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ORIGINAL ARTICLE **Experimental Models of Allergic Disease**

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

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Summary

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 (Treg cells) 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) $CD4^+$ $CD25^+$ T cells during SIT and of (ii) SIT-generated inducible $FOXP3^+$ Treg cells 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. Treg cells were quantified by flow cytometry 24 and 96 h post SIT treatment. We depleted $CD4^+CD25^+$ T cells prior to SIT, and $CD4^+FOXP3^+$ T cells prior to allergen challenges to study their contribution to the suppression of allergic manifestations by SIT treatment.

Results Our data show that depletion of $CD4^+CD25^+$ T cells at the time of SIT treatment reverses the suppression of airway hyperresponsiveness (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 Treg cells during treatment. Depletion of $CD4^+FOXP3^+$ Treg cells 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 $CD4^+CD25^+$ 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 Treg cell number or their activity during and after SIT could be of clinical relevance to improve the therapeutic effects of SIT.

Keywords allergen immunotherapy, asthma, eosinophil, FOXP3, Hyper-reactivity, IgE, regulatory T cells
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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, as it can prevent the progression of respiratory allergies to allergic asthma, as well as reduce 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 $CD4^+FOXP3^+$ Treg cells may play a role in the therapeutic effects of SIT. In clinical studies, it has been observed that the number of $CD4^+FOXP3^+$ Treg cells 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 $CD4^+CD25^+FOXP3^+$ Treg cells 1.5–2 years after the treatment [9]. Taken together, this evidence suggests that inducible Treg cells 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 (nTreg cells) 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]. Treg cells can also be derived from naïve T cells in the periphery by the acquisition of regulatory properties, inducing the outgrowth of so-called inducible Treg cells (iTreg cells) under certain conditions [11]. Both naturally occurring and inducible $FOXP3^+$Treg cells are capable of suppressing allergic asthma-like manifestations in the mouse model [12]. Interestingly, nTreg cells can facilitate the conversion of naïve $CD4^+$ T cells to different types of iTreg cells, 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 nTreg cells in maintaining a tolerogenic microenvironment during SIT injections, or for iTreg cells 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 Treg cells are critically required for the suppression of allergic manifestations by SIT. Here, we aim to test the role of Treg cells at two time points: (i) during SIT injections where $CD4^+CD25^+$ nTreg cells could facilitate the generation of allergen-specific tolerance, and (ii) at the time of allergen challenges after the completion of SIT treatment where inducible $FOXP3^+$ Treg cells could contribute to suppression of the manifestations of asthma. Previously, we have established a mouse model of experimental SIT using OVA as allergen, which is characterized by long-term suppression of antigen-induced airway manifestations of allergic asthma and up-regulation of specific IgG levels in serum [15, 16]. These suppressive effects were demonstrated to be specific for ovalbumin as treatment with an unrelated antigen, keyhole limpet haemocyanin, did not induce suppression [15]. Using this mouse model of ovalbumin-based experimental SIT, we show that depletion of $CD4^+CD25^+$ T cells during SIT partially reverses the SIT-associated suppression of airway hyperresponsiveness (AHR), but does not affect the suppression of airway eosinophilia and OVA-specific IgE in serum. Here, we demonstrate that the percentage of $CD4^+CD25^+FOXP3^+$ T cells 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.

Materials and methods

Animals

Six to eight-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. FOXP3-diphtheria toxin receptor (FOXP3-DTR) transgenic mice on Balb/c and C57BL/6 background were used to specifically deplete $FOXP3^+$ T cells *in vivo*. These mice express diphtheria toxin receptor under the control of FOXP3 promoter, and therefore administration of diphtheria toxin to these mice results in a specific depletion of all FOXP3-expressing cells [17]. The experiments using FOXP3-DTR mice have been performed twice, once on C57BL/6 and once on Balb/c background. Using two-way ANOVA and general linear model, the effects of genetic background were determined to be negligible and data were pooled. Non-transgenic littermates of FOXP3-DTR mice were used as controls.

Experimental allergic asthma, SIT

The protocol to induce experimental allergic asthma and to perform SIT is described elsewhere [18]. In brief, as shown in Fig. 1, mice received two intraperitoneal (i.p.) injections of 10 μg ovalbumin (OVA; Seikagaku Kogyo, Tokyo, Japan, endotoxin levels less than 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 10 days after the third s.c. injection, mice were exposed to OVA inhalation challenges three times. Airway responsiveness to increasing doses of methacholine was measured 24 h after the last challenge, thereafter mice were killed, bronchoalveolar lavage was performed and blood and lung samples were taken.

