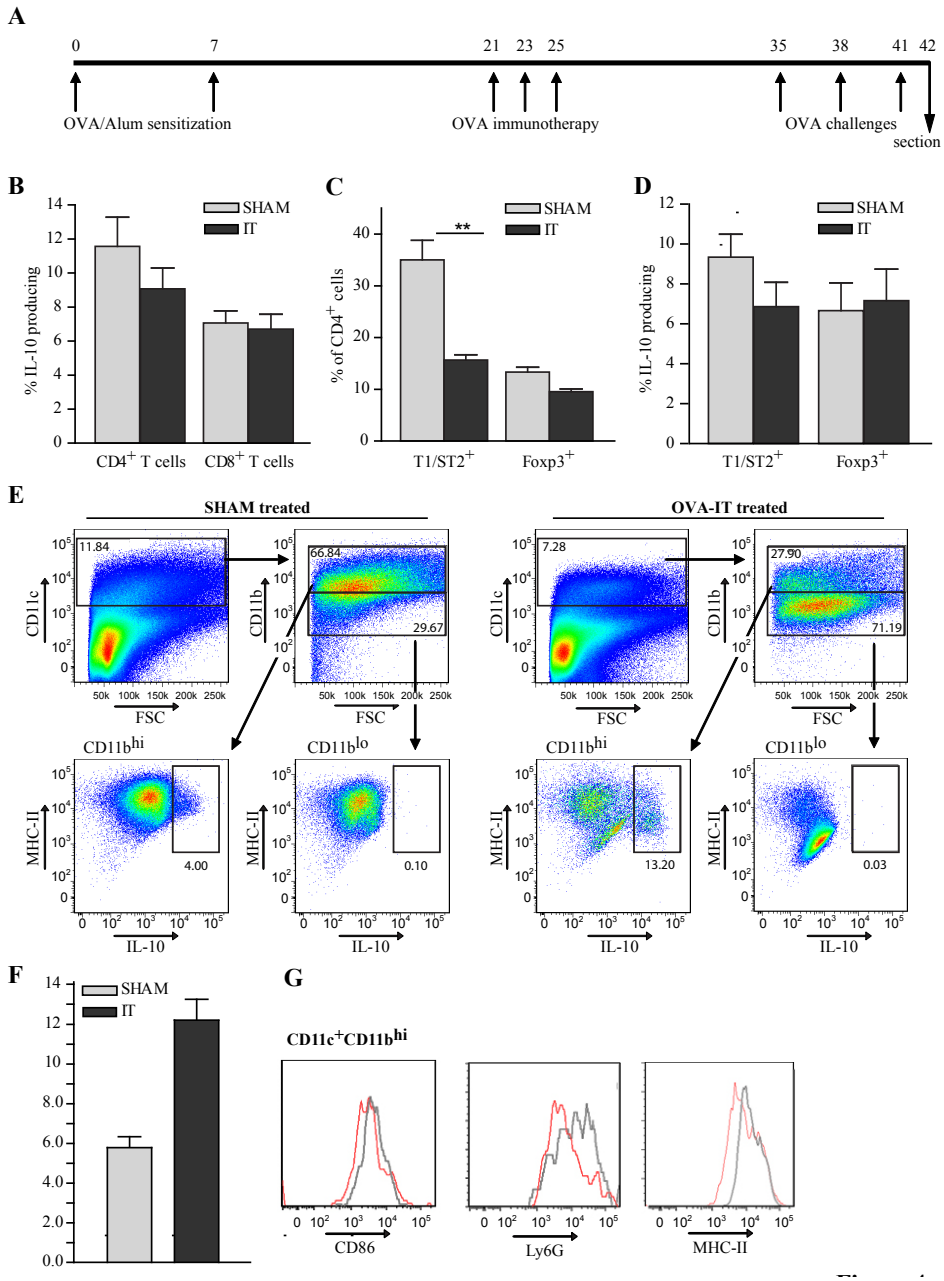

Figure 4 -(A) The experimental protocol for the identification of IL-10 secreting cells after OVA inhalation challenges in OVA-IT or sham treated mice. (B) Fraction of IL-10 secreting cells within the CD4+ and CD8+ T cell population in sham (grey bars) or OVA-IT (black bars) treated mice. (C) Fraction of T1/ST2 and FoxP3 expressing cells within the CD4+ T cell population in sham (grey bars) or OVA-IT (black bars) treated mice. (D) Fraction of IL-10 secreting cells within T1/ST2+ and FoxP3+ CD4+ T cell population in sham (grey bars) or OVA-IT (black bars) treated

mice. (E) Identification of IL-10 secreting cells with the CD11c+ dendritic cell population in lung tissue, gated for CD11blo versus CD11bhi expression, performed in either sham or OVA-IT treated mice as indicated. (E) Fraction of IL-10 secreting cells within the CD11c+CD11bhi inflammatory DC cell subset in sham (grey bars) or OVA-IT (black bars) treated mice upon OVA inhalation challenges. (F) Characterization of MHC-II, Ly6G and CD86 expression levels on CD11c+CD11bhi inflammatory DC cells from lung tissue of sham (grey lines) or OVA-IT (red lines) treated mice upon OVA inhalation challenges.

the suppression of Th2 cells. The fraction of IL-10 producing cells after OVA inhalation challenges, however, was not altered by SIT treatment in either T1/ST2$^+$ Th2 cells or FOXP3$^+$ CD4$^+$ Tregs in lung tissue or lymph nodes (figure 4B and data not shown). These data indicate that, surprisingly, T cell subsets did no longer produce increased amounts of IL-10 upon inhalation challenge in the SIT treated mice.

Therefore, we also evaluated IL-10 production in CD11c$^+$ APCs in BAL and lung tissue. Here, we observed a strongly increased fraction of IL-10 producing cells in CD11c$^+$CD11b^{hi} cells after OVA inhalation challenges in SIT-treated mice (figure 4C, 4D and data not shown). Further characterization of the IL-10-producing subset of CD11c$^+$CD11b^{hi} DCs indicated a reduced expression of CD86 and MHC-II, as well as Ly6G by this DC subset (figure 4E), reflecting a reduced maturation of the IL-10 producing DCs in lung tissue of SIT-treated mice.

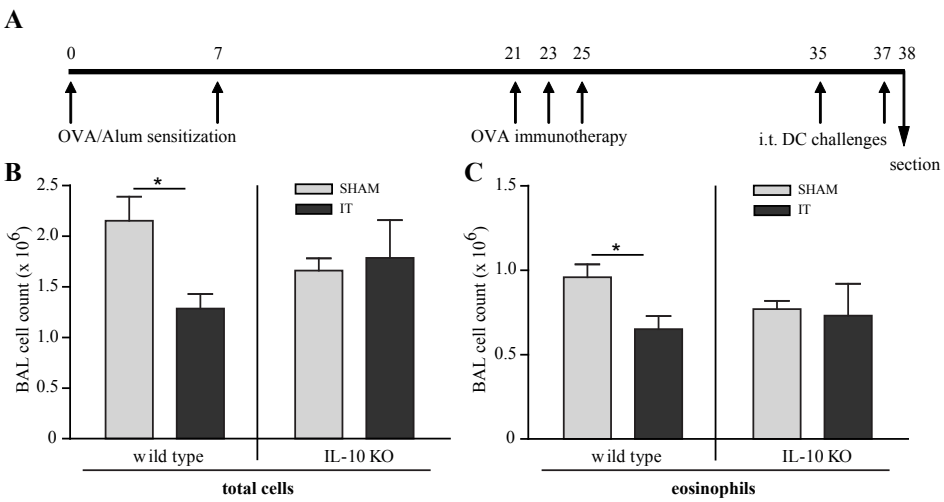

Figure 5 -(A) The experimental protocol for allergen challenge using OVA-treated bone-marrow derived DCs of OVA-IT treated mice. (B) Total cell counts in BAL of OVA-IT (black bars) or sham (grey bars) treated mice challenged with OVA-loaded IL-10-proficient (wild-type) or IL-10-deficient (IL-10 KO) bone-marrow derived dendritic cells as indicated. (C) Total numbers of eosinophils in BAL of OVA-IT (black bars) or sham (grey bars) treated mice challenged with OVA-loaded IL-10-proficient (wild-type) or IL-10-deficient (IL-10 KO) bone-marrow derived dendritic cells as indicated.

IL-10 production by DCs is required for suppression of eosinophilic inflammation.

Next, we aimed to assess whether IL-10 production by the allergen-presenting DCs was required for suppression of eosinophilic airway inflammation in SIT-treated mice. To this end, we employed a previously established model of allergen challenge using adoptive transfer of bone-marrow derived DCs[19]. Groups of OVA-sensitized and either sham- or SIT-treated mice were challenged by intratracheal transfer with OVA-pulsed DCs derived from wild-type or IL-10$^{-/-}$ bone marrow (figure 5A). Mice that had not received OVA-

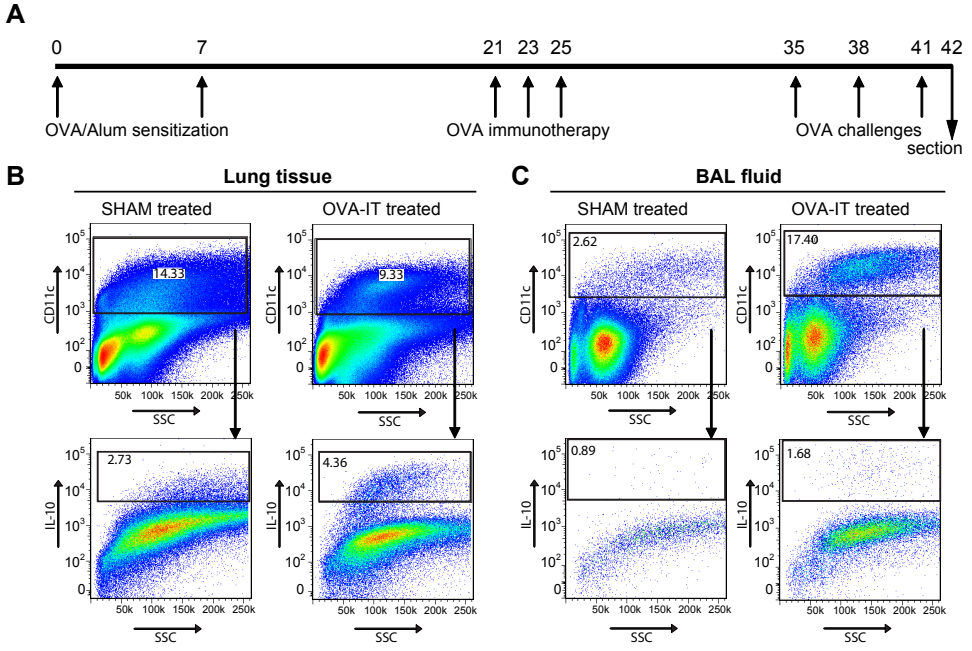

Figure S1 -(A) The experimental protocol for the identification of IL-10 secreting cells after OVA inhalation challenges in OVA-IT or sham treated mice. (B) Identification of IL-10 secreting cells with the CD11c+ dendritic cell population in lung tissue or BAL, performed in either sham or OVA-IT treated mice as indicated.

SIT treatment displayed marked infiltration of eosinophils into the airways 48 hours after instillation of OVA-pulsed wild-type DCs. This infiltration of inflammatory cells (figure 5B; left panel) and the numbers of eosinophils (figure 5C; left panel) were significantly reduced in SIT-treated mice challenged with OVA-pulsed wild-type DCs. These data demonstrate that the transfer of OVA-pulsed DCs induces an eosinophilic airway inflammation that remained sensitive to SIT treatment of the recipient mouse. Remarkably, upon challenge of the OVA-SIT treated mice with OVA pulsed IL-10-deficient DCs, neither the total cell count of the BAL nor the number of eosinophils were decreased compared to sham-treated mice, which was in marked contrast to the situation after challenge using IL-10-proficient DCs. Taken together, these data show that the production of IL-10 by the antigen presenting DCs is required for suppression of eosinophilic airway inflammation by OVA-SIT.

Discussion

Here, we show for the first time the critical role for DC-derived IL-10 in suppression of eosinophilic inflammation upon allergen challenge in SIT-treated mice. We find that IL-10 is indispensable for SIT-induced suppression of allergic inflammation (Figure 1). Although $CD4^+$ and $CD8^+$ T cells produce significant amounts of IL-10 shortly after the SIT treatments (Figure 2), most of the $CD4^+$ T cells are $T1/ST2^+$ Th2 cells[25] while the $CD8^+$ T cells are dispensable for suppression of allergic inflammation by SIT (Figure 3).

Upon allergen challenge of SIT-treated mice, we no longer observe an increased IL-10 production by $CD4^+$ or $CD8^+$ T cells or $CD4^+$ $FOXP3^+$ Tregs (Figure 4). This transient increase in IL-10 expression in T cells after SIT injections mirrors observations from patient studies, where IL-10 production in $CD3^+$ T cells can be detected already on the day of the first SIT injections[7], but tends to decrease again during maintenance phase of SIT[8,9], indicating that IL-10 production by T cells is in fact an early event.

The major IL-10 producing cell in lung tissue upon allergen challenge was the $CD11c^+$ $CD11b^+$ inflammatory DC[26]. Intratracheal delivery of BM-derived DCs from wild-type donors into OVA sensitized mice that had received either OVA-SIT or Sham treatment induced eosinophilic airway inflammation that was suppressed by prior OVA-SIT treatment of the recipient mice. In contrast, adoptive transfer of IL-10 deficient DCs failed to induce suppression of eosinophilic airway inflammation. These data also strongly indicate that OVA-SIT treatment induces the presence of an as of yet unidentified allergen-specific regulatory T cell population that induces IL-10 production by the adoptively transferred DCs, culminating in suppression of Th2 cells

and eosinophilic inflammation. Since we ruled out a role for $CD8^+$ T cells, we hypothesize the presence of a $CD4^+$ Treg cell subset to be critical for suppression of allergic inflammation in our mouse model of SIT.

Antigen-presenting cells have previously been shown to contribute to the suppression of allergen-specific pulmonary responses through the production of IL-10. For instance, induction of inhalation tolerance in naïve mice was associated with a transient IL-10 production by pulmonary DCs[27]. Moreover, it has been shown that IL-10 treated OVA-pulsed DCs are capable of suppressing airway inflammation in OVA-sensitized mice in an IL-10 dependent fashion[28]. In human studies, the majority of the IL-10 producing cells in biopsies from the nasal mucosa of grass pollen SIT-treated patients were APCs of myeloid origin[10]. Taken together, these data indicate that the APCs might very well mediate suppression of local allergic inflammation after SIT treatment by producing IL-10. The identity of the putative allergen-specific regulatory T cell subset licensing the APCs for IL-10 production, however, remains to be established.

In our experiments, we did not detect an increased number of IL-10 producing $FOXP3^+$ $CD4^+$ (or $CD8^+$) T cells. This might be due to the low frequency of allergen-specific Tregs, precluding efficient identification of these cells. Alternatively, the putative Tregs that induce IL-10 production by the OVA-presenting DCs might not express IL-10 themselves. For instance, in mice with a T cell-specific IL-10 deficiency, OVA-SIT reportedly retains the ability to suppress eosinophilic airway inflammation[29,30]. Moreover, it has been shown that adoptive transfer of OVA-specific $CD4^+CD25^+$ regulatory T cells to OVA-sensitized mice reduced airway inflammation after allergen challenge in an IL-10-dependent manner, even when IL-10 deficient Tregs were used for the transfer[31]. We hypothesize a similar allergen-specific Treg-DC interaction in SIT, leading to IL10 production by the allergen-presenting DCs.

In summary, our data for the first time show that IL-10 production by the antigen-presenting DCs is critically required for SIT-induced suppression of allergic inflammation upon allergen provocation in sensitized and SIT treated mice. We propose that allergen-SIT induces a regulatory T cell subset that is able to instruct the allergen-presenting DC to induce IL-10 production through cognate interactions. The identification of the critical cellular mechanisms driving the differentiation of this putative allergen-specific regulatory T cell subset might allow for the rational design of improved allergen preparations for SIT treatment with enhanced efficacy through the use of specific adjuvants.

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CTLA4-Ig is a potent adjuvant for experimental allergen immunotherapy

CHAPTER 7

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Abstract

Background: Allergen specific immunotherapy (SIT) is the only treatment for allergic diseases that targets allergen-specific Th2 cells which are the cause of the disease. There is an unmet requirement for adjuvants that increase the clinical efficacy of SIT allowing application of lower doses of the allergen and thereby reducing the risk of anaphylactic reactions. Cytotoxic T-Lymphocyte Antigen 4-Ig (CTLA4-Ig) has been shown to induce immunological tolerance in autoimmunity and allograft transplantation by blocking T cell co-stimulation and induction of the immunoregulatory enzyme indoleamine 2,3 dioxygenase (IDO). Previously, we showed that CTLA4-Ig treatment at the time of allergen inhalation induced tolerance to subsequent allergen exposure in a mouse model of asthma. In this study, we test whether CTLA4-Ig acts as an adjuvant for experimental SIT.

Objective: To determine if CTLA4-Ig can act as an adjuvant for SIT.

Methods: We performed a mouse model of SIT in the ovalbumin (OVA)-driven experimental allergic asthma model. Mice were sensitized by OVA/alum and two weeks later OVA-SIT was performed by three s.c. injections with CTLA4-Ig as adjuvant or control IgG. Two weeks after OVA-SIT, mice were challenged three times by OVA inhalation. We used wild-type mice as well as IDO-deficient mice to assess the role of IDO in the adjuvant effect of CTLA4-Ig.

Results: Co-administration of CTLA4-Ig strongly increased SIT-induced suppression of AHR, specific IgE in serum, airway eosinophilia and Th2 cytokine levels. Moreover, we find that CTLA4-Ig, as an adjuvant for SIT, is equally effective in IDO-deficient and wild type mice, demonstrating that the effect of CTLA4-Ig is independent of IDO expression.

Conclusions & Clinical Relevance: We show that CTLA4-Ig acts as a potent adjuvant to augment the therapeutic effects of SIT. Since the adjuvant activity of CTLA4-Ig is independent of IDO, we conclude that it acts by blocking CD28 mediated T cell co-stimulation.

Introduction

Atopic Th2 immune responses against innocuous environmental antigens are the cause of allergic diseases that impair the quality of life of a significant portion of the world's population [1;2]. Currently, allergen specific immunotherapy (SIT) is the only remedy for allergic diseases that modifies the dominant Th2 response and causes long-lasting relief of symptoms [3]. Classically, SIT is performed by repeated administration of high doses of the sensitizing allergen

for a period of 3-5 years, after an initial gradual increase of administered allergen to avoid anaphylaxis [3]. SIT not only induces a sustained relief of allergic symptoms but it can also prevent the development of new allergen sensitizations [4;5] and the progression of allergic rhinitis to allergic asthma [6]. Currently, there are concerns about the safety of using high doses of allergen and the required long-term duration of treatment [7;8]. Therefore, improvement of SIT by using clinically applicable adjuvants that achieve optimal efficacy at lower doses of allergen and leads to a safer therapy in possibly a shorter time-frame, is highly required [9].

Cytotoxic T Lymphocyte Antigen 4 (CTLA4) is an inhibitory co-stimulatory molecule expressed by naturally occurring regulatory and activated T cells[10;11]. T cell receptor signaling upon antigen presentation results in T cell activation or inhibition when accompanied by CD28 or CTLA4 co-stimulation, respectively [11;12].

CTLA4-Ig is a fusion molecule of the extracellular domain of CTLA4 and the heavy chain of human or mouse IgG [13;14]. This molecule has been shown to exhibit tolerogenic properties towards self and allograft antigens in human patients and in animal models [15-17]. CTLA4-Ig is an FDA-approved compound that has been used in the treatment of rheumatoid arthritis and prevention of allograft rejection [18;19]. Interestingly, we have previously shown that CTLA4-Ig treatment at the time of allergen inhalation in sensitized mice induced long-term tolerance to subsequent allergen-induced airway eosinophilia, but not AHR in a mouse model of experimental asthma [20].

CTLA4-Ig shows tolerogenic properties through two mechanisms: (i) sequestration of B7 and thereby inhibition of CD28 signaling [11;21] and (ii) reverse signaling into dendritic cells through B7 and subsequent activation of the alternative NFkB pathway leading to expression of the immunoregulatory enzyme indoleamine 2,3 dioxygenase (IDO) [22]. Interestingly, we have previously shown that IDO contributes to SIT-induced tolerance induction in our model [23]. Recently, an early induction of IDO has been observed after venom SIT suggesting a role for IDO in SIT-induced allergen tolerance in human patients [24].

In this study, we tested whether CTLA4-Ig can act as an adjuvant for experimental SIT. To this aim we administered CTLA4-Ig with SIT in an ovalbumin (OVA)-driven mouse model of asthma. We show that co-administration of CTLA4-Ig with SIT highly enhances the SIT-induced suppression of airway hyperreactivity (AHR), airway eosinophilia and OVA-specific IgE levels in serum. Furthermore, we show that the effect of CTLA4-Ig is independent of IDO indicating that CTLA4-Ig in our model acts by

blocking the CD28-mediated T cell co-stimulatory signal.

Methods

Animals

Specific pathogen free 6-8 week BALB/cByJ mice (Charles River laboratories, L'Arbresle, France) and IDO-knockout (IDO-KO; C.129X1(B6)-Ido1^{tm1Alm}) on BALB/c background (kindly provided by Dr. A.L. Mellor, GA, USA), were used according to the guidelines of the institutional animal care and use committee of the University of Groningen.

Experimental allergic asthma, SIT

Experimental allergic asthma was induced and SIT was performed according to the previously described protocol [25]. Concisely, as shown in figure 1, mice were sensitized by intraperitoneal (i.p.) injection of 10 μg endotoxin-free/low (<5 EU/mg) OVA (Seikagaku Kogyo, Tokyo, Japan) and 2.25 mg alum (Pierce, IL, USA) in 100 μL of pyrogen-free saline. Two weeks later, they either received 100 μg OVA in 200 μl saline per injection as OVA-SIT or 200 μl saline as placebo through three subcutaneous (s.c.) injections on alternate days. At least ten days after the 3rd s.c. injection mice were challenged by aerosolized OVA 1% in phosphate-buffered-saline 3 times every third day. Airway responsiveness to increasing doses of methacholine was measured 24 hours after the last challenge, thereafter mice were dissected, bronchoalveolar lavage was performed and blood and lung samples were taken.

Administration of CTLA4-Ig as adjuvant for SIT

In the experiment using IDO-KO mice clinical grade CTLA4-Ig (abatacept, Bristol-Myers, Woerden, the Netherlands) was used. In other experiments CTLA4-Ig was obtained as described previously [26;27]. CTLA-Ig (280 μg/injection) or control IgG (280 μg/injection) were mixed with OVA-SIT (100 μg/injection) and s.c. injected.

Measurement of lung function

Airway reactivity to methacholine was evaluated by direct measurement of airway resistance in response to increasing doses of methacholine as explained previously [23]. In brief, anesthetized mice (by i.p. injection of ketamine 100 mg/kg; Pfizer, New York, NY and medetomidine 1 mg/kg; Pfizer) were tracheotomized (20-gauge intravenous: i.v. cannula; Becton Dickinson,

Alphen a/d Rijn, The Netherlands), attached to a computer-controlled small-animal ventilator (Flexivent; SCIREQ, Montreal, Quebec, Canada), then paralyzed (i.v. injection of Pancuronium bromide: Pavulon, 50 μg/Kg Merck Sharp & Dohme, NJ, USA).Ventilation was adjusted at a breeding frequency of 300 breaths/min and a tidal volume of 10 mL/kg. Tidal volume was pressure limited at 300 mm H2O. An i.v. cannula was inserted through the jugular vein for the administration of methacholine. Airway resistance in response to intravenous methacholine (acetyl-b-methylcholine chloride, Sigma-Aldrich, Dordrecht, the Netherlands) was calculated from the pressure response to a 2-second pseudorandom pressure wave.

Determination of serum levels of specific IgE

Serum levels of OVA-specific IgE were determined by enzyme-linked immunosorbent assay (ELISA) as described previously [28] and results are expressed as experimental unit/ml.