Treg depletion

One of the widely used approaches to deplete Tregs is using anti-CD25 (PC61) that has been shown to efficiently deplete $CD4^+CD25^+$ nTreg cells. Therefore, we used this antibody for our study. However, it cannot be excluded that other subsets of T cells such as recently activated T cells are not depleted by this approach [19]. As shown in figure 1, in experiment A, $CD4^+CD25^+$ T cells were depleted before SIT using anti-CD25 (500 μg/mouse) [19] administered i.p. 1 day before every s.c. OVA SIT injection. Control mice received rat IgG (500 μg/mouse).

In experiment B, $CD4^+FOXP3^+$ Treg cells were depleted by administration of unnicked diphtheria toxin (DT, 1 μg/mouse, Merck, Darmstadt, Germany) to transgenic FOXP3-DTR mice and to their wild-type littermates (as control for DT treatment) on two consecutive days, 2 days prior to the first OVA inhalation challenge. This approach leads to removal of more than 90% of $CD4^+FOXP3^+$ Treg cells for at least 3 days [17].

Evaluation of airway responsiveness

In the experiment using anti-CD25 antibody (experiment A), airway responsiveness to inhaled methacholine (Sigma-aldrich, Zwijndrecht, Netherlands) was measured twice (1 week before and 1 day after ovalbumin inhalation challenges) in conscious, unrestrained mice using barometric whole-body plethysmography by recording respiratory pressure curves (Buxco; EMKA Technologies, Paris, France) as described in detail previously [20].

In experiment B, airway responsiveness to increasing doses of methacholine was evaluated 24 h after the last inhalation challenge by directly measuring airway resistance by invasive methods as explained in detail elsewhere [21]. Briefly, tracheotomized (20-gauge intravenous: i.v. cannula; Becton Dickinson, Alphen a/d Rijn, the Netherlands), paralyzed (i.v. injection of Pancuronium bromide: Pavulon, 50 μg/Kg Merck Sharp & Dohme,

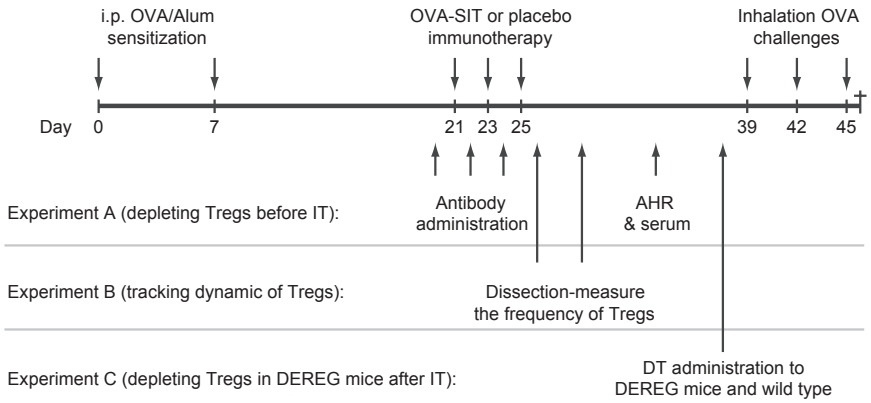

Fig. 1. Experimental set-up in evaluating the role of naturally occurring and inducible Tregs in the mouse model of SIT. Mice were sensitized by OVA (10 μg) combined with alum (2.25 mg) on day 0 and 7. SIT was performed by three s.c. injections of 1 mg OVA in SIT-treated or saline in placebo-treated mice on alternate days, 2 weeks after the last sensitization. Airway hyper-reactivity, 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. In experiment A, baseline airway responsiveness to methacholine was determined 1 week prior to OVA inhalation challenges in experiment C, a control (PBS challenge) group was used to determine baseline methacholine responsiveness. One day after the last challenge, lung function was measured and then mice were dissected. In experiment A, $CD4^+CD25^+$ nTregs were depleted using anti-CD25 (clone: PC61) antibody i.p. injected 1 day before every SIT injection. In experiment B, a group of SIT-treated and placebo-treated mice were dissected 1 day after the last SIT injection and another group of SIT-treated and placebo-treated mice were dissected 4 days after the last SIT injection. In experiment C, all $CD4^+FOXP3^+$Tregs were selectively depleted in FOXP3-DTR transgenic mice by administration of 1 μg/mouse diphtheria toxin on two consecutive days, 2 days prior to inhalation challenges.

NJ, USA) mice were attached to a computer-controlled small-animal ventilator (Flexivent; SCIREQ, Montreal, Quebec, Canada) under general anaesthesia (by i.p. injection of ketamine 100 mg/kg; Pfizer, New York, NY, USA and medetomidine 1 mg/kg; Pfizer). Mice were then ventilated at a respiratory rate 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 placed through the jugular vein for methacholine administration. Thereafter, resistance in response to intravenous administration of increasing doses of methacholine (acetyl-b-methylcholine chloride, Sigma-Aldrich) was calculated from the pressure response to a 2-s pseudorandom pressure wave.

Two different methods of measurement of AHR were used based on practical feasibility. We have previously observed that allergen challenge-induced AHR and its suppression by SIT are detectable by both direct and indirect methods of lung function measurements [21, 22].

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 [15] and results are expressed as EU/mL.

Analyses of the BAL fluid

Bronchoalveolar lavage (BAL) was performed as explained previously [21]. In brief, animals were lavaged five times through the tracheal cannula with 1-mL aliquots of saline. The first millilitre of saline contained a cocktail of protease inhibitors complete mini tablet (Roche Diagnostics, Woerden, the Netherlands) and 1% bovine serum albumin (BSA: Sigma-aldrich). BAL cells from all 5 mL were pooled, counted and cells types were indentified using flow cytometry as described elsewhere [23]. Flow cytometry data were analysed 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 [21]. 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 *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 using 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 analyse 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 six to eight animals was used in each group. Single-cell suspensions were made by gently force filtering lymph nodes through 70-μm cell strainers (BD Falcon™) using 1 mL syringe plunger. Red blood cells were destroyed by keeping the cells in ammonium chloride-containing lysis buffer for 2 min at room temperature. Cells were washed two times with ice-cold phosphate-buffered saline + 1% BSA (Sigma-aldrich) and were analysed by flow cytometry.

Flow cytometry and antibodies

PerCp labelled anti-CD4 (clone RM4-5) was purchased from BD Bioscience. PE-labelled anti-mouse FOXP3 (clone FJK-16s), eFluor450-labelled anti-mouse CD25 (clone eBio3C7) were purchased from eBioscience (San Diego, CA, USA). For flow cytometry, single cells were washed with FACS buffer (PBS + 1%BSA + 0.1% NaN_3), then incubated with antibody mixtures for 30 min on ice. Thereafter, cells were washed three times with FACS buffer. Flow cytometry was performed using LSR-II (BD bioscience) and data were analysed by FlowJo.

Statistical analysis

Data are expressed as mean ± SEM. The airway resistance curves to methacholine were statistically analysed 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. In experiments A and C, $n = 7$–9 and in experiment D, $n = 5$.

Results

Depleting $CD4^{+}CD25^{+}$T cells during SIT treatment partially reverses SIT-mediated suppression of AHR

Naturally occurring Treg cells might contribute to SIT treatment by facilitating a tolerogenic microenviron-

ment at the time of allergen injections in the sensitized host. To examine whether nTreg cells, which express high levels of CD25, are required for the induction of a suppressive allergen-specific immune response during the induction phase of SIT, mice received anti-CD25 depleting (PC61) or rat IgG control antibody, 24 h before each SIT/placebo injection (Fig. 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) (Fig. 2a). Inhalation challenges in these mice also elevated the level of OVA-specific IgE in serum (Fig. 2b), and recruited eosinophils to the airways (Fig. 2c). As expected, SIT treatment in the rat IgG control-treated group significantly suppressed AHR to methacholine (63 ± 2.4% at the peak response, $P < 0.005$, Fig. 2a), OVA-specific IgE in serum (79 ± 5.6%, $P < 0.005$, Fig. 2b) and airway eosinophilia (83 ± 3.4%, $P < 0.001$, Fig. 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 manifestations of experimental allergic asthma were efficiently suppressed by SIT treatment in this model.