Analyses of bronchoalveolar lavage fluid

Animals were lavaged five times through the tracheal cannulae with 1-ml aliquots of saline. BAL cells were pooled, counted, and cell types were indentified using flow cytometry as described elsewhere [29].

Cytokines measurement in the lung tiusse

Homogenates were made from the cardiac lobe of lung as described elsewhere[30]. The levels of IL-4, IL-5, IL-10, IFN-g and TGF-β in the lung homogenates were determined by commercially available ELISA kits according to the manufacturer's instructions (BD Pharmingen, NJ, USA).

Flow cytometry and antibodies

PerCP-anti-CD4 (BD, NJ, USA), FITC-anti-T1ST2 (MD-Biosciences, Zurich, Switzerland), PE-anti-FOXP3 and eFluor450-anti-CD25 (eBioscience, CA, USA) were used for FACS.

Statistical analysis

Data are expressed as mean ± SEM. The airway response curves to methacholine were analyzed by repeated measurements. All the other data were compared using Mann–Whitney U-test corrected for multiple comparisons. A P-value of less than 0.05 was considered significant.

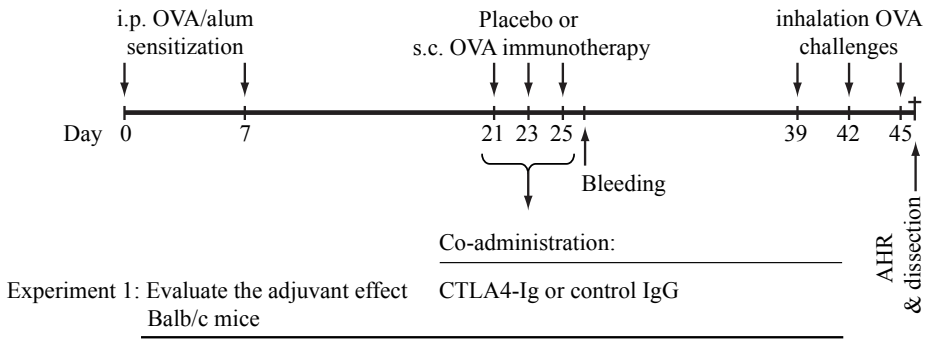

Figure 1 - Experimental layout: in experiment 1, to evaluate that whether CTLA4-Ig can enhance the suppressive effects of SIT, it was combined with OVA-SIT (100 µg/mouse) in Balb/c mice. In experiment 2, CTLA4-Ig is combined with OVA-SIT in IDO-/- mice to examine whether the effect of CTLA4-Ig is mediated by IDO. In experiment 3, to analyse the effects of CTLA4-Ig on regulatory T cells the frequency of CD4+CD25+FOXP3+ Treg cells and CD4+T1ST2+ Th2 cells were analyzed 24h after the last SIT injection and 24h after inhalation challenges.

Results

CTLA4-Ig strongly augments the therapeutic effects of SIT

CTLA4-Ig was combined with SIT to examine whether it augments the suppressive effects of SIT in a mouse model of allergic asthma (Figure 1). OVA-sensitized placebo-treated mice exhibit a strong OVA-specific IgE response, airway eosinophilia and AHR upon OVA inhalation challenges (Figure 2A-C). OVA-SIT treatment significantly reduced the level of these three basic manifestations of allergic asthma ($P<0.05$, Figure 2A-C) but did not significantly affect the levels of IL-4 and IL-5 in lung tissue (Figure 2 D, E). Co-administration of CTLA4-Ig with SIT highly augmented the SIT-induced suppression of AHR ($P<0.05$), OVA-specific IgE ($P<0.005$) and airway eosinophilia ($P<0.005$) as compared to SIT alone. Combination of CTLA4-Ig with SIT also induced a reduction in the levels of IL-4 ($P<0.05$) and IL-5 ($P<0.05$) in lung tissue, which was not observed with SIT treatment alone (Figure 2D, E).

CTLA4-Ig-mediated augmentation of SIT is independent of IDO

Since CTLA4-Ig has been shown to increase the expression of indoleamine 2,3 dioxygenase and thereby induce tolerogenic effects (31), we tested whether the augmenting effect of CTLA4-Ig on SIT in our model is dependent on IDO activity. To this aim we compared the effects of co-administration of CTLA4-Ig with SIT between IDO-KO and wild-type BALB/c mice. OVA-SIT alone

significantly suppressed AHR (P<0.05), specific IgE in serum (P<0.05) and airway eosinophilia (P<0.05) in wild-type mice (Figure 3A,C-D). Co-administration of CTLA4-Ig with OVA-SIT significantly increased the suppression level of AHR (P<0.05), OVA-specific IgE in serum (P<0.05)

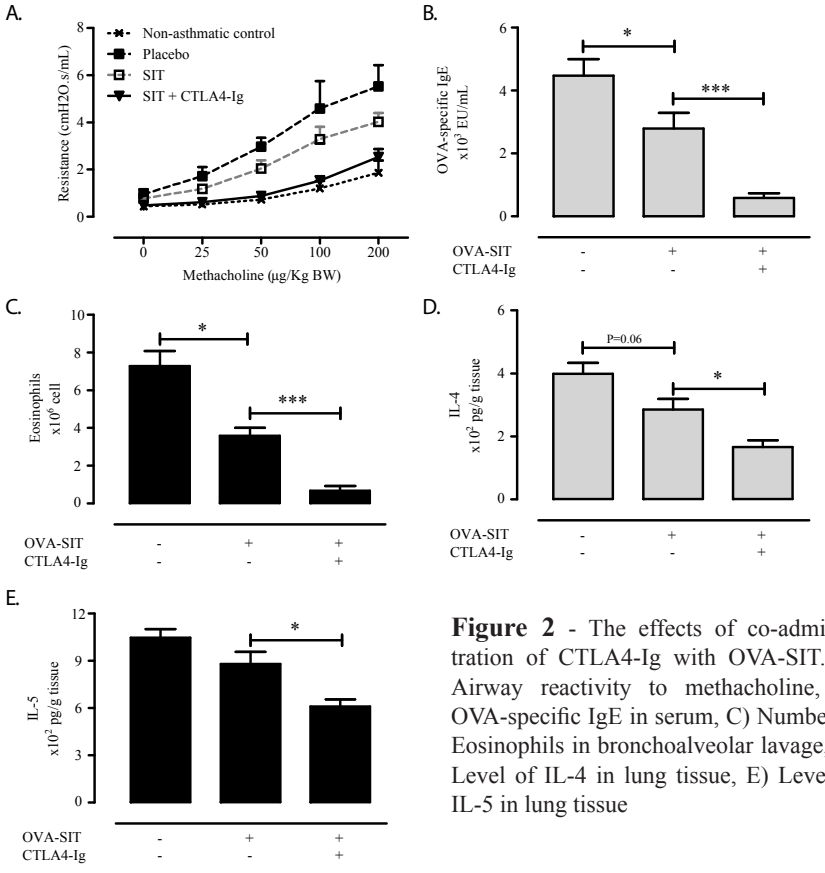

Figure 2 - The effects of co-administration of CTLA4-Ig with OVA-SIT. A) Airway reactivity to methacholine, B) OVA-specific IgE in serum, C) Number of Eosinophils in bronchoalveolar lavage, D) Level of IL-4 in lung tissue, E) Level of IL-5 in lung tissue

and airway eosinophilia (P<0.05) compared to OVA-SIT alone in wild type mice (Figure 3A,C-D). In IDO-KO mice, OVA-SIT significantly suppressed airway eosinophilia (P<0.05) but neither AHR nor specific OVA-specific IgE levels were suppressed (Figure 3B-D). Surprisingly, co-administration of CTLA4-Ig with OVA-SIT in IDO-KO mice also strongly enhanced SIT-induced suppression of the manifestation of experimental allergic asthma, resulting in significant suppression of OVA-specific IgE and AHR, which was not achieved by the OVA-SIT alone, and significantly augmented suppression of eosinophils (Figure 3B-D). These data indicate that although SIT treatment is less efficient in IDO-KO mice, CTLA4-Ig co-administration remains

effective in enhancing the suppressive effects of the OVA-SIT.

Co-administration of CTLA4-Ig with SIT reduces peripheral Treg and Th2 cells

To evaluate whether administration of CTLA4-Ig results in the induction of Treg cells, that might suppress reactivation of Th2 cells upon allergen inhalation challenge, we analyzed the frequency of $CD4^+CD25^+FOXP3^+$ Treg

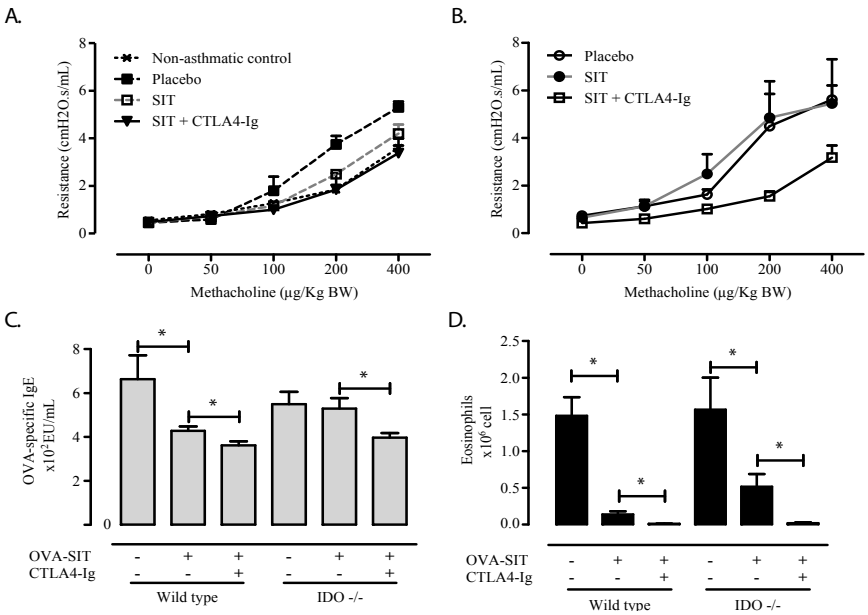

Figure 3 - The effects of co-administration of CTLA4-Ig with OVA-SIT in IDO-/- mice. A) Airway reactivity to methacholine in wild type mice , B) Airway reactivity to methacholine in IDO-/- mice , C) OVA-specific IgE in serum, D) Number of Eosinophils in bronchoalveolar lavage.

cells and $CD4^+T1ST2^+$ Th2 cells in peripheral blood 24h after OVA-SIT. Solo treatment of OVA-SIT does neither alter the frequency of $CD4^+CD25^+FOXP3^+$ Treg cells nor the frequency of $CD4^+T1ST2^+$ Th2 cells (Figure 4A-B). Surprisingly, co-administration of CTLA4-Ig with SIT significantly reduced the percentage of both $CD4^+CD25^+FOXP3^+$ Treg cells and $CD4^+T1ST2^+$ Th2 cells within the $CD4^+$ T cell population ($P<0.05$, Figure 4A-B) as compared to OVA-SIT alone. To test whether these effects of CTLA4-Ig on Treg persist after OVA inhalation challenges, the percentage of $CD4^+CD25^+FOXP3^+$ Treg cells were analyzed in the blood 24h after the last inhalation challenge. No significant differences in the percentage of $CD4^+CD25^+FOXP3^+$ Treg cells were observed between the different treatment groups at this time point (Figure 4C).

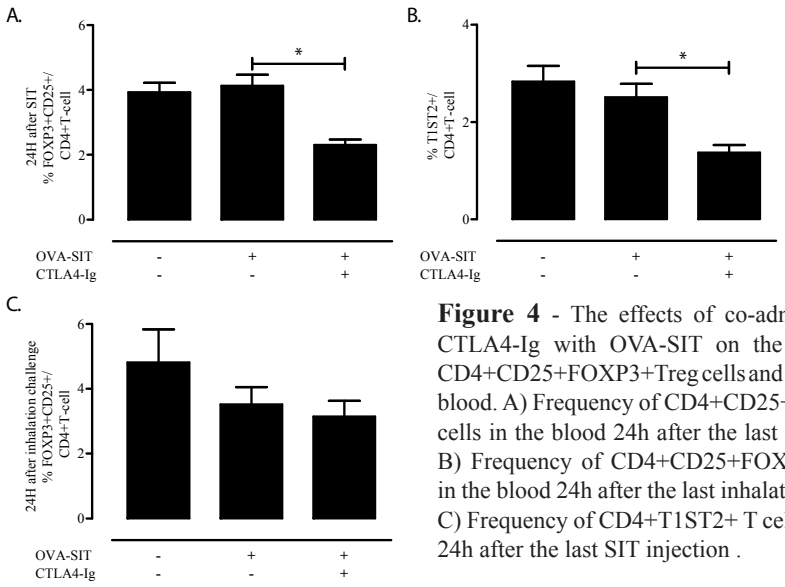

Figure 4 - The effects of co-administration of CTLA4-Ig with OVA-SIT on the frequency of CD4+CD25+FOXP3+Treg cells and Th2 cells in the blood. A) Frequency of CD4+CD25+FOXP3+Treg cells in the blood 24h after the last SIT injection , B) Frequency of CD4+CD25+FOXP3+Treg cells in the blood 24h after the last inhalation challenge , C) Frequency of CD4+T1ST2+ T cells in the blood 24h after the last SIT injection .

CTLA4-Ig reduces interferon-γ in the lung tissue after inhalation challenge

To further dissect the mechanism of the augmenting effects of CTLA4-Ig on SIT we tested whether these effects are mediated by enhancing the activity of lung-resident Treg cells or Th1 cells which can suppress Th2 and effector cells upon allergen inhalation challenge. To this aim we measured the levels of IL-10, TGF-β and interferon-γ in the lung tissue 24h after the last OVA inhalation challenge. Remarkably, the levels of interferon-γ in lung tissue were significantly reduced in the group receiving combined CTLA4-Ig and OVA-SIT compared to the group receiving only OVA-SIT ($P<0.05$, Figure 5C). No differences were observed in the levels of IL-10 and TGF-β in lung tissue between the different experimental groups (Figure 5A-B).

Discussion

In this study we demonstrate that CTLA4-Ig acts as a potent adjuvant for SIT by strongly enhancing SIT-induced suppression of the manifestations of experimental allergic asthma, including the suppression of Th2 cytokines production, which was not achieved by SIT treatment alone. The adjuvant effect of CTLA4-Ig on SIT is independent of IDO activity, indicating that it is mediated by blocking the CD28-mediated T cell co-stimulatory signal.

The tolerogenic effects of CTLA4-Ig can be mediated by two mechanisms: (i)

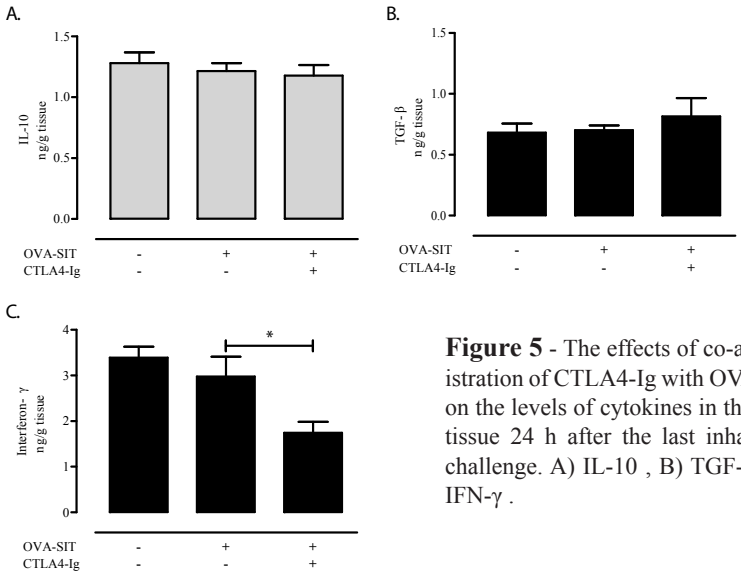

Figure 5 - The effects of co-administration of CTLA4-Ig with OVA-SIT on the levels of cytokines in the lung tissue 24 h after the last inhalation challenge. A) IL-10 , B) TGF-β , C) IFN-γ .

Signaling into dendritic cells through B7 molecules, leading to activation of the non-canonical NF-kB pathway and induction of IDO [32] and (ii) Blocking the CD-28-mediated co-stimulatory signal on T cells [12]. Here, we show that the adjuvant effect of CTLA4-Ig on SIT is independent of IDO. In agreement with our observations, David *et al* showed that CTLA4-Ig inhibits DC-dependent proliferation of human T cells *in vitro*, in an IDO independent fashion [33]. In contrast, it has also been observed that administration of CTLA4-Ig is tolerogenic in non-obese diabetic mice in a strictly IDO dependent fashion[32]. However, since NOD mice show impaired expression of CTLA4 and spontaneously develop auto-inflammatory disorders [32], these latter observation might not be very relevant to our model in which CTLA4-Ig has been used in mice without such an impaired expression of CTLA4. Moreover, IDO can only partially explain the CTLA4-dependent regulation of T cell responses, as IDO-KO mice do not show the same lymphoproliferative phenotype as CTLA4-KO mice [34].

In our model, we previously demonstrated that pharmacological inhibition of IDO contributes to SIT-induced suppression of the manifestation of allergic asthma [23]. In the present study, we confirm these observations using IDO-KO mice and show that the suppression of AHR and specific IgE induced by SIT treatment in wild-type mice is absent in IDO-KO mice. Apparently, loss of IDO changes the sensitivity to SIT-mediated suppression of asthmatic manifestations, but remains sensitive to the adjuvant effect of CTLA4-Ig since CTLA4-Ig co-administration restores the suppression of AHR and

OVA-specific IgE responses in IDO-KO mice to the level observed in wild type mice.

The adjuvant effect of CTLA4-Ig might also indicate point to other tolerogenic mechanisms such as activation of members of the forkhead box O (FOXO) family of transcription factors, or induction of nitric oxide synthase by so-called reverse signaling in DCs through B7 molecules. Interestingly, FOXO has been implicated in tolerance induction and it has been shown that CTLA4-Ig induces tolerogenic effects by activating FOXO in DCs [32;35]. Moreover, it has been observed that induction of allograft tolerance by CTLA4-Ig is dependent on both IDO and nitric oxide [36]. More studies are needed to unravel the role of other pathways induced by reverse signaling in the adjuvant effect of CTLA4-Ig towards SIT.

Although we can not yet exclude all reverse signaling pathways, it appears very likely that CTLA4-Ig acts by blocking CD28-mediated T cell co-stimulation during SIT treatment. Antigen presentation in the absence of proper co-stimulation leads to T cell anergy or induction of inducible regulatory T cells (iTreg cells) [37]. Since we found that CTLA4-Ig co-administration suppresses the frequency of both $CD4^+CD25^+FOXP3^+$ Treg and $CD4^+T1ST2^+$ Th2 cells in blood, we speculate that the augmented suppression induced by CTLA4-Ig is mediated by a FOXP3 negative Treg cell subset or the direct induction of anergy in Th2 cells. Alternatively, the reduced percentage of $CD4^+CD25^+FOXP3^+$ T cells in the blood could be due to migration of these cells to the lymph nodes as has been seen in venom SIT in human [38].

After inhalation challenges when SIT-induced tolerance suppresses the manifestation of experimental asthma, we observe no increased production of TGF-β or IL-10. In fact, at this time point, we observe suppression of both Th1 (IFN-g) and Th2 (IL-4, IL-5) cytokines in the lung tissue. This may indicate that co-administration of CTLA4-Ig with SIT leads to increased function of Treg cells which are capable of suppressing both Th1 and Th2 cell activity. Such an enhanced Treg cell function, though, appears to be independent of the production of the immunoregulatory cytokines TGF-β or IL-10 since their levels were not elevated. An alternative mode of action might entail suppression of Th1 and Th2 effector cells mediated by direct cell-cell contact[39]. However, we can not exclude that these immunoregulatory cytokines are produced at an earlier time-point or are produced locally in the lung and this is not reflected in increased levels in BAL fluid.

In conclusion, we found that CTLA4-Ig acts as an adjuvant for SIT by highly enhancing its suppressive effects on manifestations of experimental allergic asthma. Adjuvant effects of CTLA4-Ig appear to be mediated by blocking

CD28-mediated T cell co-stimulation since they are independent of IDO function. It is tempting to speculate that using CTLA4-Ig might allow a safer SIT treatment regimen with lower doses of allergen.

Interestingly, CTLA4-Ig (Abatacept) has been approved for clinical use by United States Food and Drug Administration and European Medicines Agency[40] and has been safely used in clinical trials as a treatment for rheumatoid arthritis [18] and to prevent transplant rejection [19]. Therefore, it is feasible to design clinical studies using CTLA4-Ig in combination with SIT in allergic patients to achieve enhanced efficacy of the treatment.

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TGF-β potentiates the suppressive effects of OVA-SIT in a mouse model of allergic asthma

CHAPTER

8

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Abstract

Background: Allergen specific immunotherapy (SIT) is a disease modifying treatment that has been shown to render long-lasting relief from symptoms in variety of allergic disorders such as rhinitis and insect venom allergy. There is an unmet medical need to improve SIT, given the long treatment schedule, the lack of efficacy in some allergic disorders and the severe side effects such as anaphylaxis. Induction of IL-10 and TGF-β producing regulatory T cells has been shown to occur during SIT, although their precise role is currently unclear. In this study we tested the hypothesis that administration of exogenous TGF-β during SIT treatments improves its efficacy, by expanding the induction of regulatory T cells (Tregs). In addition, we tested whether blocking of IL-6 signaling could enhance the TGF-β dependent induction of Tregs.

Methods: TGF-β and a blocking anti-IL6 receptor antibody were co-administered with suboptimal SIT in a mouse model of ovalbumin (OVA)-driven asthma.

Results: Co-administration of TGF-β with suboptimal SIT resulted in significantly increased serum levels of specific IgA, and improved the suppression of eosinophilic inflammation of the airways and Th2 cytokines in the BALF as well as OVA-specific IgE responses, but did not enhance suppression of AHR. These effects were accompanied with a trend to increased numbers of $FOXP3^+$ iTregs. Neutralizing IL-6 signaling during TGF-β/SIT treatment did not further improve the TGF-β enhanced suppressive effects of SIT, indicating an optimal induction of iTreg activity by SIT treatment in the presence of TGF-β.

Conclusion: Here, we show that co-administration of TGF-β with suboptimal SIT treatment potentiates the suppressive effects on Th2-driven allergic inflammation. Furthermore, the potentiation is associated with a trend to increased numbers of $FOXP3^+$ iTreg cells and significant induction of specific IgA responses in serum. These data clearly indicate that therapies aimed at enhancing iTreg cell function are viable therapeutic strategies for improvement of SIT efficacy.

Introduction

Allergen specific immunotherapy (SIT) is the only treatment for allergic diseases that leads to long-term relief of symptoms by altering the immunological response to the allergen, the underlying cause of the disease [1]. This treatment is performed by repeated administration of optimal high doses

of the sensitizing allergen at least for three years [2]. Although high doses of allergen are required for optimal effects of SIT, these contribute to the risk of anaphylaxis. Therefore, SIT treatment has an induction phase, during which low but gradually increasing doses of allergen are given, and a maintenance phase during which high doses of allergen are applied [3]. Even in such a treatment regimen, SIT is accompanied by the risk of anaphylaxis and has variable to low efficacy for some allergic disorders, including allergic asthma and food allergies, whilst being very efficacious for insect venom allergy and allergic rhinitis. Therefore, there is an unmet medical need to improve SIT to reduce the risk of anaphylactic reactions and to achieve efficacy of treatment for a wider range of allergic disorders. One approach to this end is the use of adjuvants to achieve a more pronounced suppressive immune response at lower doses of allergen.

Transforming Growth Factor-b (TGF-β) is an immunomodulatory cytokine which may play an important role in tolerance induction by SIT. Enhanced mucosal expression as well as increased secretion of TGF-β by PBMCs has been observed after successful subcutaneous and sublingual SIT [4-6]. Moreover, a positive correlation has been observed between the levels of TGF-β and allergen specific IgA in serum and subsequently in the improvement of clinical symptoms after grass pollen immunotherapy [6].

It has been demonstrated that TGF-β can contribute to tolerance induction by facilitating the generation of two distinct subsets of inducible regulatory T cells (Tregs), (i) TGF-β producing Th3 cells and (ii) $CD4^+FOXP3^+$ inducible Tregs (iTregs) [7;8]. For instance, TGF-β has been shown to mediate the induction of oral tolerance by generation of *de novo* iTregs [9]. Furthermore it has been shown that these, TGF-β induced $FOXP3^+$ iTregs can be generated from naïve $CD4^+$ T cells *in vivo,* express TGF-β and have a potential suppressive function that can prevent the HDM-induced allergic pathogenesis in lung[10]. The role for TGF-β in inducing Treg cell activity, however, is highly dependent on the presence or absence of other pro-inflammatory mediators in the local microenvironment. For instance, it has been demonstrated that in the presence of IL-6, TGF-β promotes the generation of Th17 cells in mice at the expense of Treg induction [11;12]. Moreover, IL-6 has been shown to hamper TGF-β-mediated generation of iTregs in humans [13], suggesting a negative regulatory role for IL-6 on TGF-β induced iTregs mediated tolerance.

We have previously found evidence that TGF-β contributes to SIT-induced allergen tolerance in a mouse model of OVA-driven asthma [14]. However, it is currently unknown what the role for TGF-β is in SIT induction, and whether increased TGF-β levels might enhance the efficacy of SIT in our mouse

model. Here, we hypothesize that while endogenous TGF-β contributes to SIT-induced allergen tolerance, administration of additional exogenous TGF-β might augment the therapeutic effects of SIT by increased induction of $FOXP3^+$ iTregs.

Using a mouse model for SIT based on the classical OVA-driven experimental asthma model, we show that administration of TGF-β during SIT treatment augments suboptimal SIT-mediated suppression of allergen induced airway eosinophilia, serum IgE as well as Th2 cytokines in the bronchoalveolar lavage fluid (BALF) but not AHR. Blocking the biological effects of IL-6 concurrent to administration of TGF-β and SIT does not enhance the potentiating effects of TGF-β suggesting that Th17 cells are likely not induced in this model.

Materials and Methods

Mice

6-8-week-old female BALB/c mice were purchased from Charles River laboratories (L'Arbresle, France) and were kept under SPF (specific pathogen free) conditions. All animal experiments were performed in accordance with the guidelines of the institutional animal care and use committee (IACUC; DEC) of the University of Groningen.

Experimental allergic asthma and OVA-SIT

The OVA-specific immunotherapy for experimental allergic asthma in mice was similar as previously described [15], only with minor modification on the dose and frequency of SIT injections. Optimal SIT performed by 3 times injection of 1 mg OVA results in the maximal suppression of all the manifestations of experimental allergic asthma [14]. In order to determine the potential enhancing effects of co-administration of TGF-β with SIT, a suboptimal SIT treatment regime was used in our studies. Sub-optimal SIT could was performed by reducing the number of injections (2 injections of 1 mg of OVA instead of 3), and in the second experiment also by reducing the dose (2 injections of 0.1 mg OVA instead of 1 mg). Briefly, for allergic sensitization mice received 2 i.p. injections of 10 μg OVA (endotoxin-free (<0.5 U/ml) Seikagaku Biobusiness Corporation; Tokyo, Japan) 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 last sensitization, mice were treated with 2 s.c. injections of either 1 mg OVA (suboptimal SIT, regarding number of injections) or 100 μg OVA (suboptimal SIT regarding dose and number of injections) in 200 μl pyrogen-free saline or 200 μl saline (sham

treatment group) on alternate days. After 10 days, mice were challenged 3 times every third day with OVA aerosols in saline (1% w/v) for 20 min. The OVA inhalation challenges were performed in a Plexiglas exposure chamber 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.

Co-administration of TGF-β with SIT: Recombinant Human Latent TGF-β1 was purchased from R&D system, the Netherlands. TGF-β was injected subcutaneously (1 μg in 100 μl saline) at the time of SIT injections in the same region. The dose of TGF-β to be used in these experiments was determined based on the utilized dose in previous studies [16;17]. Before injection of TGF-β1, the latent form was activated as described in detail previously [16]. Briefly, the latent form of TGF-β1 was activated via acidification in 4 mM HCL containing 0.1% BSA for one hour in room temperature, which followed by PH neutralization with NaOH (4M) and dissolved in saline before use.

Blocking IL-6 signaling: mice received one dose (5 mg/mouse, i.p.) of rat anti-mouse interleukin-6 receptor monoclonal antibody (MR16-1), was kindly provided by Chugai pharmaceutical co., ltd, Tokyo, Japan, just before the first suboptimal SIT injection.

Evaluation of airway responsiveness

Airway responsiveness to inhaled methacholine (acetyl-b-methylcholine chloride; Sigma-Aldrich) was measured at 2 time points in each mouse; pre-challenge (one week before the first OVA aerosol challenge) and post-challenge (24 hours after the last challenge) in conscious, unrestrained mice using barometric whole-body plethysmography by recording respiratory pressure curves (Buxco; EMKA Technologies) as described in detail previously [18]. In brief, mice were placed in whole-body exposure chambers and baseline values were measured and averaged for 3 min. Hereafter, mice were exposed to saline aerosols followed by 2-fold increasing concentrations of methacholine aerosols (ranging from 1.6 to 50 mg/ml in saline). Aerosols were generated by a Pari LC Star nebulizer. In each step mice were exposed to the aerosols for 3 min which followed by 3 min of recording to achieve average values. As a parameter of airway responsiveness, increases in enhanced pause (Penh), an index of airway obstruction were determined, as described in details previously [19]. Airway responsiveness was expressed as the Penh per dose of methacholine. Dose-response curves to methacholine were statistically analyzed by a general linear model of repeated measurements followed by Bonferroni correction for multiple comparisons.

Determination of ovalbumin-specific immunoglobulin in serum

Blood samples for serum analyses were collected 3-5 days before the first OVA aerosol challenge (via orbital puncture) and 24 hrs after the last challenge (via cardiac puncture). OVA-specific IgE, IgA and IgG1 levels in serum were measured by enzyme-linked immunosorbent assays (ELISA) as described previously [20]. Briefly, 96-well microtiter plates from Nunc A/S (Roskilde, 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, 0.5% BSA, 2mM EDTA, 136.9 mM NaCL, 50 Mm Tris, 0.05% Tween-20 [Merck, Whitehouse Station, NJ USA], PH 7.2) and left for one hour at room temperature. For OVA-specific IgA or IgG1 96-well microtiter plates were coated with 100 µl of 10 µg/ml OVA (grade V, Sigma-aldrich, Zwijndrecht, the Netherlands) over night followed by blocking with ELISA buffer containing 0.5% BSA. After multiple washings with PBS containing 0.05% v/v Tween-20, diluted serum samples and OVA-specific IgE, IgA or IgG1 reference serums were added to the wells and incubated for 2h. The reference serums was obtained by immunization of mice with OVA as described above and arbitrarily assigned a value of 1000 experimental units/ml (EU/ml). Next after multiple washing the wells, plates were incubated with 1µg/ml of DIG-conjugated OVA for 1.5 h for IgE. For IgA and IgG1 plates were coated with biotinylated rat anti-mouse IgA or biotinylated rat anti-mouse IgG1 for 1.5 h respectively. Thereafter, another multiple washing step was performed followed by incubation for 1 h with anti-DIG Fab coupled to horseradish peroxidase, according to manufacturer`s instructions (Roche Diagnostics, Basel, Switzerland) for IgE or streptavidin coupled to horseradish peroxidase (R&D systems) for IgA and IgG1. For detection, 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_2SO4. 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.

Cell counts and cytokine ELISAs in BALF

Mice were sacrificed by collecting blood through the vena cava and opening of the thoracic cavity under anesthesia. Lungs were lavaged 5 times through a tracheal cannula (23-gauge blunt needle) with aliquots of 1 ml pyrogen-free saline at 37°C. The first aliquot contained a cocktail of protease inhibitors (complete mini tablet [Roche Diagnostics, Almere, the Netherlands]) and 5% bovine serum albumin (BSA; Sigma-Aldrich) and was kept separately,

whereas the other 4 ml were pooled together after collecting the broncoalevolar lavage fluid (BALF). BALF were centrifuged and supernatants of the first ml were used to measure cytokines. Total BALF cells were pooled to determine the total number of cells using a Burker-Turk counting chamber (Karl Hecht Assistant KG, Sondheim/Rohm, Germany). To determine the cellular composition of the BALF, cells were analyzed with flow cytometry as described in details before[21] .Briefly, A cocktail of antibodies to MHCII-FITC, CD11c-APC, CD3and B220-Percp and Siglec F-PE (R&D Systems) were used to differentiate the cellular composition of the BALF. All the staining procedure was carried on ice and 2.4.G2 was used to prevent nonspecific binding.

Cytokines in the BAL fluid were determined by sandwich ELISA according to the manufacturer's instruction (Becton Dickinson, Franklin, Lakes, NJ, USA).

Flow cytometry of mediastinal lymph nodes (MLNs)

Immediately after sacrificing the mice (24 hours after the last OVA inhalation challenges), mediastinal lymph nodes were isolated and kept on the ice. Single cell suspension from lymph nodes were prepared by mechanical disruption via pressing the organs through a 70 μm nylon cell strainer (Becton Dickinson) with the piston of a 1 ml plastic syringe. Cells were stained with CD4-FITC, CD3-PE-cy5, CD25-APC and FoxP3-PE for 30 minutes on ice. Cells were acquired on a LSRII flow cytometer (BD) and FlowJo software (Tree Star, Inc.).

Statistical analysis

Data are expressed as mean ± SEM and were compared using Mann–Whitney U test corrected for multiple comparisons. A P-value of less than 0.05 was considered significant.

Results

Co-administration of TGF-β enhances suppression of airway eosinophilia by suboptimal SIT

To examine whether exogenous administration of TGF-β during suboptimal SIT will potentiate the suppression of the asthma manifestations, we first co-administered rhTGF-β (1 μg/ mouse) with suboptimal SIT treatment two injectionsof 1 mg OVA) as it depicted in the schematic view in Figure 1.To

evaluate the efficacy of the combined treatment, we compared the Penh as a substitute measure for AHR [22], as well as airway inflammation and serum levels of OVA-specific IgE and IgA between suboptimal SIT treated mice and controls.

As expected, the dose-response curve of Penh to methacholine did not vary among the different treatment groups prior to allergen challenges (data not shown). Hence, the mean value of all treatment groups is depicted as pre-challenge value in figure 2. OVA inhalation challenges induced a significant elevation of Penh in both sham treated control ($P < 0.001$) and sham/TGF-β treated ($P < 0.01$) mice (Figure 2a). Administration of TGF-β in sham treated mice induced a somewhat lower Penh at higher doses of methacholine, although this did not reach statistical significance (Fig 2a). Suboptimal SIT treated mice displayed significantly reduced responses to methacholine as compared to the sham treated control mice ($P < 0.05$). In contrast, co-administration of TGF-β with suboptimal SIT resulted in a not significantly reduced Penh response to methacholine in comparison with either sham treated mice or suboptimal SIT alone, indicating that TGF-β co-administration did not further enhance suboptimal SIT-induced suppression of Penh after OVA-challenges (Fig 2a).

A large number of inflammatory cells, especially eosinophils, were observed in BAL fluid of sham-treated mice, confirming the efficient induction of allergic inflammation in our model (Fig 2b). The total cell count in the BALF as well as number of eosinophils was not significantly (31.62% ± 15.66, $P = 0.335$ and 41.23% ± 17.68, $P = 0.46$ respectively) reduced by the suboptimal SIT treatment or by TGF-β administration alone in comparison to sham treated control mice (Fig 2b). Interestingly co-administration of TGF-β with suboptimal SIT significantly reduced the total cell count (66.3%, ± 6.82, $P < 0.05$ compared to sham treatment) as well as the number of eosinophils by 78.48% ± 5.32 ($P < 0.05$) in comparison with sham treated control mice. These findings clearly show a potentiating role for exogenous TGF-β on suppression of eosinophilic inflammation by suboptimal SIT. As it shown in Figure 2c, along with the suppression of airway eosinophilia, a significant suppression of IL-5 levels in BALF was observed after co-administration of TGF-β with suboptimal SIT (62.74% ± 11.47, $P < 0.05$ compared with sham control), while suboptimal SIT or TGF-β alone failed to suppress the IL-5 levels in the BALF. Similarly, co-administration of TGF-β with suboptimal SIT suppressed level of IL-13 by 56,02% ±14.15, ($p=0.061$) in comparison with sham treatment (supplementary figure 1). No changes on IL-10 levels in the BALF were observed among different treatment groups (supplementary figure 1).

We further examined whether concurrent administration of TGF-β during SIT would enhance the suboptimal SIT mediated suppression of serum IgE responses. In sham/control and sham/TGF-β treated mice, OVA inhalation challenges induced a significantly (89.61% ±1.76, $P < 0.001$ and 83.76%± 4.68, $P < 0.01$ respectively) increased level of OVA-specific IgE in serum compared with pre-challenge values (Fig 2d). Suboptimal SIT treatment strongly suppressed the OVA-specific IgE response in serum (74.88% ± 5.03, $P < 0.01$) in comparison with sham treated control (Fig 2d). A similar level of suppression of specific IgE was achieved when TGF-β was combined with suboptimal SIT (74.13% ± 6.28, $P < 0.01$ compared to sham control) as it shown in Fig 2d. TGF-β treatment alone did not have any significant effect on both, pre and post challenge serum IgE levels when compared with sham treated control.

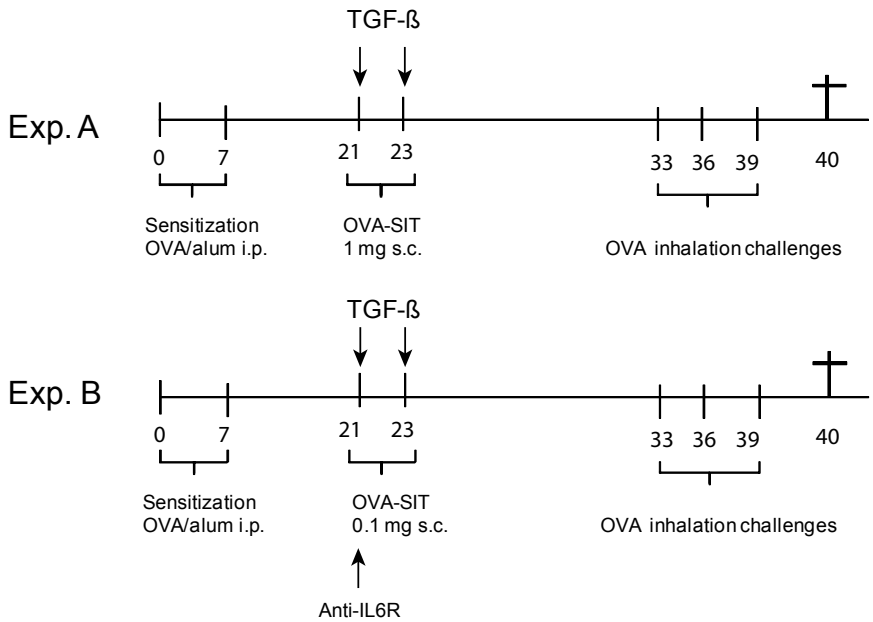


Figure 1 - Time line of experimental SIT protocol. Mice were sensitized with 2 i.p. injections of OVA/alum on days 0 and 7. (Exp A) On days 21 and 23 mice were treated with two s.c injections of either suboptimal SIT (1 mg OVA/200 ml saline) or sham treatment (200 ml saline) with and with out TGF-β, which then followed by 3 OVA-aerosols challenges on days 33, 36 and 39. (Exp B) Similar to the exp A, sensitized mice received either sham (200 ml saline) or suboptimal SIT (1 or 0.1mg OVA) which combined with/with out s.c TGF-β administration. In some groups anti IL-6R antibody was i.p injected on day 21 together with the first sham or suboptimal SIT treatment. Thereafter mice were challenged with 1% OVA aerosols every three day and 24 hours after the last OVA challenges AHR was measured and mice were sacrificed. In both experiments pre-challenge serum was collected 3-4 days after the last sham/suboptimal SIT injections and pre-challenge AHR was measured one week before the first OVA-aerosol challenges.

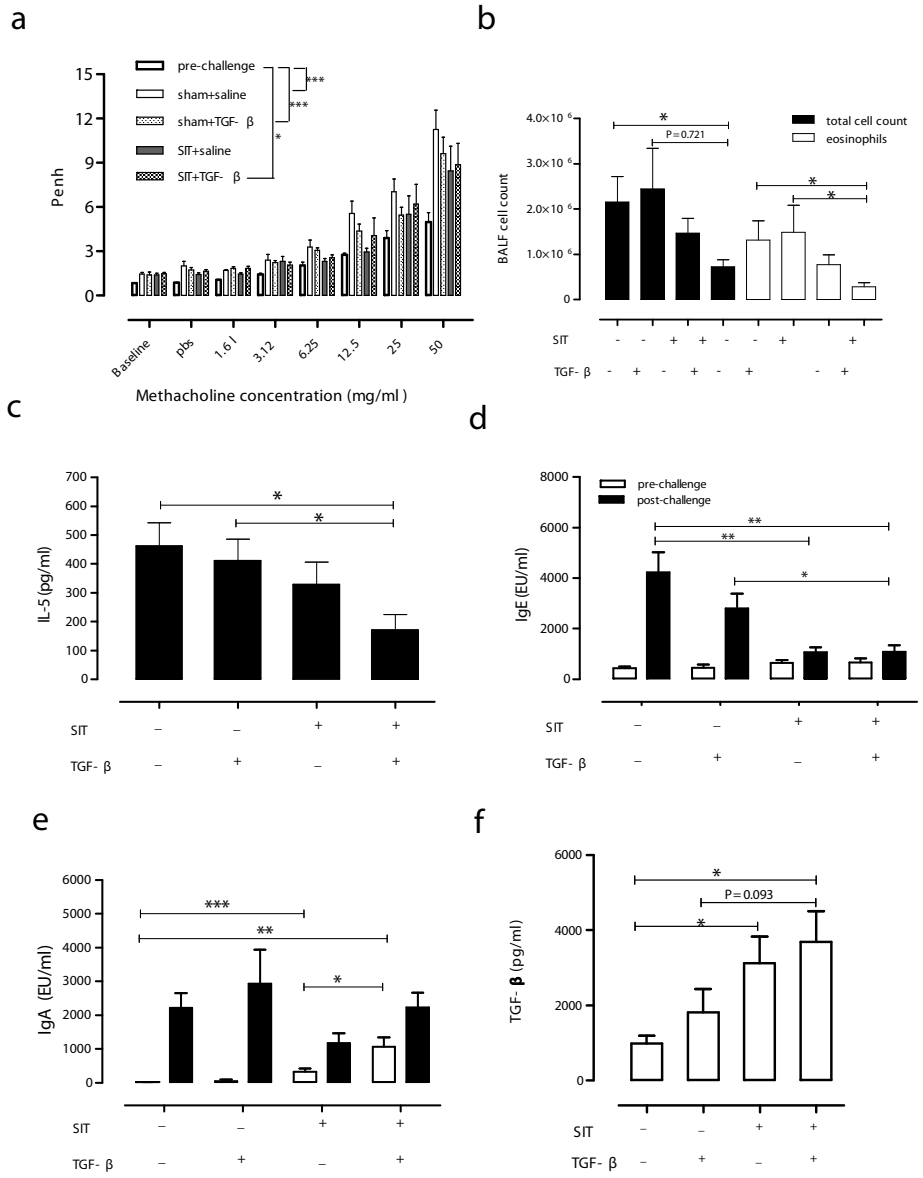


Figure 2 - Co-administration of TGF-β enhances the suppressive effects of suboptimal SIT. OVA-sensitized and sham or suboptimal (2 x 1mg) treated mice were injected with rhTGF-β or saline and challenged with OVA aerosols as depicted in Fig 1 (EXP A). (a) AHR was measured one week before the first OVA-challenge (pre-challenge) and 24 hours after the last OVA-challenge (post-challenge). (b) Total cell and eosinophils count in the BALF. (c) IL-5 in the BALF. Pre-challenge (white bars) and post-challenge (black bars) OVA-specific (d) IgE and (e) IgA in serum. (f) TGF-β levels in pre-challenge serum. Values are expressed as mean ± SEM of 6-7 mice per group. *P < 0.05, **P < 0.01 and ***P < 0.001

Since TGF-β can contribute to IgA class switch in B cells, we also analyzed the specific IgA responses in serum (Figure 2e; white bars). As expected, a remarkable induction of IgA responses was observed after suboptimal SIT treatment as compared with sham/control ($P < 0.01$, Fig 2e). Interestingly co-administration of TGF-β enhanced the suboptimal SIT induced specific IgA levels by more than 3 fold (326.99% ± 9.01, $P < 0.05$) as compared to suboptimal SIT treatment in the absence of TGF-β. This clearly demonstrates that a biologically effective dose of TGF-β was used in our experiments. As shown in Fig 2e, no significant changes in the level of IgA were observed after OVA inhalation challenges among different experimental groups, although the fold induction of specific IgA induced by the inhalation challenges was much lower in the suboptimal SIT treated groups.

Measuring the OVA-specific IgG1 in serum revealed that OVA challenges induced an increased level of OVA-specific IgG1 in sham treated mice ($P < 0.01$) as well as in sham/TGF-β treated group ($P < 0.05$) in comparison with pre challenge serum levels (table 1). Suboptimal SIT treatment induced an enhanced IgG1 responses in serum prior to OVA inhalation challenges ($P < 0.05$ compared to sham-treated control mice), which was not affected by concurrent administration of TGF-β. Moreover, in both suboptimal SIT and suboptimal SIT/TGF-β treated groups, OVA challenges did not further increased the level of IgG1 in serum compared to pre challenge values (table 1). Thus, there appears to be no potentiating effect of TGF-β co-administration on suboptimal SIT induced IgG1 responses.