To evaluate whether $CD4^+CD25^+$ Treg cells contributed to the induction of allergen tolerance during SIT treatment, we depleted these cells using CD25-specific antibodies at the time of SIT treatment [24]. Administration of anti-CD25 antibody efficiently depleted more than 90% of $CD4^+FOXP3^+CD25^+$ T cells in the spleen and blood of mice 24 h after antibody injection and that effect lasted for at least 48 h (Fig. 2d). Compared with control antibody-treated mice, anti-CD25 treatment in the placebo-treated group did not affect antigen-induced manifestations of experimental allergic asthma (Fig. 2a–c). In the SIT-treated group, however, we observed a significantly reduced suppression of AHR

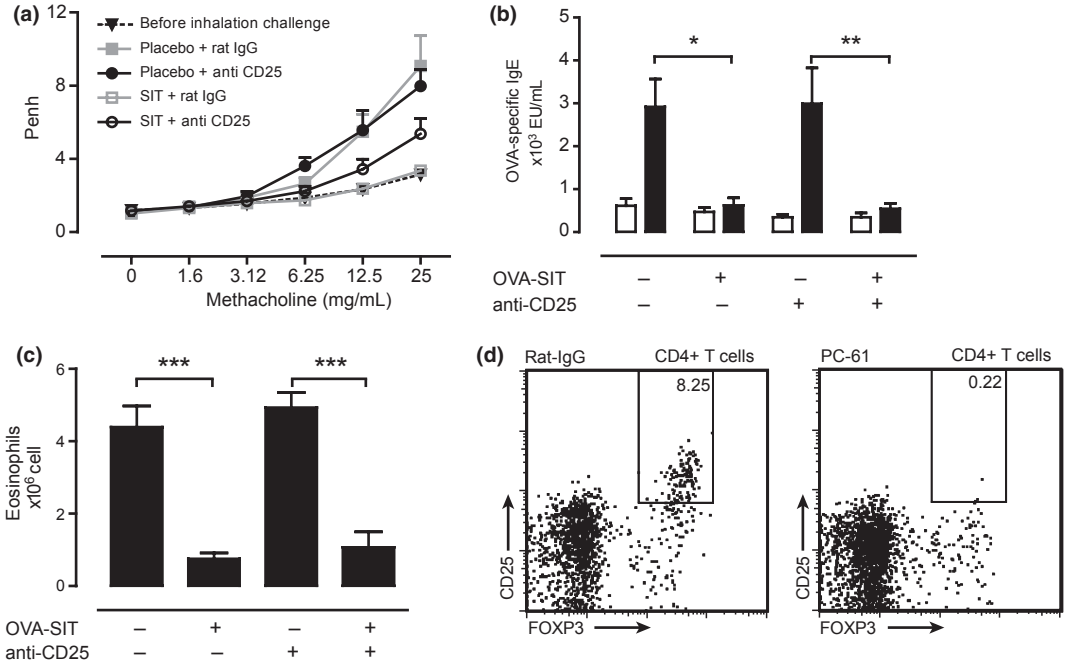

Fig. 2. The effects of depleting nTregs during SIT injections on (a) airway hyper-reactivity, (b) OVA-specific IgE in serum, (c) airway eosinophilia. ($n = 8$ per group) (a) Inhalation challenges lead to an increased airway reactivity to methacholine in placebo-treated control antibody-receiving (grey-filled squares with grey solid line, $P < 0.05$) and placebo-treated anti-CD25-receiving mice (black-filled circles with black solid line, $P < 0.05$) as compared with pre-challenge values (triangles with black dotted line). SIT treatment suppressed allergen-induced AHR in control antibody-receiving mice (unfilled squares with grey 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 with 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 with 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. (d) 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 in control vs. anti-CD25-receiving mice 2 days after antibody administration.

by SIT treatment as compared with 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 (Fig. 2a). Anti-CD25 treatment did not affect the suppression of airway eosinophilia (79 vs. 83% in control) and OVA-specific IgE levels in serum (82 vs. 79% in control) compared with control antibody receiving SIT-treated mice (Fig. 2b and c). Thus, depletion of $CD4^+CD25^+$ T cells by anti-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.