Next, we examined whether administration of exogenous TGF-β during suboptimal SIT would influence the levels TGF-β either in BALF or in serum. Interestingly, analyzing the pre-challenge serum (obtained 4 days after the last SIT injection) revealed a robust induction of TGF-β levels by suboptimal SIT ($P < 0.05$, Fig 2f) compared to sham treated controls. Co-administration of TGF-β with suboptimal SIT induced a comparable rise in the TGF-β levels as in suboptimal SIT treatment alone ($P < 0.05$ compared with sham control and $P = 0.093$ when compared with sham/TGF-β treated group), indicating that local TGF-β administration did not induce a prolonged systemic increase in TGF-β levels. Moreover, no significant increase in the serum level of TGF-β was observed in TGF-β and sham-treated groups compared with their respective controls. These data indicate that in our experimental model SIT is accompanied with induction of TGF-β in serum and administration of exogenous TGF-β had no further effect on TGF-β levels. Remarkably, the rise in TGF-β levels induced by suboptimal SIT treatment was no longer observed after OVA inhalation challenges. Although the level of TGF-β was comparable

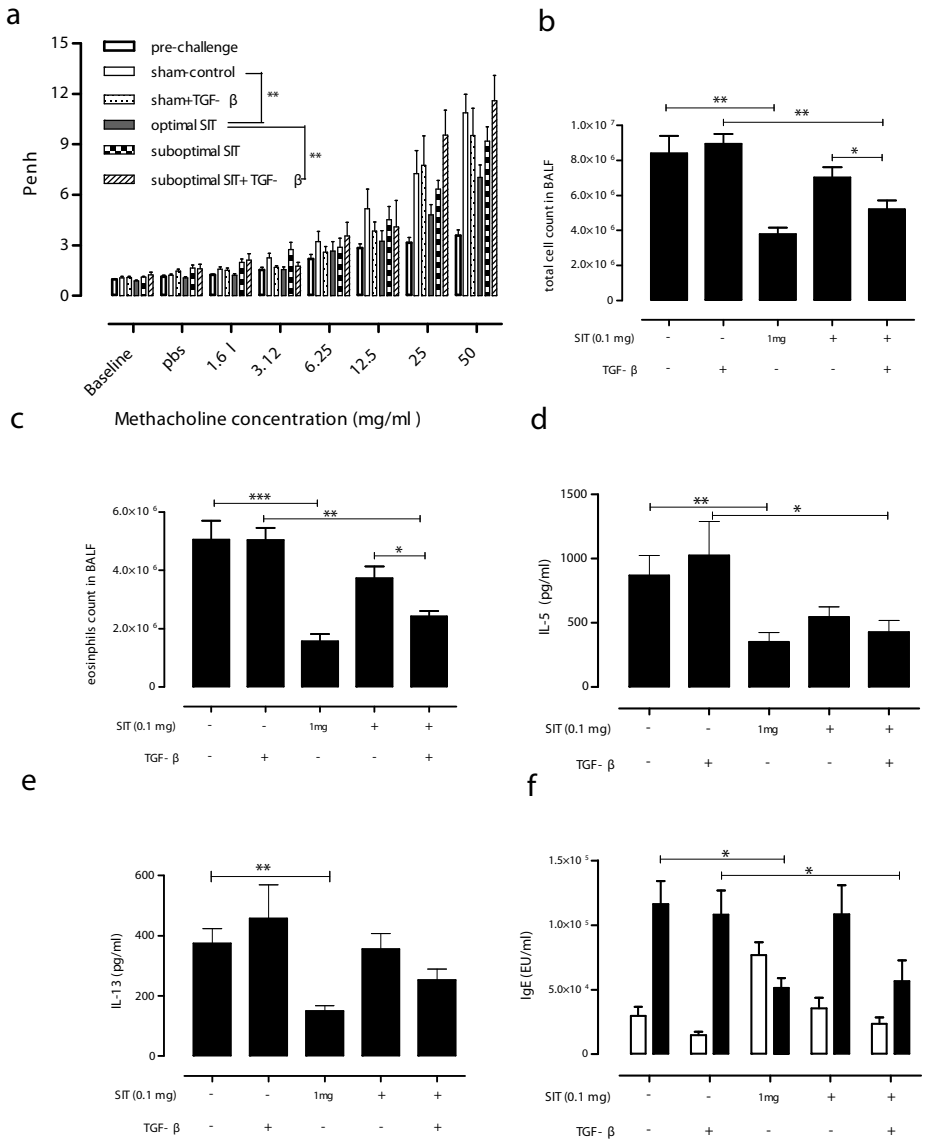


Figure 3 - Concurrent administration of TGF-β potentiates the efficacy of suboptimal SIT with lower dose of OVA. OVA-sensitized mice received sham or suboptimal SIT treatment with high dose (2 x 1 mg) or low dose (2 x 0.1 mg) of OVA. TGF-β or saline was co-administered with sham or suboptimal SIT treatment which followed by 3 OVA-aerosol challenges according the protocol depicted in Fig 1 (EXP B). (a) AHR was measured one week before the first OVA-challenge (pre-challenge) and 24 hours after the last OVA-challenge (post-challenge). (b) Total cell count and (c) number of eosinophils in the BALF. (d) IL-5 and (e) IL-13 in the BALF. (f) Pre-challenge (white bars) and post-challenge (black bars) OVA-specific) IgE in serum. Values are expressed as mean ± SEM of 6-7 mice per group. *P < 0.05, **P < 0.01 and ***P < 0.001

between all treatments groups, it dramatically increased compared with pre challenge values (supplementary data fig 5d). Measuring TGF-β levels in the BALF revealed no significant changes between experimental groups (supplementary data, fig 5c).

Co-administration of TGF-β allows the use of a low dose allergen for suppression of eosinophilic airway inflammation and specific IgE

Since co-administration of TGF-β with suboptimal SIT treatment using a high dose of allergen (1 mg OVA) readily improved suppression of eosinophilic inflammation and Th2 cytokines in BALF, we next evaluated whether TGF-β co-administration would allow SIT to suppress manifestations of allergic inflammation when a suboptimal dose of allergen (100 μg OVA) was used.

As shown in Fig 3a, OVA inhalation challenges induced a strong increase in the dose response curve to methacholine in all treated groups compared with pre challenge values. Suboptimal SIT treatment at the maximal dose of OVA (2 x 1mg) significantly ($P < 0.01$) suppressed the Penh dose response curve to methacholine in comparison to sham treated controls (Fig 3a). SIT performed with a 10-fold lower dose of allergen (2 x 0.1mg) only induced partial suppression of the Penh dose response curve for methacholine, which did not reach statistical significance in comparison with sham treated control. Co-administration of TGF-β with suboptimal SIT treatment at the reduced allergen dose did not at all enhance suppression of the Penh curves to the level achieved by suboptimal SIT at the maximal allergen dose (2 x 1mg).

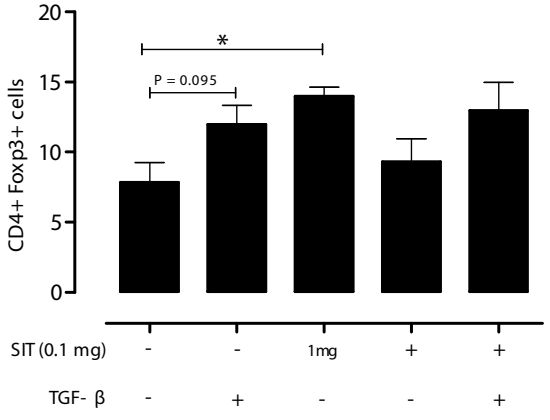


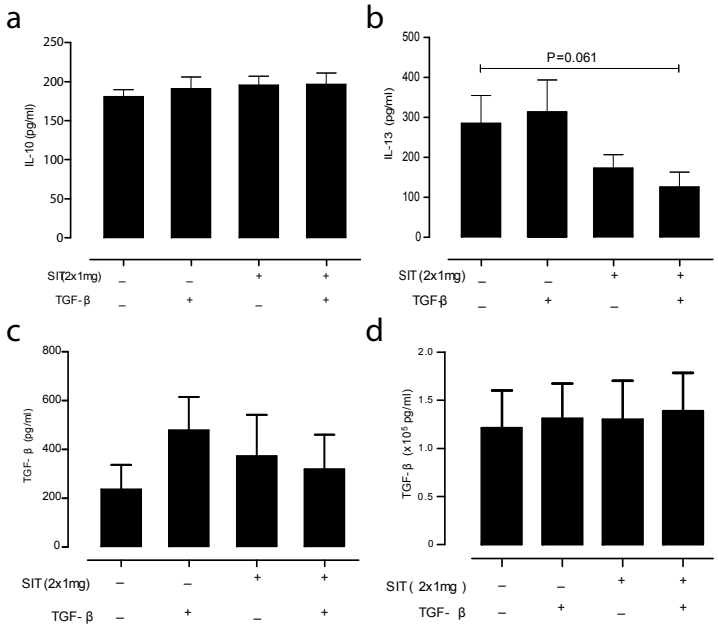
Figure 4 - Percentage of FOXP3+ T cells within CD4+ T cell population in mediastinal lymph nodes. TGF-β or saline was co-administered with sham or suboptimal SIT treatment in OVA sensitized mice. Thereafter mice were challenged with OVA-aerosols and 24 hours after the last OVA inhalation challenges, mediastinal lymph nodes were isolated. CD4+FOXP3+ T cells were analyzed with flowcytometry. Values are expressed as mean ± SEM of 6-7 mice per group. *$P < 0.05$.

In contrast, at higher doses of methacholine, TGF-β co-administration abrogated the suppression of Penh by suboptimal SIT performed with the reduced allergen dose. As in the previous experiment, we conclude that TGF-β co-administration has no effect on suboptimal SIT-induced suppression of the Penh.

In the previous experiment, we observed a remarkably enhanced suppression of eosinophilic inflammation and Th2 cytokines in BALF, when TGF-β was co-administered with suboptimal SIT at the high allergen dose (2 x 1mg). In the second experiment, the same suboptimal SIT (2 x 1mg) treatment did effectively suppress the eosinophilic airway inflammation: the total number of cells in BALF was reduced by 54.8% ±4.28 and the number of eosinophils by 69.8% ± 4.84 ($P < 0.01$ and $P < 0.001$ respectively) in comparison to sham-treated control mice (Fig 3b and 3c). In contrast, suboptimal SIT using the lower dose of allergen (2 x 0.1mg) did not achieve a significant level of suppression of airway inflammation. Interestingly, concurrent administration of TGF-β with suboptimal SIT at the lower allergen dose (2 x 0.1mg) did significantly suppress the total number of cells as well as the number of eosinophils in BALF in comparison with sham treated controls (41.72% ± 5.3 $P < 0.01$ and 57.26% ± 4.15, $P < 0.01$ respectively) as well as in comparison with suboptimal SIT alone (26.54% ± 7.34, $P < 0.05$ and 34.94% ± 5.36, $P < 0.05$ respectively), indicating that TGF-β co-administration allowed SIT efficacy towards eosinophilic airway inflammation at a 10-fold lower allergen dose. Along with the suppression of eosinophilic inflammation, suboptimal SIT at high allergen dose (2 x 1mg) largely reduced the IL-5 levels by 59.42% ± 7.74 and IL-13 by 59.8% ± 4.28 in BALF compared with sham-treated control ($P < 0.01$, Fig 3d and 3e). In contrast, suboptimal SIT at the low allergen dose (2 x 0.1mg) failed to substantially reduce the levels of either IL-5 or IL-13, whereas co-administration of TGF-β with the low-allergen dose suboptimal SIT significantly suppressed IL-5 in BALF (50.48% ± 6.78, $P < 0.05$ in comparison with sham control). Although the levels of IL-5 and IL-13 in suboptimal SIT/TGF-β treated group seems to be lower than in suboptimal alone treated group, these differences are not statistically significant.

As it shown in figure 3f, a strong induction of OVA-specific IgE was seen after suboptimal SIT treatment at the high allergen dose (2 x 1mg) ($P < 0.01$, Fig 3f), but not after suboptimal SIT with lower dose (2 x 0.1mg), when compared to sham-treated controls. Suboptimal SIT at high allergen dose (2 x 1mg) also suppressed the challenge-induced OVA-specific IgE response in serum compared with sham control (48.46% ± 6.18, $P < 0.05$), which

was not observed with suboptimal SIT treatment at the lower allergen dose. However, co-administration of TGF-β with the low allergen-dose suboptimal SIT (2 x 0.1mg) significantly suppressed specific IgE after OVA challenges, to a level comparable to that observed after suboptimal SIT treatment with the full dose of 1mg OVA (56.17% ± 10.03, $P < 0.05$ in comparison with sham-treated control), even though the induction of an OVA-specific IgE by the SIT treatment - as observed after 1 mg suboptimal SIT - was not observed by the combination of suboptimal SIT and TGF-β. Although the level of IgE was



OVA-specific IgG1 (x 10_3 EU/ml, Mean ± SEM)		
	Pre-challenge	Post-challenge
sham/saline	7.371 ± 0.7	12.7 ± 0.49 *
sham/TGF-β	8.665 ± 1.02	11.803 ± 0.34
SIT(2x1mg)/saline	10.965 ± 0.51	11.755 ± 0.69 #
SIT(2x1mg)/TGF-β	11.045 ± 0.62	10.945 ± 0.36 #

Figure 5 - Effects of co-administration of TGF-β with suboptimal SIT (2 x 1mg). OVA-sensitized and sham or suboptimal (2 x 1mg) treated mice were injected with rhTGF-β or saline and challenged with OVA aerosols as depicted in Fig 1 (EXP A). (a) IL-10, (b) IL-13 and (c) TGF-β in the BALF. (d) level of TGF-β in post challenge serum. (e) OVA-specific IgG1 in serum. Values are expressed as mean ± SEM of 6-7 mice per group. *$P < 0.01$ compared to pre-challenge value of sham-treated mice and #$P < 0.05$ compared to the pre-challenge value of sham-treated mice.

much lower when TGF-β was co-administered with suboptimal SIT, it did not reach the level of significance when compared with suboptimal SIT at the low-dose allergen (2 x 0.1mg) alone.

Taken together, these data indicate that co-administration of TGF-β enhanced suppression of Th2 cytokines, eosinophilic airway inflammation and serum OVA-IgE levels by suboptimal SIT at a 10-fold lower allergen dose, at the point where this treatment by itself was no longer efficacious.

$CD4^+$ $FOXP3^+$ Tregs in mediastinal lymph nodes

To study the mechanisms underlying these activities of co-administration of exogenous TGF-β, we analyzed the number of $FOXP3^+$ Tregs in MLN by flowcytometry. As shown in Fig 4, suboptimal SIT at high-dose allergen significantly increased the percentage of $CD4^+$ $FoxP3^+$ iTregs compared to sham treated controls ($P < 0.05$), whilst suboptimal SIT using the low dose of allergen failed to induce enhanced levels of CD4+ $FoxP3^+$ T cells in MLN. However, co-administration of TGF-β induced a trend ($P < 0.1$) towards increased numbers of iTregs, both in sham and in suboptimal SIT treated mice (Fig 4). These data indicate that co-administration of TGF-β induced outgrowth of a $FOXP3^+$ T cell subset independently from the SIT-induced effects.

Neutralizing IL-6 does not further enhance suppression of allergic manifestations by suboptimal SIT

Since the effects of TGF-β on $FOXP3^+$iTreg cell induction (Figure 4) might be thwarted by the presence of pro-inflammatory cytokines such as IL-6 at the time of allergen presentation during SIT [23], we also assessed whether neutralizing IL-6 at the time of allergen injections might further substantiate the TGF-β -induced enhancement of suppression of allergic manifestations by suboptimal SIT. To this end, we neutralized the IL-6 signaling *in vivo* by injecting the mice with a neutralizing antibody against IL-6R [24], with or with out TGF-β co-administration during suboptimal SIT treatment at the low dose of allergen.

As shown in figure 6, blocking IL-6R alone or in combination with TGF-β treatment, did not have any effect on the suppression of the Penh dose response curve to methacholine after OVA challenges. Moreover, neutralizing the IL-6R by antibodies did not enhance the suppression of BALF eosinophils, or levels of IL-5 and IL-13 in BALF by suboptimal SIT at low-dose allergen, and in fact did even abolished the augmentation observed by TGF-β co-administration (Fig 6).

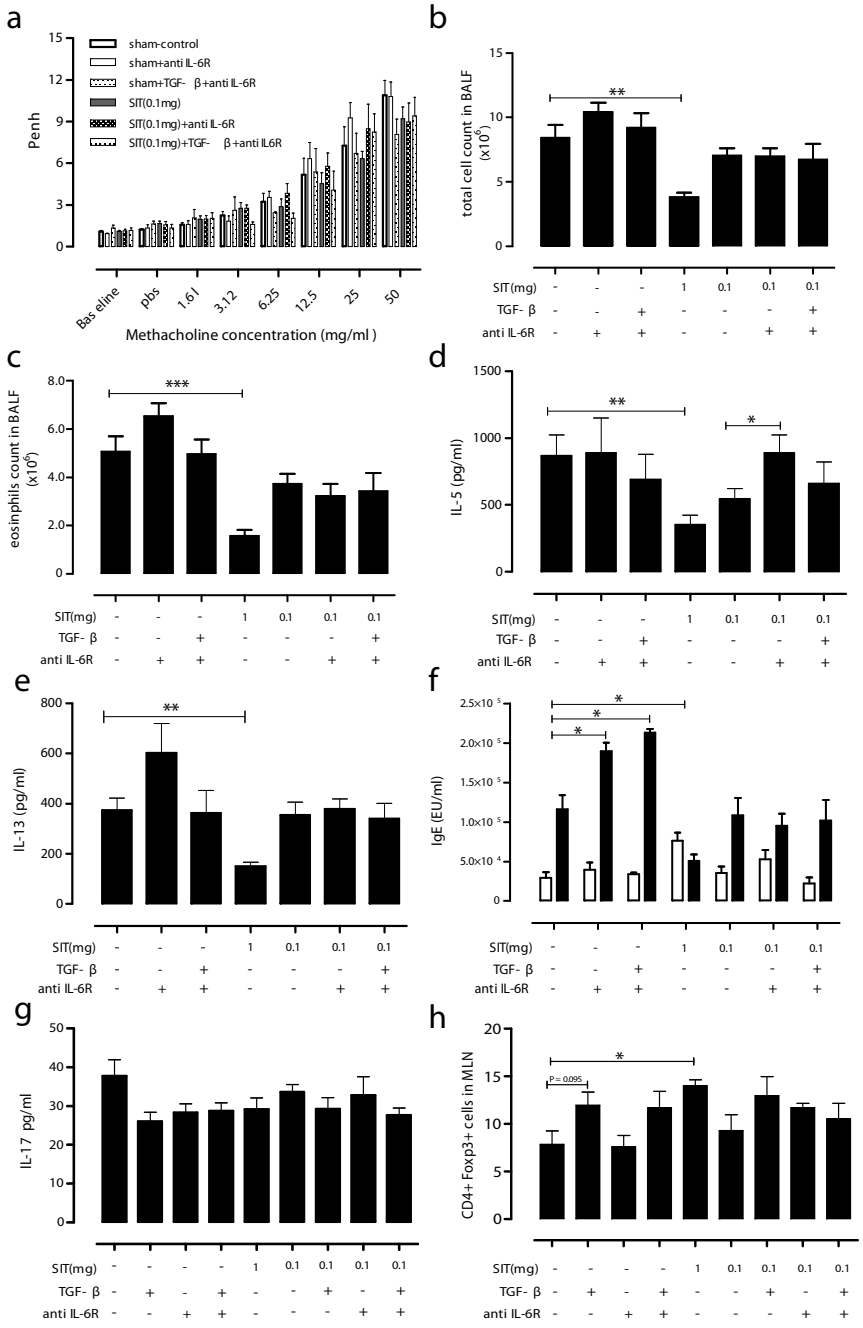


Figure6 - Neutralization of IL-6R did not enhance the suppressive effects of co-administration of TGF-β with suboptimal SIT. OVA-sensitized mice received sham or suboptimal SIT treatment (high dose (2 x 1 mg) or low dose (2 x 0.1 mg)) with or with out TGF-β and anti IL-6R. (a) AHR, (b) total cell count and (c) number of eosinophils in the BALF. Level of (d) IL-5 and (e) IL-13 in the BALF. (f) Pre-challenge (white bars) and post-challenge (black bars) OVA-specific) IgE in serum. (g) Level of IL-17 in the BALF. (h) Percentage of CD4+FOXP3+ T cells in mediastinal lymph nodes. Values are expressed as mean ± SEM of 6-7 mice per group. *P < 0.05, **P < 0.01 and ***P < 0.001

Remarkably, blocking IL-6R significantly increased the OVA inhalation challenge-induced IgE in serum ($P < 0.05$ compared with sham-control) when administered alone or combined with TGF-β in sham treated mice (Fig 6f). However, blocking IL-6 signaling during co-administration of TGF-β with suboptimal SIT reversed the TGF-β mediated decline of serum IgE after challenges. Finally, neutralization of IL-6 signaling during administration of TGF-β did not alter the induction of iTregs by suboptimal SIT or by TGF-β (supplementary data, Fig 6h). Finally, we assessed whether Th17 cell activity was influenced by TGF-β treatment and neutralization of IL-6 signaling, by measuring IL-17 in the BALF. To our surprise level of IL-17 was not different between any of the treatment groups (supplementary data, Fig 6f).

Discussion

In this study, we show that administration of exogenous TGF-β during SIT treatments augments suboptimal SIT-induced suppression of airway eosinophilia as well as Th2 cytokines in the BALF when suboptimal SIT (at high doses of allergen) is given. Furthermore, we observe an enhanced suppression of airway eosinophilia, Th2 cytokines in BALF and OVA-specific IgE when a 10fold lower dose of allergen is applied. We demonstrate that the beneficial effect of TGF-β in this model is associated with a trend of an enhanced number of $FOXP3^+$ iTregs in the mediastinal lymph nodes and significantly increased levels of OVA-specific IgA in serum. Finally, neutralization of IL-6 signaling did not increase the induction of iTregs by TGF-β and had no effect on improving the TGF-β enhanced suppressive function of SIT, indicating that concurrent administration of TGF-β with blocking IL-6 signaling aiming at induction of more iTregs at the expenses of Th17 cells is not applicable here in our model. Taken together, these data indicate that exogenous TGF-β, used as an adjuvant at the time of allergen injection, is able to enhance suppression of allergic manifestations, allowing the use of lower doses or reduced frequency of immunotherapy injections to achieve these effects.

Transforming Growth Factor-β (TGF-β) is an important immunomodulatory cytokine with a potential capacity to augment the effects of SIT [25]. Here we show that co-administration of exogenous TGF-β with suboptimal SIT strongly augmented the beneficial effects of SIT. TGF-β can contribute to SIT-induced tolerance induction by at least four known mechanisms. The first two mechanisms are facilitating the induction of TGF-β producing Th3 cells and $CD4^+FOXP3^+$ iTregs [26]. We observed that sub-optimal SIT increases the

levels of TGF-β in the serum, so TGF-β producing Th3 cells may contribute to the induction of allergen tolerance by SIT alone. However we have no evidence that administration of exogenous TGF-β further potentiates this mechanism in our model. Secondly, we observed a trend towards an increase in the number of $CD4^+FOXP3^+$ iTregs in MLN after TGF-β treatment in both sham and SIT treated mice, which indicates that administration of TGF-β might promote generation of iTreg cells *in vivo* in our experimental mouse model. Nevertheless, in the absence of the relevant antigen, these $FOXP3^+$ iTregs were not able to promote any specific suppression since we did not observe any dampening of asthma symptoms on sham/TGF-β treated mice despite the increased number of $FOXP3^+$ iTregs. An explanation for this observation might lie in the absence of the cognate antigen in the Sham/TGF-β treated group. The TGF-β might induce $FOXP3^+$ iTreg differentiation, but these will not be specific for OVA in the absence of the allergen at the time of their induction.

The third mechanism of contribution of TGF-β to SIT-induced allergen tolerance is by instructing B cells to produce antigen-specific IgA, which can contribute to mucosal protection upon allergen challenge [27;28]. Increased level of TGF-β after different SIT treatments have been reported in several clinical studies [6;29]. Moreover it has been demonstrated that SIT treatment is associated with elevated IgA responses in serum which positively correlates with the enhanced mucosal expression and serum production of TGF-β after successful SIT treatments [6;30]. In line with these findings, we also observed an enhanced TGF-β response in serum, shortly after SIT treatment in our study. Moreover we find that the enhancement of SIT mediated suppression of allergic manifestations by TGF-β is accompanied by increased production of IgA in serum, indicating that IgA might contribute to the suppression of allergic manifestations. Nevertheless, we have previously shown that SIT remains effective in polymeric IgR and in B cell deficient mice, precluding a critical role for IgA in the tolerance induction by SIT [13]. However, these data do not exclude the possibility that allergen-specific IgA may contribute to the SIT-enhancing effects of TGF-β as observed in the current study.

The fourth mechanism of suppressive function of TGF-β is its direct effect on dendritic cells (DSs). TGF-β can regulate the maturation and differentiation of DCs [31]. For example in the study by Mou et al [32], it has been shown that TGF-β treated DCs are resistant to maturation and fail to up regulate the co-stimulatory molecules upon stimulation with LPS. Additionally, it has been shown that autocrine TGF-β is essential in maintaining the tolerogenic function of indoleamine 2, 3-dioxygenase (IDO)-competent $CD8^+$ DCs and

converting immunogenic $CD8^-$DCs to tolerogenic cells by induction of IDO[33]. Intriguingly we have previously shown that IDO signaling contributes to the tolerance induction by SIT in our mouse model of asthma [18]. Although in the current study, we haven't investigated the possible effects of TGF-β on dendritic cells, but the potential role of TGF-β on promoting tolerogenic DCs during SIT should not be disregarded.

In our study, despite the presence of a clear allergen/antigen-induced AHR to methacholine, we did not see an effect on AHR by TGF-β co-administration. Within contrast to our findings, a regulatory function for TGF-β in directly suppressing AHR has been described in literature [34]. The major difference with our study is the time of administration of TGF-β. In our experiment, TGF-β is administered at the time of SIT injections and is no longer present at the time of OVA inhalation challenges. Although, suboptimal SIT significantly increases the levels of TGF-β in the serum, there are no additional effects for administration of exogenous TGF-β in this regards. Therefore, there may be a role for TGF-β producing T cells in suboptimal SIT alone but this mechanism is not potentiated by administration of exogenous TGF-β. Here, we do not find evidence for an effect of subcutaneously administered TGF-β on TGF-β levels in the BALF, indicating that the peripheral administration of TGF-β did not alter local TGF-β levels in the lungs, and likely did not induce an effect on AHR.

It has been shown that IL-6 can prevent the suppressive function of $CD4^+CD25^+$ Tregs and induce expansion of Th2 cells thereby inhibiting the induction of peripheral tolerance [10]. Furthermore it has been demonstrated that presence of IL-6 inhibits the induction of Treg cells by TGF-β and drives the induction of Th17 cells [13]. As the number of $FOXP3^+$ iTregs was increased but did not reach the level of statistical significance, by administration of TGF-β, we also tested whether blocking the IL-6R concurrent with co-administration of TGF-β with SIT would induce a more pronounced outgrowth of $FOXP3^+$ iTregs. However, no improvement was achieved on the induction of $FOXP3^+$ iTregs by neutralizing the IL-6 signaling. Furthermore the level of the prototypic Th17 cytokine IL17 was not changed in the BALF with or without blocking IL-6R during administration of TGF-β by SIT. These data indicate that deviation of $FOXP3^+$iTregs into Th17 cells does not appear to hamper TGF-β induced potentiation of SIT-induced suppressive responses.

In summary, our findings clearly demonstrate a potential suppressive function for TGF-β on the beneficial effects of SIT in our experimental mouse model. Co-administration of TGF-β with suboptimal SIT strongly suppressed the eosinophilic inflammation together with Th2 cytokines in the BALF as

well as serum IgE responses. These suppressive effects were accompanied with an induction of IgA responses in serum and a rise in the number of $FOXP3^+$ iTregs in MLN. Overall our data, implicate an immunosuppressive function for TGF-β in tolerance induction by SIT in an experimental mouse model of asthma. More studies are required to exactly clarify the mechanisms underlying its suppressive function to achieve optimal effects and be considered as an adjuvant for future immunotherapy strategies in clinic.

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CHAPTER

9

Absence of maternal transfer of protection by allergen immunotherapy in a mouse model of allergic asthma

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Abstract

Background: Experiments in rodents suggest that prenatal exposure to allergens affect the susceptibility of the developing embryo for allergic disorders later during life, in part through active immunological protection, including materno-fetal transfer of allergens and neutralizing antibodies by allergen sensitized mothers. Interestingly, allergen provocation of sensitized mice during lactation was recently shown to confer protection to allergic sensitization of the offspring by transfer of allergen/IgG immune-complexes (IC) through the milk. We hypothesize that allergen-specific immunotherapy (SIT), a treatment known to strongly increase the allergen-specific IgG levels in allergen sensitized individuals, may induce materno-fetal transfer of allergen tolerance.

Objective: In this study we aimed to address whether OVA-SIT treatment of allergen sensitized mice prior to pregnancy allows the transfer of allergen tolerance to the offspring by either pre-or post-natal maternal transfer.

Methods: OVA sensitized female mice were sham or SIT-treated before mating. Offspring was nursed for 3 weeks either by their own mothers or by naïve foster mothers. In a parallel group naive pups were nursed by sham or SIT-treated mothers to exclusively determine post-natal transfer of protection. At 7 weeks of age, offspring were OVA/Alum sensitized followed by OVA-inhalation challenges. Eosinophilic inflammation of airways and serum OVA-specific IgE responses were assessed.

Results: Despite transfer of OVA-specific IgG and IC via breast milk from sensitized and SIT-treated mothers to the offspring, SIT treatment failed to convey tolerance to the offspring and protect them from allergic asthma. In contrast, offspring from sensitized and sham treated mothers was protected from allergic asthma and this protection critically required involvement of both pre-and post-natal transfer of tolerance by sensitized and sham-treated mothers to the progeny.

Conclusion: Our data using an experimental mouse model of OVA-SIT show that SIT treatment of the mother prior to the pregnancy did not protect the offspring from Th2 mediated airway inflammation. However, SIT-treated mothers did protect the offspring against allergic sensitization by placental transfer which was only apparent if nursed by naïve foster mothers. Moreover prenatal sensitization of mothers was sufficient to induce protection against allergic asthma in offspring, which required both pre and postnatal maternal transfers.

Introduction

Allergic asthma is a Th2 mediated inflammatory disease of the airways and results from an inappropriate immune response to innocuous airborne environmental allergens [1-4]. Genetic studies have reported that the susceptibility to allergic asthma has a strong genetic component, especially for childhood asthma [5]. The prevalence of allergic asthma is rapidly increasing in industrialized countries, which is most likely attributed to changes in the environment or life style. These environmental and life style factors, especially those present during early life, are thought to contribute to the increased prevalence of allergic asthma by inducing epigenetic changes, thereby influencing the asthma susceptibility [6]. For example it has been shown that prenatal exposure to a farming environment conveys protection against respiratory allergies, in part through exposure to different species of cattle and the consumption of raw, unpasteurized milk [7;8]. Epidemiological studies assessing the protective effect of other early life exposures, such as breastfeeding during the first year of life, have yielded conflicting data [9-11]. Mechanistic studies using mouse models, however, have clearly indicated that allergen-transfer through the breast milk of mothers exposed to an airborne allergen during lactation rendered a protective effect in the offspring against subsequent induction of allergen-specific IgE and manifestations of experimental allergic asthma [12-14]. Interestingly, the involvement of allergen-specific immunoglobulins was only observed in case of allergen sensitized mothers exposed to the airborne allergen during lactation [13]. The symptoms of allergic asthma can be efficiently, but transiently, repressed by a combination of bronchodilators and corticosteroids in most patients [15]. In contrast to these pharmacological treatments for allergic disorders, which provide only temporary relief of symptoms, allergen-specific immunotherapy (SIT) is the only long-lasting treatment available for allergic disorders, that alters the immunological response to allergen exposure and prevents the occurrence of new sensitizations [16;17]. After the first successful application of SIT by Noon et al in 1911 [18], SIT has become an efficient therapy for many allergic disorders including allergic rhinitis and insect venom allergy [19]. Successful SIT treatment is associated with a strong increase in allergen-specific IgG in serum, the induction of IL-10 expression in allergen-stimulated PBMCs and the differentiation of allergen-specific regulatory T cells [20-22]. Since the protection to manifestations of allergic asthma rendered by allergen exposure of lactating mothers was most pronounced in case of allergen sensitized mothers and was associated with the presence of allergen-IgG immune complexes in the breast milk, we hypothesized that SIT treatment, associated with strongly

increased allergen-specific IgG responses, would likewise allow the transfer of allergen-specific tolerance upon lactation by SIT-treated mothers.

To test this hypothesis, we employed our previously developed mouse model of OVA-SIT in which SIT induces a long-lasting suppression of different aspects of OVA induced allergic asthma, including airway eosinophilia, Th2 cytokines, airway hyperresponsiveness (AHR) and serum immunoglobulins. In parallel to clinical observations, SIT injections in our mouse model are accompanied by the induction of strong allergen-specific IgG and IgA responses in serum [23]. Using SIT-treated or naïve mothers, we employed a cross-over design (allowing SIT-treated mothers to feed their own pups as well as pups from naïve mothers, and *vice versa*) to assess whether allergen-specific immunotherapy might in fact induce maternal transfer of allergen-IgG immune complexes and protection from allergic asthma to the progeny.

Material and methods

Mice

BALB/c mice were purchased from The Centre d'Elevage Janvier (France) and housed under specific pathogen-free conditions.

Experimental OVA-SIT protocol

The OVA-SIT treatment has been performed as previously described [24] with elimination of the OVA-inhalation challenges (Fig 1a). Briefly, female mice were sensitized with two i.p. injections of 10µg OVA (endotoxin-free, < 0.5 U/ml; Seikagaku Kogyo, Tokyo, Japan) adsorbed onto 2.25 mg alum (ImjectAlum, Pierce, Rockford, IL) in 100µl pyrogen-free saline (B.Braun, Melsungen, Germany) on days 0 and 7. Two weeks after the last OVA- sensitization, mice received 3 s.c. injections of either 1 mg OVA in 200 µl saline (pyrogen-free) as SIT or 200 µl saline as sham control treatment every other day. One week after the SIT/sham treatment, mice were mated with naïve males. In parallel, naive females were also mated with naïve males and used as a control group. After birth, female pups from SIT/sham treated mothers were breastfed by either, their own or by naïve foster mothers and vice versa. To evaluate the sensitivity to OVA-induced allergic asthma, 6 weeks old pups were i.p. sensitized on days 0 and 7 which followed by 3 OVA-inhalation challenges on days 21, 24 and 27 for 20 minutes. Challenges were conducted using an ultrasonic nebulizer (Ultramed, Medicalia) connected to a 13,000-cm^3 box that served as the deposition chamber for the mice. 24 hours after the last inhalation challenges mice were sacrificed and BALF and serum were obtained to further analyze the asthmatic phenotype (Fig 1a).

Determination of level of immunoglobulins (Igs) in serum and milk

Serum levels of OVA-specific IgE and IgG1 were measured by ELISA as described previously [25]. In brief, 96-well microplates (Greiner Bio-One, Hannover, Germany) were coated for 16h with 2 µg/ml anti-mouse IgE antibody (BD bioscience, Breda, the Netherlands), washed using 0.005% Tween20 (Sigma-Aldrich) in PBS, flowed by a blocking step using 3% bovine serum albumin (Sigma-Aldrich) in PBS for 2 h. Serial dilutions of reference serum as well as samples were added and incubated for 1.5h at room temperature (RT). Then, plates were washed again and digoxigenin-labeled (Roche Diagnostics, Almere, the Netherlands) OVA was added and incubated for 1h at RT. Anti-digoxigenin-POD (Roche Diagnostics) was added after washing the plate and incubated for 1h at RT. O-Phenylenediamine (Thermo-scientific, IL, USA) was used to detect PO activity by measuring optical density at 490 nm using ELx808™ microplate reader (BioTek, Friedrichshall, Germany). Concentrations were calculated according to the standard curve drawn based on serial dilutions of reference serum and results are expressed as experimental units (EU)/ml.

For IgG1, Maxisorp plates (Nunc) were coated with OVA saturated with 10% FCS in PBS, and incubated with serial dilution of sera followed by biotinylated anti-IgG1 antibody (BD553-441, BD, France).

Immune complexes content in milk and serum

Breast milk was collected from the stomach of 2-week-old pups and diluted in 1 volume of PBS. Samples were spun down at 10,000 g for 10 min and the supernatant was collected and stored at − 20° C until analysis. Milk and serum were analyzed for the presence of OVA-IgG1 immune complexes by ELISA. Maxisorp plates were coated with a rabbit anti-OVA polyclonal antibody (AB1221, Abcam, U.K), saturated with 3% FCS in PBS-Tween 0.05%, and incubated with serial dilutions of milk or serum followed by biotin-conjugated anti-IgG1 antibody (BD553-441, BD, France)

Determining the cellular composition of the BAL fluid

After sacrificing the mice, lungs were washed trough a tracheal cannula for three times with 1 ml of PBS. For differential BAL cell counts, cells were stained with mAbs to CCR3 (R&DSystems), Gr-1, CD3 and CD19 (Becton Dickinson) and analyzed by FACS using a FACSCalibur flow cytometer and CellQuest software. Different cells were defined with expression of CD markers as defined below; eosinophils as $CCR3^{+}CD3^{-}CD19^{-}$, neutrophils as

Gr-1^{hi}CD3^{-}CD19^{-}, lymphocytes as CD3^{+}CD19^{+} and alveolar macrophages as large autofluorescent cells.

Statistical analysis

All data are expressed as mean ± SEM. Statistical significance was assessed using a two-tailed P value calculated with the Mann-Whitney *U test* for nonparametric data and $P < 0.05$ was considered significant.

Results

We hypothesized that SIT induced allergen-specific IgG responses allowed the transfer of tolerance to OVA-induced airway eosinophilia through the formation of allergen-IgG immune complexes in the breast milk. To test our hypothesis, female mice were either sham or SIT-treated, and mated to induce pregnancy. After birth, the lactating SIT- or sham-treated mothers nursed their progeny for three weeks as depicted in Fig 1a.

OVA-specific IgG1 in mother's serum and milk

We have previously shown that SIT injections induce strong antigen-specific IgG1 responses in serum [25]. Though these increased IgG levels are not critically required to the suppression of late-phase allergic responses by SIT, they might contribute to maternal transfer of allergen-IgG immune complexes through the breast milk, and thereby induce allergen-specific tolerance in the offspring. To confirm the efficient induction of OVA-specific IgG responses in the SIT-treated female mice, OVA-specific IgG1 levels were measured in serum as well as in breast milk of the lactating mothers. As shown in Fig 1b, OVA-sensitized and SIT-treated mothers have significantly increased OVA-specific IgG1 levels in serum in comparison to OVA-sensitized and sham treated mothers (1.4 ± 1 mg/ml versus 0.005 ± 0.002 mg/ml respectively, $P < 0.05$). Similarly, OVA-specific IgG1 levels in milk were also strongly increased in OVA-sensitized and SIT–treated mice, compared to OVA-sensitized and sham-treated controls (Fig 1c). In line herewith, a strongly increased level of allergen-IgG immune-complexes (IC) was present in milk from OVA-sensitized and SIT-treated mothers when compared to OVA-sensitized and sham-treated treated or naïve mothers (Fig 1d). Overall, these data indicate that OVA-specific IgG1 is efficiently induced by SIT treatment and was transferred as allergen-IgG immune-complexes to the milk.

Maternal transfer of tolerance to OVA-induced airway eosinophilia in offspring

Since maternal transfer of allergen-IgG IC via the breast milk was previously shown to be associated with a strongly increased protection of the pups to manifestations of experimental allergic asthma [13], we OVA-sensitized and challenged the pups born to OVA-sensitized and OVA-SIT treated mothers, as well as the pups born to OVA-sensitized and sham-treated or naïve control mothers (Fig 1a). As expected, we observed a strong influx of inflammatory cells, especially eosinophils, into the airways in the control group which was born to and nursed by naïve mothers (Fig 2a, b). Interestingly, and in contrast to our hypothesis, offspring from OVA-sensitized and sham treated mothers

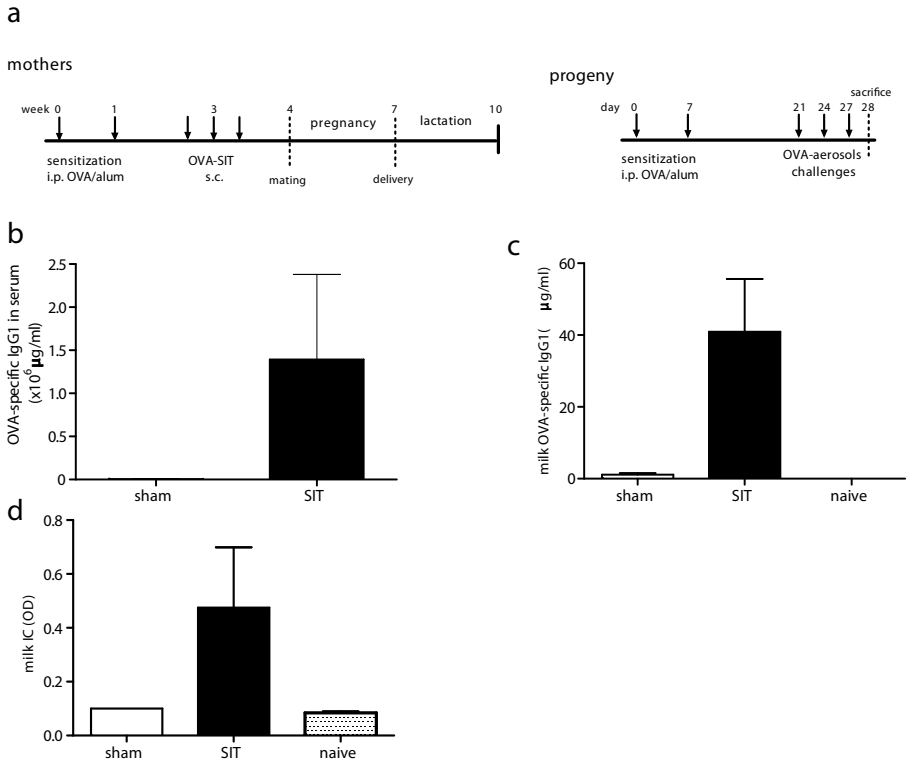

Figure 1 - OVA-specific IgG levels in mother's sera and milk. (a) Schematic overview of experimental protocol. Sensitized mothers were sham or SIT-treated before mating. Serum samples were obtained from mothers on day 21 and IgG1 levels were measured by ELISA. (b) OVA-IgG1 in serum, (c) OVA-IgG1 in milk. Breast milk was collected from pup's stomach on day 14 and analyzed for presence of IgG1 and ICs. (d) IgG-IC in milk. Data are expressed as mean ± SEM.

had a significantly reduced number of eosinophils in BAL compared to mice born to naïve mothers ($P < 0.05$). In addition, mice born to sensitized and OVA-SIT treated mothers displayed a similar number of eosinophils compared to mice born to naïve mothers (Figure 2B). These data are especially intriguing given the presence of OVA-IgG immune complexes only in the milk from the OVA-SIT treated mothers (Figure 1).

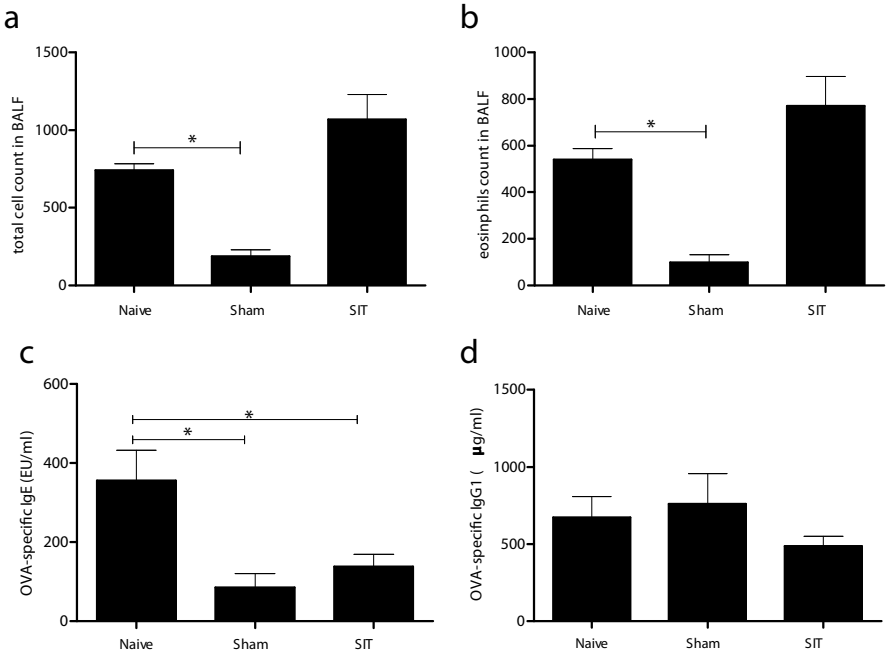

Figure 2 - Cellular composition of the BAL fluid in the offspring. BAL was collected from offspring 24 hours after the last OVA inhalation challenge analyzed by FACS. (a) Total number of cells and (b) number of eosinophils in the BAL fluid. Data are expressed as Mean ± SEM and *$P < 0.05$.

In addition to eosinophilic airway inflammation we measured serum levels of OVA-specific IgE and IgG1 after the inhalation challenges. Here, the mice born to OVA sensitized mothers all had a reduced level of OVA-specific IgE in serum, irrespective of OVA-SIT or sham treatment, although the effect was slightly more pronounced in the sham-treated group (Fig 2c). Levels of OVA-specific IgG1 did not show differences between the three groups (Fig 2d).

The effect of placental versus breast milk maternal transfer

Since we did not observe an association of allergen-IgG immune complexes in the breast milk with protection against eosinophilic airway inflammation in the pups, but an effect of sensitization of the mothers could be seen for allergen-specific IgE responses, we aimed to ask whether the suppression of IgE was transferred through the breast milk. To this end, we also performed an experiment in which mice born to sensitized and sham- or OVA-SIT treated mothers were weaned by naïve foster mothers, and *vice versa*. Remarkably, the suppression of eosinophilic inflammation observed in the mice born to sham-treated sensitized mothers was not observed in any of the groups of this cross-over experiment (Fig 3a, b). OVA-specific IgE levels in serum, however, were strongly reduced (24.55 ± 12.02 EU/ml) in mice born to SIT-treated mothers when they were weaned by naïve mothers (Fig 3c), and

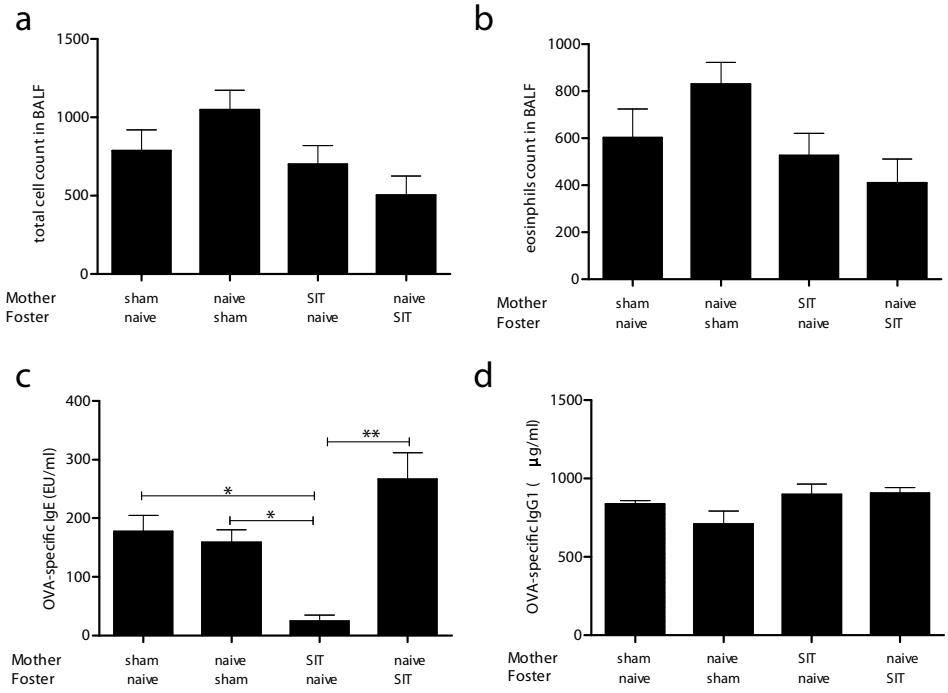

Figure 3 - OVA-specific Ig responses in serum from offspring. Progeny was OVA/alum sensitized and challenged with OVA aerosols. Serum was collected 24 hours after the last OVA inhalation challenge and (a) OVA-IgE and (b) OVA-IgG1 were measured by ELISA. Data are expressed as Mean ± SEM and *P < 0.05, **P < 0.01.

this effect was much stronger than in the mice that were weaned by their own SIT-treated mothers (138.08 ± 34.96 EU/ml). For mice born to sham-treated mothers, weaning by naïve mothers did not enhance suppression of IgE responses any further. Remarkably, mice born to naïve mothers that were weaned by sensitized mothers only displayed a reduced allergen-specific IgE response (by 59.88%, P = 0.095) in case they were weaned by sham, but not when weaned by OVA-SIT treated mothers. Again, OVA-specific IgG1 levels did not show differences between the different groups (Fig 3d).

Discussion

In the present study we tested whether OVA-SIT treatment of allergen sensitized female mice induced an enhanced formation of allergen-immune complexes in the breast milk and contributes to protection of the pups from manifestations of allergic asthma.