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

Next, we addressed whether SIT treatment induces an increased frequency of $CD4^+CD25^+FOXP3^+$ T cells, as has been observed in human studies [6]. To this aim, the frequency of $CD4^+CD25^+FOXP3^+$ T cells was measured in spleen, blood and pooled head and forelimb draining lymph nodes 1 and 4 days after the last SIT injection. Interestingly, we found that SIT significantly increases the number of $CD4^+CD25^+FOXP3^+$ T cells in the spleen by 98% and in the blood by 39% of placebo the first day after the last SIT injection (Fig. 3). This difference in the number of $CD4^+CD25^+FOXP3^+$ T cells between SIT-treated and placebo-treated control mice was no longer evident 4 days after the last SIT injection. Analysing the number of $CD4^+CD25^+FOXP3^+$ T cells 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 $CD4^+CD25^+$ Treg cells by the treatment.

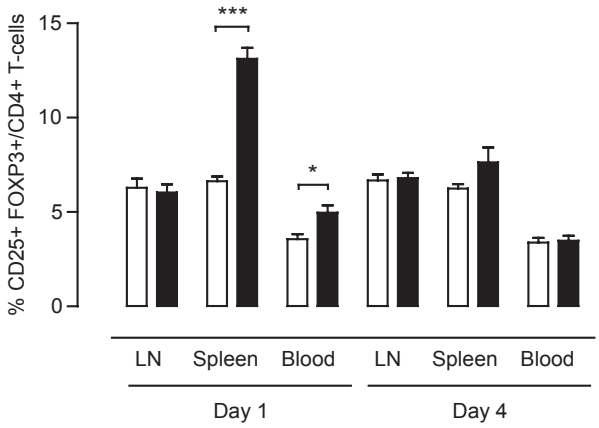

Fig. 3. Frequency of $CD4^+CD25^+FOXP3^+$ Tregs in head and forelimb draining lymph nodes, spleen and blood, 24 and 96 h after the last SIT injection. The number of $CD4^+CD25^+FOXP3^+$ Tregs in the spleen and blood were significantly increased in SIT-treated mice as compared with placebo-treated group ($P < 0.005$ and $P < 0.05$ respectively) ($n = 5$ per group).

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

To test the hypothesis that SIT induces $CD4^+FOXP3^+$-inducible Treg cells that contribute to the suppressive effects of SIT, DT was administered to FOXP3-DTR mice to selectively deplete $CD4^+FOXP3^+$ Treg cells 24 h prior to allergen challenges in SIT- or control-treated mice (Fig. 1: experiment C). DT administration in transgenic FOXP3-DTR mice resulted in more than 90% depletion of $CD4^+FOXP3^+$ regulatory T cells as compared with their wild-type littermates (Fig. 4d). DT administration did not affect the asthma manifestations in wild-type mice. 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 FOXP3-DTR transgenic mice and their wild-type littermates (Fig. 4a–c). SIT resulted in a significant level of suppression of AHR that was similar between wild-type (59%) and FOXP3-DTR mice (48%) (Fig. 4a, $P < 0.05$). Interestingly, depletion of $CD4^+FOXP3^+$ Treg cells in FOXP3-DTR mice resulted in slightly increased levels of OVA-specific IgE in serum compared with wild-type mice, while the level of suppression achieved by SIT treatment was similar between the two groups (Fig. 4a and b). In contrast, SIT caused only a mild reduction of airway eosinophilia in FOXP3-DTR mice (57.23%, $P = 0.12$), whereas the suppression in wild-type mice was much more pronounced (81%, $P < 0.05$) (Fig. 4c). Moreover, the level of airway eosinophilia in FOXP3-DTR mice was significantly higher compared with their wild-type littermates (7.8×10^5 vs. 1.9×10^5, $P < 0.01$). Thus, specific depletion of $CD4^+FOXP3^+$ Treg cells 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 $CD4^+CD25^+$ T cells for generating allergen-specific tolerance during SIT injections as well as the role of SIT-generated $CD4^+FOXP3^+$ T cells in the suppression of allergic manifestations upon allergen exposure. Using a mouse model of allergic asthma, we show that during SIT injections, $CD4^+CD25^+$ T cells are partially required