We show that as expected, SIT treatment induced increased level of OVA-specific IgG1 in serum. Interestingly, we could confirm that SIT treatment also resulted in the presence of high levels of OVA-specific IgG1 as well as IgG-ICs in breast milk from sensitized and SIT treated mothers. The levels of OVA-specific IgG1 in breast milk, however, only reached about 30% of those observed in OVA-sensitized mice that were exposed to OVA aerosols during lactation [13]. Surprisingly, the presence of OVA-specific IgG1 and ICs in breast milk from SIT-treated mothers failed to protect the offspring of these mothers against airway eosinophilia and only partially protected against OVA-specific IgE responses in serum upon OVA-sensitization and challenge of the offspring. Intriguingly, we did observe a protection of the progeny from sensitized and sham-control treated mothers to airway eosinophilia upon OVA sensitization and challenge. In the original study of Mosconi and colleagues using OVA-sensitized mice, the control group lacking the OVA inhalation challenges during lactations also did display a slightly reduced level of airway eosinophilia and allergen-specific IgE, but these differences were not statistically significant, and far less pronounced than the suppression achieved in pups from OVA-sensitized mice challenged with OVA during lactation[13]. Taken together, these data seem to indicate that allergic sensitization of female mice prior to pregnancy might render a low level of protection against airway eosinophilia in the progeny, which is not associated with the presence of allergen-IgG immune complexes in the breast milk, and which is abrogated by SIT treatment of the sensitized mothers. One reason for this abrogation could be an increase in allergen-IgG2a ICs in the breast milk of SIT-treated mothers. We have previously shown that SIT treatment induces high levels of allergen-

specific IgG2a in the serum besides allergen-specific IgG1 [25]. Although not measured in the present study, increased levels of IgG2a in the serum of SIT-treated mothers could be reflected in the breast milk and thereby abrogate the beneficial effects of allergen-IgG1 ICs. In line with this hypothesis, it has been shown that maternal transfer of antigen-IgG1 ICs conveys protection to antigen immunization in progeny through FCγRIIb [26], while antigen-IgE and antigen-IgG2a ICs potentiate T cell responses to the cognate antigen [27]. More studies are needed to unravel the precise contribution of these different ICs in the SIT-induced abrogation of maternal tolerance by allergically sensitized mothers.

Allergen provocation during lactation results in stronger protection of offspring from allergic asthma which is mediated by OVA-IgG IC [13]. To assess the contribution of the OVA-IgG immune complexes in the breast milk, we also analyzed the asthma manifestations in mice born to sensitized and sham control or OVA-SIT treated mice but weaned by naïve mice, and *vice versa*. Here, we clearly show that the presence of allergen-IgG immune complexes in the milk, present in sensitized mother that were SIT-treated, did not confer protection to airway eosinophilia or allergen-specific IgE. Mice born to naïve mothers displayed in fact a higher level of IgE after weaning by SIT-treated mice compared to sham-treated mice, whilst airway eosinophilia was not affected at all by weaning by either sham or OVA-SIT treated mothers. In contrast, mice born to sensitized and OVA-SIT, but not sham-control, treated mothers and weaned by naïve mothers were protected against the induction of high OVA-specific IgE levels in.

Taken together, these findings seem to suggest that sensitization of mothers to the model allergen OVA before pregnancy was sufficient to induce a certain level of tolerance in the offspring and protect them from OVA-induced airway inflammation and allergen-specific IgE responses. However, OVA-SIT treatment of the sensitized mother prior to pregnancy abrogated the protection against airway eosinophilia, even though this was the sole condition that induced significant levels of OVA-immune complexes in the breast milk. In a previous study by Mosconi et al, the presence of allergen-immune complexes in the breast milk was associated with a strong suppression of the parameters of allergic inflammation in the pups in a TGF-β-independent manner [13], whereas the presence of free allergen in the breast milk induced a TGF-β-dependent repression of allergic inflammation in the pups [12]. The main difference between these studies and our experiments is the allergen challenges of the mother during lactation, which likely strongly increased the levels of allergen and allergen-ICs present in the breast milk. In addition,

the level of allergen-specific IgG1 in breast milk in our study was only one third of that of the previous study, which in the absence of allergen challenges during lactation might result in an even lower fraction of immune complexes compared to the previous study. In our studies, tolerance was induced prior to conception and no further allergen challenges of the mothers were performed during pregnancy or lactation. So we tend to interpret the data in our study to reflect the level of maternal transfer of allergen-specific tolerance in the absence of free allergen in the milk and a relatively low level of allergen-immune complexes.

It has been clearly shown that maternal IgG, mainly IgG1, has strong inhibitory effects on eliciting allergen-specific IgE responses in offspring[4;28;29]. Maternal IgG can be transferred to the offspring either via breastfeeding or via placenta by transfer via Fc receptor (FcRn) [30].

It has also been shown that oral tolerance induction in female mice during pregnancy can induce allergen-specific tolerance towards asthma manifestations in the pups, which was exclusively dependent on breastfeeding of the tolerized pups, and was associated with the presence of allergen-specific IgG in the breast milk [31]. Interestingly, in a study using a similar approach but inducing oral tolerance prior to conception, tolerance induction was also observed, but under these conditions breastfeeding was not critically required for the effects, although it remained sufficient in the absence of placental IgG transfer in FcRn-deficient mice [30]. The main difference between these two studies is the timing of tolerance induction relative to pregnancy and breastfeeding, where tolerance induction during pregnancy might possibly not induce sufficiently high levels of allergen-IgG immune-complexes to efficiently allow placental transfer of IgG-allergen ICs and induction of tolerance. For tolerance induction by breast feeding, transfer of free allergen might contribute in a TGF-β dependent fashion [12]. Since in our experiment, SIT treatment was performed preconceptionally, and no allergen challenges were given to the pregnant or lactating mothers, transfer of tolerance to the progeny was anticipated to also include placental transfer of IgG-allergen immune complexes. Indeed, mice born to SIT-treated mothers but fostered by naïve mice show a strongly reduced allergen-specific IgE upon sensitization and challenge, indicating that tolerance towards antigen-specific IgE has indeed been induced in these mice. Remarkably, eosinophilic airway inflammation was not suppressed under these conditions, in contrast to the other models of placental tolerance transfer [13;30]. The cause for this discrepancy between the individual parameters of our experimental allergic asthma model is unknown, although we and others have previously shown the IgE-independence of

the eosinophilic airway inflammation in the OVA-driven asthma model [25;32]. Intriguingly, according to our data on suppression of IgE responses, breast milk from SIT-treated mothers, despite containing high level of IgG and IC, actually did not confer suppression of IgE responses to the offspring, since pups born to naive mothers but nursed by SIT-treated fosters had unaltered IgE responses compared to pups born to and nursed by naive mothers. Hence, we conclude that either the level of allergen-IgG ICs in the breast milk of the SIT-treated mothers was not sufficiently high or the nature of allergen-IgG ICs is different in the milk of SIT-treated versus untreated allergically sensitized mothers. Therefore tolerance towards the induction of OVA-specific IgE responses in the pups is not achieved is SIT-treated mothers. In our study, we observe that offspring from OVA-sensitized and sham treated mothers were protected against eosinophilic airway inflammation and allergen-specific IgE, indicating an effect of prior sensitization of the mother. Interestingly, a trend towards a similar protective effect of prior allergen sensitization of the female mice was observed in the study by Mosconi et al, where airway inflammation, allergen-specific IgE and Th2 cytokines were all reduced in pups born to sensitized compared to non-sensitized mothers, irrespective of OVA challenges during lactation [13]. These effects, however, were far less pronounced than the effect of allergen challenge during lactation, and failed to reach statistical significance. Nevertheless, these and our data might indicate that IgG responses induced by allergen sensitization also have a, albeit limited, contribution to the transfer of tolerance to the pups. Our data on the cross-fostering experiment indicate that both placental and breast-milk transfer of IgG might contribute to these effects.

In summary, we have shown here that despite induction and transmission of IgG and IC via breast milk from sensitized and OVA-SIT treated mothers to the offspring, SIT induced tolerance was not conferred to the offspring through the breast milk, although placental transfer of tolerance to allergen-specific IgE, but not airway eosinophilia could be observed. Our findings also indicate that allergen sensitization prior to conception might also contribute to allergen tolerance in the pups, and tolerance induction in this manner strictly requires contribution of both pre and postnatal transfers. Future experiments involving allergen provocation of SIT-treated mothers during pregnancy or lactation as well as performing SIT injections during pregnancy or lactation might be helpful to elucidate the possibility of SIT induced maternal transfer of tolerance to the offspring.

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General summary and discussion

CHAPTER

10

Allergy

Allergy is caused by a specific Th2 immune response to innocuous environmental antigens and the subsequent production of allergen-specific immunoglobulin E (IgE) to these antigens [1;2]. The prevalence of allergic diseases has continuously increased over the last four decades, especially in industrialized countries [3]. The underlying causes of this increased prevalence of allergic diseases are not understood to date. Epidemiological data indicate that increased prevalence of allergic diseases has been associated with changes in environmental factors and lifestyle, such as: (i) reduced exposure to microbial infection as stated in the hygiene hypothesis [4], (ii) increased exposure to perennial allergens [5], (iii) decline in physical exercise and changes in diet [5] e.g. increased usage of iron intake by infants in affluent societies[6-8]. However, supportive data for mechanisms underlying each hypothesis is lacking to date.

The question whether increased iron intake by infants offers a causal explanation for the increased prevalence of allergic diseases has not been tested by experimental approaches to date. In our OVA -driven mouse model we asked whether increased iron intake can contribute to an increased severity of manifestations of experimental allergic asthma. To this end, we evaluated whether high levels of orally and parenterally administered iron altered the asthma parameters in our mouse model of OVA-driven allergic asthma (chapter 2). Surprisingly, we found that high levels of oral iron administration in fact reduce the allergen-induced airway eosinophilia, whereas parenteral iron administration suppresses allergen-induced airway hyperreactivity and reduces airway eosinophilia. We did not find evidence supporting our hypothesis that iron administration could augment the manifestation of allergic disease. We examined the effects of systemic as well as orally administered iron on allergic sensitization and the severity of asthma-like manifestations in our mouse model. However, the contribution of increased usage of iron supplements to the increased prevalence of allergic diseases in human populations, which more likely reflects an increase in allergic sensitization than an increased severity of symptoms, remains to be elucidated. Nevertheless, our data indicate that in a sensitized individual, increased iron intake might have beneficial effects on alleviating the symptoms of the disease.

The importance of a causal treatment for allergic diseases that leads to long-lasting relief of the disease symptoms by changing the immunological response to the allergen, which is the cause of the disease, is underscored by the lack of practical options for preventive measures despite the high prevalence of allergic diseases [9]. Allergen specific immunotherapy (SIT)

is currently the only causal treatment for allergic diseases rendering long-lasting relief of symptoms. There is requirement for improvement of SIT [10;11]. A detailed understanding of the mechanisms of action of SIT, a prerequisite for the rational improvement of the treatment, is lacking to date.

Allergen Specific Immunotherapy

Noon and colleagues were the first who documented the clinical efficacy of allergen specific immunotherapy (SIT) in alleviating the allergic symptoms in 1911 [12]. This treatment has been in clinical use, ever since, for different allergic diseases with vrariable efficacies. SIT is efficacious in allergic rhinitis and venom allergies, while its efficacy to suppress manifestations of allergic asthma remains variable at best [13-16]. There is an unmet medical need for improvement of SIT, which requires a detailed understanding of the mechanisms by which SIT induces its therapeutic effects. During the last few decades, significant progress has been made in our understanding of the SIT-induced modifications in immunological and clinical parameters [17-26]. For instance, increased production of allergen specific IgA and IgG has been reported after SIT treatment in allergic patients [27-31]. This might indicate a role for blocking immunoglobulins which sequestrate the allergen on the one hand and induce inhibitory signals through low affinity Fcg-RIIB on the other hand. Besides production of immunoglobulins, B cells may also play a tolerogenic role in SIT by acquisition of regulatory capacities [32]. Increased frequency of peripheral $FOXP3^+$ as well as IL-10 producing inducible regulatory T cells (iTreg cells) have also been observed after SIT treatment in clinical studies, indicating a possible role for a heterogeneous population of iTreg cells in SIT-induced allergen tolerance [28;33]. In agreement with the induction of IL-10 producing T or B regulatory cell subsets, increased plasma levels of IL-10 and increased allergen-induced production of IL-10 by PBMCs of allergic individuals was observed shortly after the initiation of SIT treatment [34]. Production of IL-10 after SIT, however is not limited to B and T cells and it has been shown that monocytes produce IL-10 after SIT as well [35]. The long-term efficacy as well as the allergen specificity of SIT suggests that long-lived memory B or T cell subsets rather than short-lived monocytes govern the SIT-induced allergen-specific tolerance induction [13;36;37]. Collectively, these studies have stressed the importance of several subpopulations of innate and adaptive immune cells and their mediators. However, due to the limitations of the clinical studies towards experimental interventions during treatment, these studies have remained largely descriptive in nature. To allow the undisputed identification of those immune cell subsets and mediators critically required for the suppression

of allergic responses by SIT, we and others have established experimental mouse models of allergen-specific immunotherapy[38;39]. In our model, we treat OVA-sensitized mice with allergen injections (SIT treatment), followed by allergen provocation by inhalation, to assess suppression of manifestations of allergic asthma in comparison to a control group. The value of these models has been underscored by the identification of IL10 receptor signaling as an event critically required for the suppression of allergic inflammation by IT in the mouse model [39]. Notwithstanding the fact that the mouse model has allowed us to begin to unravel the immunological basis of SIT, the exact molecular and cellular mechanism of action has to be elucidated yet.

In this dissertation we aimed to (i) dissect the mechanism of SIT-mediated generation of allergen-specific tolerance in a sensitized host, and to (ii) identify efficient approaches to improve the safety or efficacy of SIT. We have approached these aims by addressing the following questions:

1) What is the role of B cells and secreted allergen-specific IgG or IgA in SIT-induced suppression of allergic manifestations upon allergen provocation?
2) Are plasmacytoid dendritic cells during SIT treatment crucially required for SIT-induced suppression of allergic manifestations?
3) What is the role of $CD4^+FOXP3^+$ Treg cells during SIT treatment for the generation of SIT-induced allergen tolerance?
4) Are $CD4^+FOXP3^+$ Treg cells critically required for SIT-induced suppression of the manifestation of allergic asthma during allergen provocation?
5) Which cell types produce IL-10 during SIT treatment as well as upon allergen provocation, and do these cells play a critical role for the suppression of allergic manifestations?
6) Can CTLA4-Ig be used as an adjuvant for SIT to improve the efficacy of SIT in suppressing the manifestations of allergic asthma?
7) Can TGF-β delivered at the time of SIT treatment improve the efficacy of SIT to suppress the manifestations of allergic asthma through the generation of iTreg cells?
8) Can SIT-induced allergen tolerance be transferred to the next generation?

The role of B cells and blocking antibodies in SIT

B cells are the only cells of the immune system that produce and secrete antibodies. They play a pivotal role in the pathogenesis of allergic diseases by Th2-induced production of allergen specific IgE that binds to mast cells and basophils and thereby sensitizes these cells [40]. B cells can potentially

contribute to the SIT-induced allergen tolerance through two mechanisms. The first mechanism entails the production of allergen specific IgG and IgA. Increased production of allergen specific IgA and IgG (especially IgG4) has been frequently observed after SIT treatment in allergic patients [27-31] and in experimental mouse models of SIT [41-43]. The observation of increased levels of allergen-specific IgG after SIT treatment has lead to the blocking IgG hypothesis as an explanation for the mechanism of action of SIT. The blocking IgG hypothesis states that allergen specific IgG antibodies have a pivotal role in SIT-induced tolerance induction [44;45]. These antibodies on the one hand can bind to the allergen and block its binding to IgE antibodies, thereby preventing IgE cross-linking on effector cells such as mast cells and basophils, IgE facilitated antigen presentation by dendritic cells and effectively preventing the allergic reaction induced by allergen provocation[46]. On the other hand, IgG can bind to the inhibitory IgG receptor (FcγRIIB) on the same effector cells and through the intracellular ITIM motif inhibit IgE-induced FceRI signaling upon allergen-mediated cross-linking [47]. The neutralizing capacity of IgG antibodies for allergen-IgE interactions has been elegantly shown by Pree and colleagues [48]. These investigators showed that SIT with recombinant Bet V1, a major allergen of birch pollen, increases the level of IgG in serum, which correlates with reduced binding of allergen-IgE complex to low affinity IgE receptor, CD23, and thereby to a reduced allergen specific T cell response[48]. Some studies have indeed observed a negative correlation between the level of IgG after SIT and improved clinical symptoms or skin prick test reaction[49-51], while other studies did not [52;53]. Some studies have suggested that the affinity for the allergen, rather than the concentration of immunoglobulin in the serum, determines the beneficial effects and neutralizing capacity of SIT-induced IgG antibodies [46;54;55].

The second potential mechanism by which B cells might contribute to SIT is by acquiring regulatory properties such as production of IL-10. It has been shown that helminth infection can induce such regulatory B cells, and that these have suppressive effects on allergic inflammation [32;56] suggesting that regulatory B cells may have therapeutic implication in allergic diseases [32].

In the studies described in chapter 3 of this dissertation we aimed to address the role of B cells in SIT-induced allergen tolerance in our mouse model of allergic asthma. Since, in parallel to the clinical situation, SIT in our model induces strongly increased levels of allergen-specific IgG1 and IgG2a, our experimental mouse model efficiently allows these mechanistic studies. First, we show that mice lacking the inhibitory FcγRIIB did not display an altered sensitivity to suppression of allergic manifestations by SIT, ruling out

a critical role for neutralizing IgG antibodies by binding to its inhibitory Fc receptor. Moreover, using mice lacking all mature B cells, we show that neither regulatory B cells nor the production of allergen-specific IgG is required for SIT-induced tolerance in our model. We further showed that local production of IgA at the mucosal surfaces, a feature retained in the mature B cell deficient mice, is dispensable for the therapeutic effects of SIT by using mice that lack the polymeric-immunogobulin receptor that uniquely transports IgA across the mucosal barrier. In conclusion, we show that production of allergen specific IgG or IgA, or B cell regulatory function is not required for SIT-induced allergen tolerance in our mouse model.

These data are in striking contrast to a study taking a similar approach towards SIT treatment in a Fel d1-driven mouse model of acute anaphylaxis[57]. Here, the authors show that signaling through FcγRIIB is critical for suppression of the systemic, basophil-mediated, anaphylactic response upon i.v. administration of the allergen, but not of the tissue mast cell degranulation upon local administration of the allergen. In our study using the B cell deficient mice, allergen-specific IgE is entirely absent, and consequently the asthma manifestations in our model exclusively reflect the allergen-induced late phase response induced by Th2 cell activity [58]. Hence, we cannot draw any conclusions regarding the requirement of blocking IgG or B cell activity for the suppression of the early allergic response, unlike the Fel d1 mouse study, which exclusively assesses the early allergic response. Taken together, production of allergen-specific IgG, as observed in human studies of SIT, may not be necessary for suppression of late-phase allergic responses, but they might still critically contribute to the effects of SIT on the early phase response, and have a limited contribution to the suppression of the late phase response by SIT through sequestration of allergen, inhibitory signaling through FcγRII and blocking IgE mediated allergen presentation.

Role of dendritic cells

Antigen presentation is a key step in eliciting an efficient T cell mediate immune tolerance. Surprisingly, there is only limited knowledge available about antigen presentation during SIT. Classically, SIT is performed by subcutaneous administration of the allergen where it can be captured by a variety of antigen presenting cells (APCs) including Langerhans cells, dermal dendritic cells, skin macrophages and plasmacytoid dendritic cells (pDCs) that are recruited to the skin [59-61]. Langerhans cells reside in the epidermal layer of the skin and show tolerogenic properties in the absence of danger signals [62]. These cells have high migratory capacities, and might therefore contribute to

allergen presentation in secondary lymphoid organs during SIT[63]. However, in SIT allergen is applied to hypodermis layer of the skin which is not the anatomical location of the Langerhans cells. Instead, Langerhans cells reside in the epidermis layer of the skin and thus the contribution of these cells to tolerance induction might be rather limited [64]. Nevertheless, Langerhans cells can be found in the hypodermis layer of the skin during their migration from epidermis to the regional lymph nodes [65]. These migratory Langerhans cells, however, undergo a maturation process leading to reduced capacity of antigen capture [66]. This makes allergen capture by Langerhans cells an unlikely event, especially during the low allergen up-dosing phase characteristic of early-phase SIT treatment. Langerhans cells might, however, contribute to the generation of SIT-induced tolerance when other routes of allergen application are employed, such as sublingual SIT [67] or SIT with skin patches where the patches are applied on the epidermis layer of the skin [68].

Conventional DCs also have the capacity of inducing immune tolerance [69;70]. It has been shown that dermal DCs have high migratory capacity *in vivo* [71] and have the capacity to generate inducible Tregs *in vitro* [72] and *in vivo* [73]. Dermal DCs (dDCs) also reside in an anatomical compartment that is located in the deeper layers of the skin [74], making them more likely candidates for the presentation of the subcutaneously administered allergen. Interestingly, it has been shown that dDCs preferentially induce FOXP3$^-$IL-10 producing regulatory T cells while Langerhans cells induce FOXP3+ regulatory T cells[75]. The role of dermal DCs in the generation of SIT-induced allergen tolerance, however, remains to be determined.

Skin-resident macrophages can possibly play tolerogenic roles during antigen presentation [60]. It has recently been demonstrated that macrophage-like cells play a critical role in antigen presentation and tolerance induction during sublingual SIT in a mouse model [76]. However, the route of antigen administration and potentially the mechanism of tolerance induction in sublingual SIT are different from subcutaneous SIT. The importance of skin macrophages for SIT-induced allergen tolerance has yet to be elucidated.

pDCs also seem to be good candidates for playing a tolerogenic role during SIT since these cells can be rapidly recruited to the skin upon skin stimulation [61] and they can contribute to allergen tolerance induction through at least two mechanisms. The first mechanism is by expression of IDO, which has been found to be expressed in particular by pDCs [77]. We have previously shown that IDO partially contributes to the therapeutic effects of SIT in our mouse model [42]. The second mechanism of pDC contribution to allergen tolerance is by facilitating the generation of IL-10 producing Tr-1

cells through expression of ICOS-L [78]. We have previously shown that SIT-induce allergen tolerance in our mouse model is IL-10 dependent [39]. In the studies described in chapter 4 of this dissertation we investigated the role of pDCs in the induction of allergen tolerance during SIT in our mouse model of allergic asthma. We show that pDCs contribute to antigen presentation during SIT by capturing the subcutaneously applied allergen followed by functional antigen presentation in the draining lymph nodes. However, these cells play dispensable roles for the induction of allergen tolerance in our model as evident by a similar efficacy of SIT in suppressing the manifestation of experimental allergic asthma in the presence or absence of pDCs. These results indicate that other sub-types of DCs or skin macrophages can play a critical role in the generation of allergen tolerance during SIT treatment. We also show that the second major population of DCs that capture OVA during SIT in our model displays a phenotype similar to dermal DCs. Since we found that pDCs are redundant for SIT-induced allergen tolerance, we suggest that dDCs play a crucial tolerogenic role during SIT. Yet, it remains to be addressed whether antigen presentation leading to tolerance induction during SIT is in fact limited to a specific subset of APCs or whether tolerance induction is instead a redundant activity, shared by different subsets of APCs and merely depends on the activation state of the APC presenting the allergen upon the SIT injections.

Role of regulatory T cells

There is a large body of evidence suggesting a pivotal role for regulatory T cells (Treg cells) in the induction of SIT-mediated allergen tolerance. Treg cells are key players in the induction of peripheral immune tolerance [79]. Central tolerance ensures that self-reactive T cells are eliminated during T cell differentiation in the thymus by the process of negative selection. Peripheral tolerance enables the immune system to control the immune responses to pathogens, to prevent unnecessary immune reactions to harmless antigens and to suppress self reactive T cells that escaped central tolerance induction. Peripheral tolerance acts through the induction of T cell anergy and through active immune suppression by Treg cells [79-82].

There is evidence suggesting that SIT generates forkhead box P3 (FOXP3) expressing inducible regulatory T cells (iTreg cells) [28;83;84]. However, it is unknown whether these *de novo* $FOXP3^+$ Treg cells play a critical role in mediating the suppression of allergic manifestations in human patients. Moreover, it is not known whether $FOXP3^+$ naturally occurring Treg cells (nTreg), a separate lineage of T cells originated from a distinct precursor in

thymus, are required for maintaining a tolerogenic microenvironment during the SIT treatment, allowing the induction of allergen-specific tolerance.

In the studies described in chapter 5 of this dissertation we first tested the possibility that during SIT, FOXP3$^+$Treg cells are required for the generation of SIT-induced allergen tolerance. Regardless of the mechanism by which SIT suppressed the manifestation of experimental allergic asthma upon allergen provocation, we addressed whether FOXP3$^+$Treg cells are required for the induction of this suppressive mechanism. It has been shown that nTreg cells can facilitate the generation of iTreg cells, a process known as infectious tolerance [85;86]. Since nTreg cells express high levels of interleukin-2 receptor α-chain (CD25) [80] we used a CD25-depleting antibody at the time of SIT injections, thereby applying an approach that has been widely used to study the function of nTreg [87-89]. Interestingly, we found that depleting nTreg cells during SIT partially impaired SIT-mediated suppression of allergen induced AHR while it did not influence the suppression of airway eosinophilia or serum levels of specific IgE. These findings indicate that the mechanism by which SIT suppresses allergen-induced AHR is, at least in part, dependent on nTreg cells present at the time of SIT treatment.

One possible mechanism of the contribution of nTreg cells at the time of SIT treatment is the generation of TGF-β producing iTreg cells (Th3), which contribute to the suppression of AHR upon allergen exposure. This explanation is supported by two lines of evidence from literature. First, it has been shown that nTreg cells can induce a subset of iTreg cells that show TGF-β dependent suppressive effects [86]. Second, it has been shown that Treg cells engineered to produce TGF-β are capable of suppressing AHR in mouse model of OVA-driven asthma [90]. Hence, we hypothesize that the induction of a TGF-β producing iTreg cells subset during SIT treatment contributes specifically to suppression of AHR.

In chapter 5, we further examined whether SIT generates *de novo* FOXP3$^+$ Treg cells and whether these FOXP3$^+$ Treg cells are critically required for SIT-induced suppression of the manifestation of allergic asthma upon allergen provocation. We show that SIT generates inducible CD4$^+$CD25$^+$FOXP3$^+$ Treg cells as evident by the transient increased number of CD4$^+$CD25$^+$FOXP3$^+$ Treg cells only in spleen and blood shortly after the injections. We found that SIT increases the number of CD4$^+$CD25$^+$FOXP3$^+$ Treg cells in the spleen and blood at 24h but not 96 h after the treatment. These data are in line with observations in human studies, where increased number of CD4$^+$CD25$^+$FOXP3$^+$ Treg cells was observed in the circulation during venom SIT [28].

Finally, we demonstrate that SIT-induced suppression of airway eosinophilia

but not of AHR and specific IgE is partially mediated by $CD4^+FOXP3^+$ Treg cells. Here, we used DEREG mice to specifically deplete $CD4^+FOXP3^+$ T cells, 24 h prior to inhalation challenges in our mouse model of SIT. This approach enabled us to selectively deplete $CD4^+FOXP3^+$ Treg cells after SIT treatment, before inhalation challenges [91]. Interestingly, we found that depletion of $CD4^+FOXP3^+$ Treg cells after SIT abrogated suppression of airway eosinophils whereas suppression of AHR and specific serum IgE remained intact. These data indicate that airway eosinophilia in our model is suppressed by an induced subset of $CD4^+FOXP3^+$ Treg cells. In other mouse models, $FOXP3^+$ T cells were also found to suppress eosinophilic inflammation. For instance, in a mouse model of nasal tolerance induction, it has been shown that depletion of $CD4^+CD25^+FOXP3^+$ Treg cells before allergen exposure abrogates the suppression of airway eosinophilia [88]. Grainer and colleagues have shown that helminth secretions lead to generation of $CD4^+FOXP3^+$ iTreg cells can effectively suppress airway inflammation in a mouse model [92]. In a clinical study it was observed that number of circulating $CD4^+FOXP3^+$ Treg cells negatively correlates with specific IgE in serum and eosinophils in allergic patients, suggesting a role for $CD4^+FOXP3^+$ Treg cells in suppressing the levels of IgE and eosinophils in the human situation, too.

Taken together, we find that $CD4^+FOXP3^+$ Treg cells contribute to the generation of tolerance towards AHR during SIT, most likely by the induction of a $FOXP3^-$ TGF-β producing regulatory T cell subset, while SIT generates $CD4^+FOXP3^+$ iTreg cells that suppress airway eosinophilia upon allergen challenge.

Source of IL-10 production

Interleukin 10 has been suggested to play a crucial role in SIT-induced allergen tolerance. Increased IL-10 levels have been frequently observed after successful SIT in patients [28;35;93-95]. Some studies have reported that SIT increases the number of peripheral $IL\text{-}10^+CD4^+$ T cells [35;93;94] while some studies report increased levels of IL-10 in serum [27;95;96]. Also in tissue biopsies, $IL\text{-}10^+$ T cells and APCs have been observed during season in grass-pollen SIT treated subjects [97]. We have previously shown that neutralizing IL-10 using IL-10-receptor neutralizing antibody completely abrogated the therapeutic effects of SIT in our model [39], indicating that IL-10 is crucially required for SIT-induced allergen tolerance in our model. In the experiments described in chapter 6, we tested which cells were producing IL-10 during SIT treatment and subsequent allergen provocation, and which of these IL-10 producing cells were critical for suppression of allergic inflammation. Here, we found

that SIT increased the number of IL-10 producing Th2 cells and $CD8^+$ T cells at the time of SIT injections. Depletion of $CD8^+$ T cells, however, indicated that these cells were dispensable for suppression of allergic inflammation by SIT. Our data demonstrating increased production of IL-10 by Th2 cells are in line with the finding of Aslam and colleagues, who showed that SIT initially activates Th2 cells which are subsequently gradually suppressed as Treg cells are induced [25].

Interestingly, at the time of allergen challenge, T cell subsets no longer produced enhanced IL-10 levels. Instead, we identified a subset of DCs that produces IL-10 upon allergen exposure subsequent to SIT in our mouse model. This DC subset expressed CD11c and CD11b, identifying it as an inflammatory DC, and intermediate levels of MHCII indicating a reduced maturation of these cells. By performing adoptive transfer experiments using OVA-pulsed DCs from IL-10 knockout mice or from wild-type controls into wild-type, OVA-sensitized and OVA-SIT or sham control treated acceptor mice, we show that IL-10 production by the allergen-presenting DCs was critically required for suppression of allergic airway inflammation at the time of allergen provocation in this model.

In our mouse model of allergic asthma, SIT induces a long-term IL-10 dependent tolerance that lasts for at least 20 weeks [39]. In the experiments described in chapter 6 we have observed that IL-10 secretion by DCs is pivotal for SIT-induced suppression of airway eosinophilia. Since long-term allergen-specific memory cannot be maintained by short-lived DCs, we postulate that a subset of allergen-specific regulatory T cells instructs the allergen-presenting DCs to produce the IL-10 required for suppression of allergic inflammation. A similar observation has been made by Kearly and colleagues, who have found that transfer of $CD4^+CD25^+$ T cells to sensitized mice suppresses allergen-induced airway inflammation and AHR in an IL-10-dependent fashion, the IL-10 secretion from the transferred $CD4^+CD25^+$ T cells themselves is in fact not required for the observed suppressive effects.

Overall, we conclude that DC-derived IL-10 mediates SIT-induced suppression of airway eosinophilia. However, the short lifespan of DCs and long term efficacy of SIT indicates that SIT-induced allergen tolerance is maintained by a yet to be identified sub-type of Treg cells. Given the data described in chapter 5, where we show that depletion of $FOXP3^+$ Treg cells at the time of allergen challenge, but not of all Treg cells at the time of allergen injections is sufficient to suppress eosinophilic airway inflammation, we postulate that this enigmatic Treg cell subset is a $FOXP3^+$ T cell subset induced during the allergen injections.

Improvement of SIT: CTLA4-Ig, a potent adjuvant for SIT

SIT has three main disadvantages, being the need for (i) high doses of the sensitizing allergen, and thereby the risk of severe side-reactions, including anaphylaxis, (ii) the long-term treatment required for sustained protection, and (iii) the variable efficacy in treated patients. Identifying clinically applicable adjuvants that increase the efficacy for allergic disorders in which SIT is currently not efficacious, or that result in the same efficacy of SIT at lower doses of allergen, or that allow a reduced treatment period, are urgently needed. In experiments described in chapter 7 we examined whether CTLA4-Ig can act as an adjuvant for SIT using our mouse model. Cytotoxic T Lymphocyte Antigen 4 (CTLA4) is an inhibitory costimulatory molecule expressed by nTreg cells and activated T cells, and has been implicated in terminating T cell responses [98]. CTLA4-Ig is a fusion molecule consisting of extracellular domain of CTLA4 with Fc regions of human or mouse IgG[99]. CTLA4-Ig has tolerogenic effects, and has been used in the treatment of rheumatoid arthritis and prevention of allograft rejection in humans [100-102].

Two mechanisms are involved in tolerogenic effects of CTLA4-Ig. First, CTLA4-Ig can sequester B7 and thereby block CD28:B7 interactions and prevent the T cell costimulatory signal required for acquisition of full effector functions [98]. Second, CTLA4-Ig can signal into the DC through B7 molecules, activate the non-canonical NF-kB pathway and induce the expression of IDO[99;103;104].

We tested whether CLTA4-Ig has an adjuvant effect on SIT in our mouse model. We found that co-administration of CTLA4-Ig with SIT highly enhances SIT-induced suppression of AHR, airway eosinophilia and allergen-specific IgE in the serum indicating that CTLA4-Ig can act as a potent adjuvant for SIT. Testing the effects of co-administration of CTLA4-Ig with SIT in mice lacking the indoleamine 2,3 dioxygenase (IDO) gene revealed that although SIT in these mice fails to suppress AHR and airway eosinophilia, CTLA4-Ig restores and highly enhances the suppressive effects of SIT. These results suggest that: First, IDO contributes to the mechanism of induction of allergen tolerance confirming our previous findings using a chemical inhibitor of IDO [42]. Second, the likely mechanism of action for CTLA4-Ig in our model is blocking CD28 costimulatory signal in $CD4^+$ T cells and not induction of IDO through reverse signaling. Since memory Th2 cells in the sensitized host are less dependent on CD28 for their activation, blocking CD28-mediated costimulation most likely affects naïve $CD4^+$ T cells leading to the induction of clonal anergy and regulatory properties in these otherwise activated cells [98;105]. It has been shown that lack of costimulation leads to the induction of anergized human T

cells which show regulatory properties through expression of inducible T cell costimulator (ICOS) and IL-10 [106]. So, co-administration of CTLA4-Ig with SIT through blocking CD28-mediated T cell costimulation may enhance the induction of anergized $CD4^+$ T cells with regulatory properties that suppress the manifestation of experimental allergic asthma upon inhalation challenges. Lack of costimulation due to antigen presentation by immature DCs might also play a role in the induction of allergen tolerance in SIT without adjuvant.

Overall, given the high efficacy of CTLA4-Ig in potentiating the effects of SIT, and approved clinical application of CTLA4-Ig by Food and Drug Administration and its European counterpart we suggest that CTLA4-Ig might be used as an adjuvant for SIT in clinical trials.

Can TGF-β augment the therapeutic effects of SIT?

Increased TGF-β levels in serum have been observed after subcutaneous SIT, and TGF-β levels correlate with the alleviation of allergic symptoms in human patients [29;107;108]. TGF-β can potentially contribute to SIT-induced allergen tolerance through four mechanisms. The first two mechanism involve facilitating the induction of TGF-β producing Th3 and $FOXP3^+$ iTreg cells in the absence of IL-6 [109-111] or in the presence of all-trans retinoic acid [112]. The third mechanism of action of TGF-β is by instructing B cells to produce IgA[113]. The fourth mechanism by which TGF-β can contribute to the induction of allergen tolerance is generating tolerogenic properties in DCs by induction of IDO [114]. While endogenous TGF-β may contribute to the therapeutic effects of SIT, we were interested to test whether exogenous TGF-β can potentiate the therapeutic effects of SIT. In the studies described in chapter 8, we examined whether administration of TGF-β during SIT injections could potentiate the effects of SIT, and if so, whether an enhanced induction of iTreg cells was observed in our mouse model of allergic asthma. Indeed, we found that TGF-β augmented SIT-induced suppression of allergen induced airway eosinophilia as well as Th2 cytokines when co-administered with suboptimal SIT (2x1 mg OVA-SIT). Interestingly, TGF-β also augments the suppressive effects of a 10-fold lower dose of sub-optimal SIT (2x 0.1 mg OVA-SIT) on airway eosinophilia, OVA-specific IgE in serum and Th2 cytokines in BAL. Moreover, we found that the enhancing effects of TGF-β on SIT is accompanied by an increased level of specific-IgA in the serum and an increased percentage of $CD4^+FOXP3^+$ Treg cells in the mediastinal lymph nodes. The latter effect was observed regardless of antigen administration. We observed that sub-optimal SIT increases the levels of TGF-β in the serum indicating a role for Th3 cells in the suppression of the manifestation

of experimental allergic asthma by SIT alone. However, administration of exogenous TGF-β does not seem to enhance the induction of Th3 cells as serum TGF-β levels were not further increased. Sub-optimal SIT-induced suppression of allergen-specific IgE, airway eosinophilia but not of AHR was augmented by co-administration of TGF-β. This suppression was associated with increased levels of allergen-specific IgA and percentage of $CD4^+FOXP3^+$ T cells in draining lymph nodes in our study. While the mechanism of SIT-induced suppression of allergen-specific IgE remains to be determined, suppression of airway eosinophilia seems to be mediated by $CD4^+FOXP3^+$ T cells revisiting our previous findings that SIT-induced suppression of airway eosinophilia is mediated by $CD4^+FOXP3^+$ T cells. As we suggested earlier, induction of a subset of $CD4^+FOXP3^-$ T cells which is not augmented by administration of exogenous TGF-β might be responsible for the suppression of AHR.

Finally, we found that blocking IL-6 receptor during SIT treatment does not enhance the adjuvant effect of TGF-β on suppression of allergic manifestations by SIT, indicating that induction of Th17 cells does not seem be an important component of SIT in our model. Taken together we provide evidence that co-administration of TGF-β with SIT augments the therapeutic effects of SIT. Since systemic administration of TGF-β may induce undesirable effects, it is suggested that indirect induction of TGF-β in APCs, for instance by administration of vitamin D3 [75], during SIT-treatment will be useful in clinical trials to improve the clinical efficacy of SIT. Interestingly, we have previously shown that co-administration of vitamin D3 with SIT potentiates the therapeutic effects of SIT through induction of IL-10 and TGF-β [43].

Absence of maternal transfer of protection by allergen immunotherapy in a mouse model of allergic asthma

The prevalence of allergic diseases has been and still is increasing worldwide[3]. There is evidence indicating that asthma is heritable and that offspring born to asthmatic mothers have a bigger chance to develop asthma [115;116]. Despite having a higher susceptibility to the disease, experimental data are now indicating that allergic mothers might in fact transfer protection to allergic sensitization to their progeny during pregnancy and lactation [117]. It has been shown that pups born to allergically sensitized mice show reduced level of allergen induced airway inflammation, allergen-specific IgE and Th2 cytokines [117]. However, the observed reduction is more far more pronounced when mothers received inhalation challenges during lactation [117]. Maternal transfer of allergen-IgG immune complexes are implicated in this maternal

transfer of protection against experimental allergic asthma [117]. Interestingly, induction of allergen tolerance by SIT is associated with the induction of blocking IgG antibodies in humans [45] raising the question whether maternal transfer of therapeutic of SIT is possible. While the process of SIT treatment is time consuming and requires at least a few years of treatment to show efficient suppression of the symptoms of allergic diseases, it will be hitting two targets by one bullet if the beneficial effects of SIT can be transferred to the next generation through maternal transfer. In the experiments described in chapter 9 we examined whether SIT-induced allergen tolerance can be transferred to the progeny of the treated mothers through allergen-immune complexes in the breast milk. As expected, we found that SIT, induces high levels of allergen-specific IgG1 in the serum of treated mothers. We discovered that enhanced allergen-specific IgG1 and allergen-immune complexes are present in the breast milk of sensitized as well as SIT-treated mothers. Interestingly, we observed that induction of experimental asthma in the progeny of all OVA-sensitized mice resulted in a lower level of OVA-specific IgE in the serum compared to the progeny of naïve mice. Surprisingly, only progeny of OVA-sensitized Sham-treated but not SIT-treated mice showed lower level of airway eosinophilia compared to the progeny of naïve mice upon induction of experimental asthma. This is in accordance with the study conducted by Mosconi and colleagues, where they showed that allergen sensitization in mothers convey a low-level of protection to the progeny [117], however, our results indicate that SIT-treatment abrogates this protection against airway eosinophilia. Although it was not evaluated directly, one reason for this abrogation could the induction of allergen-IgG2a immune complexes in the milk in SIT-treated mothers. We have previously shown that SIT treatment induces high levels of allergen-specific IgG2a in the serum in our model [58]. Induction of allergen-IgG2a complexes in the milk might abrogate the low-level protection in progeny induced by low concentration of allergen-IgG1 complexes. More studies are required to unravel the details of allergen tolerance that is transferred to the progeny of SIT-treated mothers.

It has been shown that allergen-specific IgE responses in offspring are strongly inhibited by maternal IgG [118-120]. Maternal IgG can be transferred to the offspring either via breastfeeding or via placenta by transfer via Fc receptor (FcRn) [121]. To assess the impact of transfer of OVA-IgG immune complexes through either breastfeeding or placenta, we analyzed the asthma manifestations in mice born to sensitized Sham-control or OVA-SIT treated mice but weaned by naïve mice, and *vice versa*. Intriguingly, we found that pups born to SIT-treated mothers but weaned by naïve mice show tolerance towards the induction of specific IgE in serum. However, this tolerance

towards specific IgE is absent in the pups born to naïve mothers and weaned by SIT-treated fosters. These results suggest that the placental, rather than the milk, route of transfer of allergen-IgG complexes conveys the SIT-induced maternal protection towards allergen-specific IgE in the serum. Lack of transfer of SIT-induced maternal protection through milk could be due to a relatively lower level of allergen-IgG complexes in the milk compared to the study of Mosconi and colleagues. The level of allergen-IgG complexes in the milk in our study was approximately one third of the levels observed by Mosconi and colleagues. These investigators exposed the mothers to airborne allergen during lactation while in our study SIT treatment was done preconceptionally and there was no further exposure to allergen during pregnancy or lactation. Absence of allergen exposure during pregnancy and lactation, not only leads to reduced level of allergen-IgG complexes but also reduces the transfer of free allergen through the milk which has been shown to transfer protection to allergen induced airway inflammation in a TGF-β dependent fashion [122]. In a study conducted by Polte and colleagues, they observed that preconceptional induction of oral tolerance is transferred to the progeny independent of breast milk [121]. Although this protection remained sufficient in the absence of placental IgG transfer in FcRn-deficient mice [121]. More studies are needed to determine the transferability of effects of SIT from mothers to pups and to unravel the possible mechanism of transfer.

Summarizing conclusion

In our mouse model of SIT, the sequence of events can be virtually divided into two phases of action including induction and effector phase. Induction phase is defined as the period of SIT-treatment when administration of allergen leads to alterations in allergen specific immune response and induction of allergen tolerance. Effector phase is the period of allergen provocation subsequent to SIT-treatment when the mediators of allergen tolerance suppress the manifestation of experimental allergic asthma. We contribute to unraveling the mechanisms of SIT-induced allergen tolerance by investigating the induction as well as the effector phase of SIT, focusing largely in the suppression of the late-phase allergic response, and by evaluating the role of B cells, pDCs, $CD8^+$ T cells, $CD4^+FOXP3^+$ T cells and IL-10 producing DCs. A hypothetical mechanism of action of SIT in our model is demonstrated in figure 1. Interestingly, we find no contribution of B cells or blocking IgG to the suppression of allergic asthma manifestations (chapter 3), while we do observe a dependence on IL-10 (chapter 6) and subsets of regulatory T cells (chapter 5). These data are in remarkable conflict to the data presented by

the group of Bachmann [57] who showed that blocking immunoglobulins are critically required for the suppression of anaphylaxis upon systemic delivery of Fel d1 in sensitized and SIT-treated mice. Remarkably, suppression of anaphylaxis in this model is entirely independent on the presence of Tregs or $CD4^+$ T cells altogether. Since we find a complete redundancy for B cells and IgG for the suppression of the allergic inflammation by SIT, we can conclude that SIT suppresses the early and the late phase allergic response through radically divergent mechanisms: suppression of the early phase response requires the presence of blocking Ig and B cells, but is independent on Tregs, whereas the situation is reversed for the suppression of the late phase response, that is dependent on the presence of Tregs, but entirely independent on B cells and blocking IgG.

The critical antigen-presenting cell during the induction phase of SIT remains to be established. By ruling out the role of pDCs we propose that dDCs capture the administered allergen and transport it to the skin draining lymph nodes during the induction phase. In skin draining lymph nodes allergen is presented to $CD4^+$ Th2 cells and naïve Th cells in the presence of nTregs that maintain a tolerogenic microenvironment and are relevant for the suppression of AHR at the time of allergen challenges. We postulate the hypothesis that during this process, the presence of $CD4^+CD25^+FOXP3^+$ Treg cells lead to the generation of $CD4^+FOXP3^-$ Treg cells that suppress AHR likely through expression of TGF-β, ICOS or programmed death-1 (PD-1) in the effector phase. The evidence for the possible role of ICOSL or PD-1 has been provided by McGreen and Agrawal who showed that transfer of either $CD25^+$ or $CD25^-$ $CD4^+$T cells suppresses allergen induced AHR and airway inflammation in a mouse model of allergic asthma, and proposed a role for PD-1 and ICOS expressed by $CD4^+CD25^+$ Treg cells [123]. Moreover, It has been shown that ligation of PD-1 or ICOS to their corresponding ligands on DCs induces IL-10 secretion in the DC [124]. Hansen and colleagues provided evidence for the suppressive effects of TGF-β on airway eosinophilia and AHR in a mouse model of allergic asthma [90].

During the induction phase, allergen-specific $CD4^+CD25^+FOXP3^+$ Treg cells are also generated, likely through expression of TGF-β by allergen presenting APCs, or through the absence of costimulation. It remains to be established whether these cells originate from de novo differentiated naïve T cells or from immune deviation of the pre-existing Th2 memory cells. The large amounts of allergen required for efficient induction of allergen tolerance argues in favor of a model where the pre-existing memory pool of Th2 cells needs to be saturated, allowing for the concurrent activation of naïve

T cells that will then adopt a regulatory T cell fate. Our data in chapters 4 and 6 clearly show the activation of the pre-existing Th2 cells during the induction phase. Our data in chapter 5 show that the pre-existing nTregs are not required for the induction of this iTreg subset, while our data in chapter 7 indicate that full blockage of co-stimulatory activity can further enhance the suppression of allergic manifestations. Taken together, the cumulative data presented in this thesis strongly argue in favor for the *de novo* induction of iTregs during the induction phase through the absence of costimulatory molecules by the relevant APC subset. We postulate that these iTreg cells suppress airway eosinophilia by instructing $CD11b^{hi}CD11c^{hi}$ $MHC\text{-}II^{int}$ DCs to produce IL-10 during the effector phase. The IL-10 production by the DCs then critically suppresses the induction of a late phase response during the allergen provocation in SIT treated mice, resulting in strongly reduced airway eosinophilia and reduced presence and activity of Th2 cells. Exogenous TGF-β enhances the generation of *de novo* $CD4^{+}CD25^{+}FOXP3^{+}$ Treg cells and enhances SIT-induced suppression of airway eosinophilia, indicating the possibility for further enhancement of the efficacy of SIT by augmenting the induction of these iTregs.