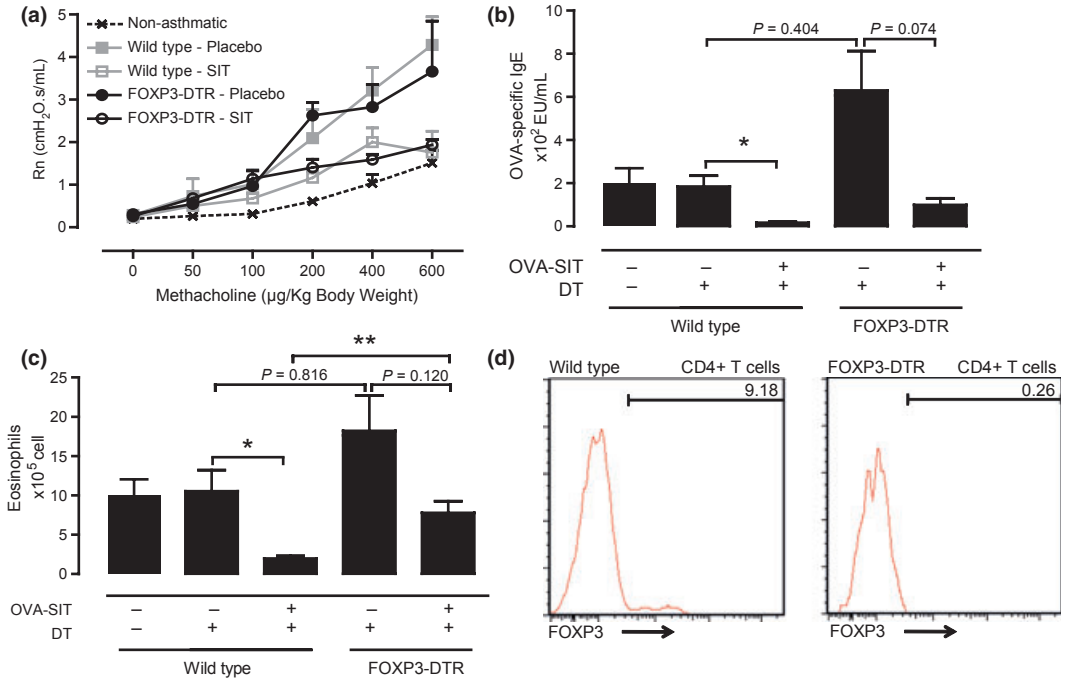

Fig. 4. The effects of selective depletion of $CD4^+FOXP3^+$Tregs in FOXP3-DTR mice after SIT on, (a) airway hyper-reactivity, (b) OVA-specific IgE in serum, (c) airway eosinophilia. (n = 7–9) (a) Inhalation challenges resulted in an increased airway reactivity to methacholine in placebo-treated DT-receiving non-transgenic (grey-filled squares with grey solid line, $P < 0.05$) and placebo-treated FOXP3-DTR mice (black-filled circles with black solid line, $P < 0.05$) as compared with pre-challenge values (triangles with black dotted line). AHR was significantly reduced in SIT-treated non-transgenic as well as FOXP3-DTR mice (unfilled squares with grey solid line and unfilled circles with black solid line, $P < 0.05$ compared with 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 with 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 FOXP3-DTR 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 with placebo-treated non-transgenic mice ($P < 0.05$). Bronchoalveolar eosinophilia in $CD4^+FOXP3^+$Treg-depleted FOXP3-DTR mice was not suppressed by SIT as compared to placebo-treated mice and it is significantly higher as compared with SIT-treated non-transgenic mice ($P < 0.01$). (d) Depletion efficacy by diphtheria toxin administration to FOXP3-DTR mice, histogram figures show the frequency of $CD4^+FOXP3^+$ T cells in the spleen of DT-treated wild-type mice vs. FOXP3-DTR mice, 2 days after administration.

for developing the SIT-induced suppression of AHR. Furthermore, we show that SIT transiently increases $CD4^+CD25^+FOXP3^+$ T cells in spleen and blood, indicating that SIT generates inducible allergen-specific Treg cells. Finally, we demonstrate that $CD4^+FOXP3^+$ T cells partially mediate SIT-induced suppression of airway eosinophilia, but not of AHR and specific IgE upon allergen provocation.

Considering that nTreg cells express high levels of CD25 and they can facilitate the generation of iTreg cells [13, 25], we evaluated the role of $CD4^+CD25^+$ T cells in tolerance induction during SIT injections. Tolerance in our model was evaluated based on the suppression of AHR, airway eosinophilia and specific IgE in serum [15]. Interestingly, when $CD4^+CD25^+$ T cells 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 [18], these data clearly demonstrate that suppression of different manifestations of experimental allergic asthma in mice including AHR, eosinophilia and specific IgE are differentially regulated by SIT. Although $CD4^+CD25^+$ nTreg cells appear to be involved in the suppression of AHR after SIT, we cannot completely exclude that other CD25-expressing T cells, e.g. recently activated T cells, were depleted by anti-CD25 and are implicated in this suppressive effect. Nevertheless, the mechanism by which $CD4^+CD25^+$ T cells specifically induce tolerance towards antigen-induced AHR remains to be elucidated. In contrast to our findings that $CD4^+CD25^+$ T cells are not critical for SIT-induced suppression of airway eosinophilia, Boudousquie and colleagues [26] 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 using s.c. injections. Different mechanisms are involved in tolerance induction through different routes of antigen administration [27, 28]. For instance, while plasmacytoid DCs are essential for tolerance induction through inhalation [27], conventional DCs play a major role in oral tolerance [28].

Although it has been shown that SIT increases the number of $CD4^+FOXP3^+$ Treg cells 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 $CD4^+FOXP3^+$ iTreg cells 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 $CD4^+CD25^+FOXP3^+$ T cells in the spleen and blood in our mouse model. Our results show that in SIT-treated mice, the frequency of $CD4^+CD25^+FOXP3^+$ T cells is increased in the spleen and blood 24 h after SIT injections, followed by a subsequent return to the level of placebo-treated mice 96 h 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 $CD4^+CD25^+FOXP3^+$ T cells to regional lymph nodes [29]. We observed no increased percentage of $CD4^+CD25^+FOXP3^+$ T cells in skin draining (axillary and accessory axillary) lymph nodes. One reason for this observation could be that upon their expansion these rapidly migrate out of the skin draining lymph nodes. Another possibility is that soluble OVA in the circulation is captured by local splenic DCs and presented to T cells in a non-inflammatory environment leading to the generation of $CD4^+CD25^+FOXP3^+$ T in the spleen. Increased $CD4^+CD25^+FOXP3^+$ T cells in the blood are most likely spleen-originated cells on their migratory path through the body. More studies are required to substantiate these speculations.

Using FOXP3-DTR mice, we show that selective depletion of $CD4^+FOXP3^+$T cells after SIT partially abrogates the suppressive effects of SIT on airway eosinophilia, suggesting that induction of $CD4^+FOXP3^+$ iTreg cells 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 $CD4^+FOXP3^+$Treg cells, again confirming that the three different cardinal features of allergic asthma namely, AHR, eosinophilia and specific IgE are differentially regulated by SIT.

Besides $CD4^+FOXP3^+$ Treg cells, TGF-β-producing Th3 cells and IL-10-producing Tr1 cells have been also implicated in SIT [30, 31]. 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 [32]. 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 [33] demonstrating that TGF-β-producing $CD4^+$ T cells are capable of suppressing 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 [30]. 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 [18]. It remains to be determined if, in addition to FOXP3 Treg cells, TGF-β producing Treg cells are involved in our mouse model of SIT.

Production of IL-10 has been frequently observed during and after SIT in clinical studies [31, 34–39], suggesting that IL-10 plays important roles in SIT in human patients. The induction of IL-10-producing Treg cells was first shown in peripheral blood mononuclear cells, which were stimulated *ex vivo* 28 days after venom SIT [32, 34]. Similar results were found after grass pollen SIT in patients with systemic anaphylaxis [40] 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 ([16] 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 iTreg cells, 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 [41]. 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 this study, we show that $CD4^+FOXP3^+$ iTreg cells are only partially involved in SIT-induced tolerance induction indicating that another, as yet unknown, subtype of $CD4^+$ $FOXP3^-$ Treg cells 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.

It is worth mentioning that while our mouse model of SIT reflects some of the aspects of clinical SIT, the experimental protocol is substantially shorter as compared with human allergen-specific immunotherapy. In line with human SIT, we have previously shown that SIT in our model induces a long-term antigen-specific suppression of the manifestation of allergic asthma [15, 16].

In summary, we show that $CD4^+CD25^+$ T cells are partly required for establishing tolerance during SIT injections. Furthermore, we show that SIT transiently increases the number of $CD4^+FOXP3^+$Treg cells in the spleen and blood and provide evidence that $CD4^+FOXP3^+$Treg cells are partially responsible for the beneficial effects of SIT. It is suggested that generation of a subtype of FOXP3-negative Treg cells is a complementary mechanism underlying the induction of full tolerance by SIT.

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