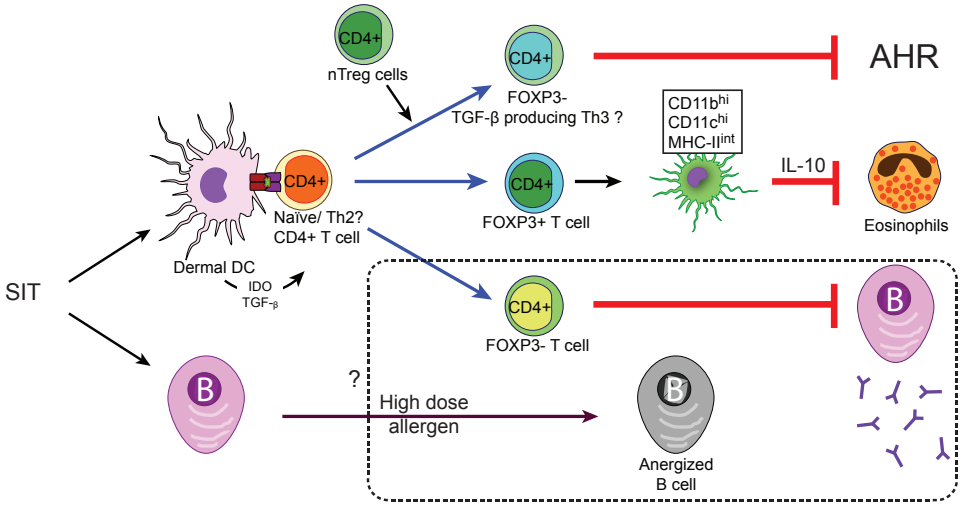

Figure 1 - Proposed mechanism by which SIT suppresses the manifestation of experimental allergic asthma in our mouse model of SIT. Antigen presentation to Th2/naïve CD4+ T cells by dermal dendritic cells in the presence of CD4+ FOXP3+ Treg cells leads to the generation of TGF-β producing Th3 Treg cells. These Th3 cells suppress allergen induced AHR. Antigen presentation during induction phase also results in the generation of de novo CD4+FOXP3+ Treg cells which suppress airway eosinophilia during effector phase. SIT-induced suppressed levels of IgE in the serum could be due to either: (i) generation of a subset of CD4+FOXP3- Treg cells during induction phase which suppress the levels of allergen-specific IgE in the serum during effector phase, or (ii) induction of anergy in B cells caused by high level of antigen administrations during induction phase.

SIT-induced suppression of allergen-specific IgE in the serum could be mediated by a subset of $CD4^{+}FOXP3^{-}$ Treg cells which are generated during induction phase, since the levels of allergen-specific IgE in the serum remained unchanged in the presence or absence of $CD4^{+}FOXP3^{+}$ Treg cells. It is also possible that administration of high levels of allergen during induction phase causes clonal anergy in the allergen-specific B cells. The exact mechanism of SIT-induced suppression of specific IgE in the serum remains to be further elucidated.

Interesting points to address in the future studies

The followings are interesting points to be addressed in future studies:

- Determining the functional role of dermal DCs, langerhans cells and skin macrophages during SIT for the induction of allergen tolerance
- Identify the exact mechanism of SIT-induced suppression of allergen-specific IgE in the serum.
- Identify which cell types produce TGF-β during the induction phase and whether TGF-β plays a crucial role during the induction phase for the therapeutic effects of SIT
- The contribution of Programmed Death-1 mediated signaling during the effector phase of SIT-induced allergen tolerance
- Identifying Treg cell sub-types that mediate SIT-induced allergen tolerance
- Testing the adjuvant efficacy of CTLA4-Ig in SIT in clinical trials
- Identifying the mechanism of transfer of beneficial effects of SIT through the milk
- Identifying whether administration of SIT injections or inhalation exposure of SIT-treated mothers during pregnancy leads to the transfer of SIT-induced allergen tolerance to the next generation

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Nederlandse samenvatting

CHAPTER

11

Allergie

Allergische ziekten zoals hooikoorts en astma zijn chronische ontstekingsziekten die worden gekenmerkt door regelmatig terugkerende klachten zoals tranende ogen, niezen en benauwdheid, telkens wanneer men in contact komt met een allergeen zoals stuifmeel of huisstof. De onderliggende oorzaak van deze ziekten is een overdreven reactie van het afweersysteem op allergenen waardoor IgE antilichamen worden aangemaakt. Deze IgE antilichamen passen als een sleutel op een slot op het betreffende allergeen, of anders gezegd ze binden er specifiek aan. Dit IgE wordt geproduceerd door de zogenoemde B-cel, een bepaald type witte bloedcel. De geproduceerde IgE-antilichamen circuleren in het bloed en binden aan het oppervlak van mestcellen die zich in de weefsels, zoals de long en neus, bevinden. Hierdoor kunnen deze mestcellen na hernieuwd contact met het betreffende allergeen heel specifiek hierop reageren en komt o.a. histamine vrij. Het vrijgezette histamine leidt tot de karakteristieke allergische klachten zoals hiervoor genoemd. Naast deze acute klachten veroorzaakt chronische blootstelling aan een allergeen zoals tijdens het stuifmeelseizoen, chronische ontsteking van de luchtwegen (long en neus). Bij astmatische patiënten ontstaat hierbij ook een toename van de gevoeligheid van de luchtwegen voor luchtwegvernauwende prikkels. Dit laatste wordt hyperreactiviteit genoemd. De ontsteking wordt gekenmerkt door de aanwezigheid van grote aantallen eosinofiele granulocyten en wordt daarom ook wel eosinofiele ontsteking genoemd. Uit onderzoek bij mensen en muizen is de laatste decennia duidelijk geworden dat zowel de aanmaak van IgE-antilichamen als de chronische klachten van ontsteking en hyperreactiviteit worden gedirigeerd door een bepaald type witte bloedcel, de type 2 T-helper-cel, kortweg Th2-cel genoemd. Th2 cellen worden gekarakteriseerd door het aanmaken van cytokinen zoals interleukine-4 (IL-4), IL-5 en IL-13 die betrokken zijn bij IgE aanmaak door B-cellen, eosinofiele ontsteking en luchtweg hyperreactiviteit.

Allergeen immunotherapie

Ruim een eeuw geleden werd allergeen specifieke immunotherapie (SIT; ook wel hyposensibilisatie of allergeen vaccinatie genoemd) voor het eerst toegepast voor de behandeling van hooikoorts. Het principe van deze behandeling is grotendeels nog steeds hetzelfde. Uit een ruwe grondstof (stuifmeel afkomstig van gras) wordt een extract gemaakt, het SIT preparaat. De behandeling met deze SIT preparaten bestaat uit een opdoseringsfase van wekelijkse onderhuidse injecties met een toenemende dosis van het allergeen extract totdat een maximale dosis wordt bereikt na ongeveer twee maanden. Na

deze eerste fase volgt een onderhoudsfase die bestaat uit maandelijkse injecties met deze hoogste dosis die wordt voortgezet gedurende drie tot vijf jaar. SIT wordt toegepast bij allergische ziekten zoals hooikoorts en allergisch astma, en kan leiden tot vermindering van de allergische klachten. Het voordeel van SIT in vergelijking met alle andere behandelingen voor allergieën (o.a. steroïden en antihistaminica) is dat de werkzaamheid van SIT aanhoudt gedurende een zeer lange tijd (> 15 jaar) na het stopzetten van de therapeutische behandeling. Dit langdurig onderdrukken van allergische klachten ondanks blootstelling aan allergeen wordt tolerantie genoemd. Belangrijke andere aspecten van SIT zijn de verminderde kansen op sensibilisatie voor andere allergenen en op het ontwikkelen van astma bij allergische kinderen. Belangrijke nadelen van SIT zijn de variabele effectiviteit, de duur van de behandeling en de vereiste klinische monitoring tijdens de opdoseringsfase om in te kunnen grijpen bij ernstige allergische reacties. Deze nadelen hebben er helaas toe geleid dat deze behandeling nog steeds niet op grote schaal wordt toegepast. Er is dus alle reden om SIT te verbeteren zowel wat betreft de effectiviteit alsook de veiligheid en de duur van de behandeling zodat deze behandeling aantrekkelijk wordt voor een grote groep allergische patiënten.

Over het werkingsmechanisme van allergeen immunotherapie in het lichaam bestaat nog veel onduidelijkheid. In de loop van de 20^{e} eeuw is 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 ná de sensibilisatie maar voorafgaande aan 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 en IgA in allergeen immunotherapie? (hoofdstuk 3)

B-cellen zijn de enige cellen van het afweersysteem die antilichamen aanmaken en uitscheiden en spelen een belangrijke rol in de pathogenese van allergische ziekten door de aanmaak van IgE antilichamen. B-cellen dragen mogelijk bij aan de tolerantie voor allergeen na SIT door twee mechanismen. Ten eerste neemt de bloedspiegel van allergeen-specifieke IgG en IgA antilichamen in het bloed sterk toe na SIT, zowel in de mens als in ons muismodel. Deze waarnemingen hebben geleid tot de zogenaamde "blokkerende antilichamen" theorie. Deze IgG antilichamen zouden de binding van het allergeen aan IgE kunnen blokkeren en daardoor allergische reacties kunnen afremmen. Daarnaast kunnen deze IgG antilichamen mogelijk via remmende IgG receptoren (FcγRIIB) allergische reacties onderdrukken. Ten tweede kunnen B-cellen een regulerende functie aannemen door de aanmaak van het afweeronderdrukkende interleukine-10 (IL-10).

In de experimenten beschreven in hoofdstuk 3 hebben we de rol van B-cellen in de tolerantie na SIT onderzocht in ons muismodel. Ten eerste toonden wij aan dat in muizen die de remmende IgG receptor niet hebben, SIT evengoed werkt als in kontrole muizen met deze receptor. Ten tweede toonden wij aan dat in muizen die geen B-cellen hebben, SIT evengoed werkt om de eosinofiele ontsteking te onderdrukken als in muizen die wel B-cellen hebben. Tenslotte toonden wij aan dat in muizen die IgA niet kunnen transporteren naar de mucosale oppervlakken zoals het slijmvlies de longen, SIT evengoed werkt om de eosinofiele ontsteking te onderdrukken als in kontrole muizen.

Samenvattend kan dus geconcludeerd worden dat de aanmaak van specifiek IgG en IgA alsook regulerende B-cellen niet essentieel zijn voor de ontstekingsonderdrukkende werking van SIT. Wij kunnen echter niet uitsluiten dat deze mechanismen een rol spelen in de remming van mestcel activatie of luchtweg hyperreactiviteit na SIT.

Is er een rol voor plasmacytoide dendritische cellen in allergeen immunotherapie? (hoofdstuk 4)

Dendritische cellen (DCs) zijn onderdeel van het afweersysteem en bevinden zich overal in het lichaam waar ze voortdurend lichaamsvreemde materialen uit de omgeving opnemen zoals bacteriën maar ook ingeademde allergenen.

Hierna zoeken de DCs T-cellen op in lymfeklieren en presenteren de opgenomen materialen aan deze T-cellen. Behalve deze presenterende rol hebben DCs ook een dirigerende rol doordat ze stofjes aanmaken die bepalen wat de T-cel vervolgens moet gaan doen. Dat kan betekenen dat de T-cel de Th2 cytokinen gaat produceren zoals bij allergische personen maar ook dat de T-cel een afweeronderdrukkende rol aanneemt. Deze afweeronderdrukkende T-cellen worden kortweg Treg cellen genoemd. Het is opmerkelijk dat er nagenoeg niets bekend is over de soort DCs en hun specifieke rol bij de tolerantie die optreedt na SIT.

Er zijn verschillende soorten DCs en één daarvan wordt de plasmacytoide DC genoemd (pDC). Het is algemeen bekend dat pDCs een belangrijke rol spelen bij de inductie van Treg cellen en tolerantie. Een van de manieren waarop de pDC dit doet is door gebruik te maken van een enzym, indoleamine 2,3 dioxygenase (IDO), dat het aminozuur tryptofaan omzet in kynurenine en andere metabolieten. Voorheen is door ons aangetoond dat na remming van dit IDO enzym, de tolerantie die optreedt na SIT in ons muismodel grotendeels afwezig is.

In hoofdstuk 4 laten we zien dat pDCs in belangrijke mate het allergeen opnemen na SIT waarbij het allergeen onderhuids wordt ingespoten. Hierna gaan deze DCs naar de lymfeklier om het allergeen aan T-cellen te presenteren. Echter als we deze pDCs uitschakelen, door het inspuiten van een antilichaam waardoor deze cellen dood gaan, en vervolgens SIT geven dan treedt nog steeds tolerantie op voor de allergische klachten in ons muismodel. Dit experiment toont aan dat de pDC geen kritieke rol speelt bij de tolerantie na SIT. Verder onderzoek is nodig om vast te stellen welke soort DC wél van kritisch belang is voor de tolerantie na SIT.

Zijn Treg cellen betrokken bij allergeen immunotherapie? (hoofdstuk 5)

Uit studies naar het werkingsmechanisme van SIT bij allergische personen blijken Treg cellen toe te nemen in het bloed na SIT. Of deze Treg cellen ook betrokken zijn bij de allergie onderdrukkende werking van SIT is moeilijk zo niet onmogelijk vast te stellen in de mens. In de muis bestaat deze mogelijkheid wel. In hoofdstuk 5 tonen wij in ons muismodel aan dat Treg cellen toenemen in het bloed en in de milt kort na de SIT injecties. Vervolgens hebben wij onderzocht of het uitschakelen van Treg cellen van invloed is op het ontstaan van de tolerantie na SIT. Hierbij is gebruik gemaakt van een zogenaamde "knock-out" muis en door antilichamen die de Treg cellen elimineren. Hieruit blijkt dat de rol van Treg cellen in de tolerantie na SIT complex is. Ze spelen

namelijk wel een rol bij de onderdrukking van de eosinofiele ontsteking maar niet bij de luchtweghyperreactiviteit en IgE antilichaam productie.

IL-10 producerende dendritische cellen zijn essentieel voor allergeen immunotherapie. (hoofdstuk 6)

Uit studies naar het werkingsmechanisme van SIT bij allergische personen blijkt de productie van het afweeronderdrukkende cytokine IL-10 door T-cellen verhoogd te zijn. Voorheen hebben wij in het allergische muismodel voor SIT aangetoond dat IL-10 een kritische rol speelt in de allergie onderdrukkende werking van SIT. In hoofdstuk 6 hebben wij nu in detail onderzocht welke lymfocyten en andere afweercellen IL-10 produceren na SIT. We hebben aangetoond dat kort na SIT het aantal IL-10 producerende CD8 lymfocyten toeneemt in de milt. Om een rol van deze IL-10 producerende CD8 lymfocyten bij SIT aan te tonen dan wel uit te sluiten, hebben wij de CD8 lymfocyten geëlimineerd kort vóór de SIT injecties. Dit bleek echter geen gevolg te hebben voor de tolerantie na SIT en kan dus geconcludeerd worden dat deze IL-10 producerende CD8 lymfocyten geen rol spelen bij SIT.

Aangezien IL-10 in staat is afweerreacties direct te onderdrukken, hebben wij in SIT behandelde muizen onderzocht welke cellen er nu IL-10 produceren lokaal in de long na de blootstelling aan het allergeen. Opmerkelijk genoeg vonden wij dat het niet de T-cellen zijn die verhoogd IL-10 maken, maar er bleek een groep van DCs te zijn die verhoogd IL-10 maakt indien de muizen SIT hebben ontvangen. Om te onderzoeken of deze IL-10 producerende DCs daadwerkelijk van belang zijn voor de allergie onderdrukkende werking van SIT in het muismodel, hebben wij gebruik gemaakt van zogenaamde adoptieve overdrachtstudies. In deze studies krijgen DCs in een reageerbuis allergeen aangeboden dat vervolgens wordt opgenomen. Deze DCs worden dan vervolgens in de luchtpijp van een allergische muis toegediend alwaar ze lokaal in de long de Th2 cellen activeren die dan de eosinofiele ontsteking veroorzaakt. Door nu DCs te gebruiken die al dan niet in staat zijn IL-10 te produceren, konden wij aantonen dat IL-10 geproduceerd door allergeen-presenterende DCs essentieel is voor de remming van de eosinofiele ontsteking na SIT. Omdat DCs een relatief kort leven hebben in het lichaam en de tolerantie na SIT langdurend aanhoudt, vermoeden wij dat er een bepaalde, nog niet nader bekende, T-cel moet zijn na SIT die deze DCs instrueert om IL-10 te gaan produceren. Het is heel goed mogelijk dat deze T-cel een Treg cel is waarvan we in hoofdstuk 5 hebben laten zien dat die betrokken is bij de onderdrukking van de eosinofiele ontsteking. Het is van belang om op te merken dat er ook bij hooikoorts patiënten die SIT hebben gehad, er lokaal in de

neus IL-10 producerende cellen zijn aangetoond die grotendeels niet bestaan uit T-cellen maar overeen lijken te komen met allergeen-presenterende cellen. Verder onderzoek zal nodig zijn om aan te tonen dat dit werkingsmechanisme van SIT in de muis ook plaatsvindt in allergische patiënten na SIT.

CTLA4-Ig is een potent adjuvans voor allergeen immunotherapie. (hoofdstuk 7)

SIT bij de mens heeft een drietal nadelen. (i) De noodzaak van toediening van een hoge dosis allergeen. (ii) De noodzaak van langdurige onderhoudsinjecties van 3-5 jaar. (iii) De grote variatie in de allergie onderdrukkende werking tussen verschillende patiënten. Daarom is er een grote behoefte om SIT te verbeteren en één van de mogelijkheden is door gebruik te maken van een adjuvans. Een adjuvans is een stof die de werking van SIT sterk bevordert terwijl deze stof alléén geen langdurende allergie-onderdrukkende werking heeft. In hoofdstuk 7 hebben wij onderzocht of het molecuul CTLA4-Ig bruikbaar is als adjuvans voor SIT in ons muismodel. Van CTLA4-Ig is aangetoond dat het tolerantie kan bevorderen op twee manieren. (i) CTLA4-Ig kan binden aan de receptor B7 op DCs en daarmee voorkomen dat het molecuul CD28, aanwezig op T-cellen, kan binden met deze receptor waardoor deze T-cellen langdurig inactief worden. (ii) CTLA4-Ig kan na binding aan de receptor B7 op DCs de hoeveelheid van het enzym IDO in deze DCs verhogen. Wij laten in het muismodel zien dat het toedienen van CTLA4-Ig tegelijk met SIT leidt tot een sterke verbetering van de allergie-onderdrukkende werking van SIT op de hyperreactiviteit, eosinofiele ontsteking en IgE aanmaak. Hetzelfde experiment uitgevoerd in muizen die het enzym IDO missen, liet een even sterke verbetering van SIT zien wat aantoont dat het enzym IDO niet betrokken is bij deze versterkende werking van SIT door CTLA4-Ig. Hoewel we dit niet formeel hebben aangetoond, ligt het dus voor de hand dat het werkingsmechanisme van CTLA4-Ig komt door het blokkeren van de B7 receptor waardoor T-cellen langdurig inactief kunnen worden. Aangezien het gebruik van CTLA4-Ig voor klinische toepassingen is toegestaan door de Europese en Amerikaanse autoriteiten (FDA, EMEA) zou een klinische studie naar de adjuvans werking CTLA4-Ig voor SIT in allergische patiënten van groot belang kunnen zijn.

TGF-β bevordert de allergie-onderdrukkende werking van allergeen immunotherapie. (hoofdstuk 8)

Er is gerapporteerd dat de bloedspiegel van het afweeronderdrukkende cytokine TGF-β toeneemt na SIT in allergische patiënten. TGF-β kan op 3

verschillende mogelijkheden een rol spelen in de tolerantie na SIT. (i) TGF-β kan het aantal Treg cellen doen toenemen. (ii) TGF-β kan B-cellen aanzetten tot de productie van IgA antilichamen waarvan bekend is dat het een allergie-onderdrukkende werking kan hebben. (iii) TGF-β kan de hoeveelheid van het enzym IDO in DCs verhogen. In hoofdstuk 8 waren wij in het bijzonder geïnteresseerd om te vast te stellen of TGF-β ook bruikbaar is als een adjuvans voor SIT in ons muismodel. Het bleek dat het toedienen van TGF-β tegelijk met SIT leidt tot een sterke verbetering van de allergie-onderdrukkende werking van SIT op de eosinofiele ontsteking en IgE aanmaak maar niet op de hyperreactiviteit. Dit ging gepaard met een duidelijke verhoging van de bloedspiegel van specifiek IgA antilichaam en een toename van het percentage Treg cellen in lymfeklieren. Dit laatste lijkt in overeenstemming met onze eerdere conclusie dat Treg cellen betrokken zijn bij de onderdrukking van eosinofiele ontsteking na SIT. Omdat systemische toediening van TGF-β ongewenste bijwerkingen zou kunnen hebben, zou het van belang kunnen zijn om te trachten DCs aan te zetten tot de aanmaak van TGF-β, door bijvoorbeeld de toediening van de actieve vorm van vitamine D. In dit kader is het interessant om te noemen dat wij voorheen al hebben aangetoond dat vitamine D als adjuvans de werking van SIT sterk bevordert.

Afwezigheid van maternale overdracht van tolerantie na allergeen immunotherapie. (hoofdstuk 9)

In muismodellen van allergisch astma is aangetoond dat via de moedermelk allergenen en allergeen-specifieke IgG antilichamen kunnen worden overgebracht naar de pups, wat bij kan dragen aan de inductie van tolerantie tegen het allergeen in de pups. Omdat de serum spiegels van de allergeen-specifieke IgG antilichamen bij SIT sterk verhoogd zijn, hebben we onderzocht of de tolerantie die SIT wordt geïnduceerd ook overdraagbaar is via de moedermelk. We konden inderdaad aantonen dat de moedermelk van SIT behandelde muizen allergenen en specifieke IgG antilichamen bevatte. Dit gaf echter maar een zeer beperkte bescherming tegen de aanmaak van IgE in de pups, en geen bescherming tegen eosinofiele luchtwegontsteking. Vreemd genoeg waren pups van moeders die wel allergisch waren maar niet behandeld met SIT wel enigszins beschermd tegen eosinofiele luchtwegontsteking, ook al was er geen aanwezigheid van specifieke IgG antilichamen in de moedermelk van deze muizen. In onze experimenten waren de spiegels van de specifieke IgG antilichamen in de moedermelk aanzienlijk lager dan bekend uit gepubliceerd onderzoek. Dit komt waarschijnlijk omdat wij in ons experiment de moedermuizen tijdens de zwangerschap en de lactatie

niet aan allergeen hebben blootgesteld, wat in de voorgaande studies van andere onderzoekers wel het geval was. In de toekomst zullen we verder gaan onderzoeken wat nu precies de bijdrage is van de neutraliserende IgG antilichamen in de moedermelk aan de bescherming in de pups, en of SIT van de moeder hier nu een extra bescherming kan bieden voor het nageslacht.

Conclusies

In ons onderzoek hebben we laten zien dat SIT de verschillende aspecten van een allergische reactie op meerdere manieren onderdrukt. De meeste vooruitgang hebben we geboekt in het ontrafelen van het mechanisme waarmee SIT de allergische ontsteking remt. Wij hebben aangetoond dat neutralizerende IgG antilichamen en IL-10 productie door B cellen van belang zijn voor de onderdrukking van de eosinofiele ontsteking van de luchtwegen na SIT. Daarentegen zijn Treg cellen en de productie van IL-10 door antigeen-presenterende cellen tijdens een allergeen-provocatie juist wel belangrijk voor de onderdrukking van de eosinofiele luchtwegontsteking. We hebben ook laten zien dat deze Treg cellen niet al aanwezig hoeven te zijn tijdens de SIT behandeling, maar dat er juist door de SIT behandeling meer Treg cellen uitgroeien. Wij denken dus dat de SIT behandeling zelf nieuwe Treg cellen induceert, die bij allergeen provocatie vervolgens belangrijk zijn om de allergeen-presenterende cellen aan te zetten tot IL-10 productie, wat essentieel is voor het onderdrukken van de allergische ontsteking. We hebben ook aangetoond dat aanpassingen van de SIT behandeling die de uitgroei van Treg cellen verhogen sterk kunnen bijdragen aan de effectiviteit van SIT. Hierin liggen dus mogelijkheden voor toekomstige verbetering van de klinische behandeling met SIT.

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