

Selective depletion of Foxp3⁺ Treg during sensitization phase aggravates experimental allergic airway inflammation

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Recent studies highlight the role of Treg in preventing unnecessary responses to allergens and maintaining functional immune tolerance in the lung. We investigated the role of Treg during the sensitization phase in a murine model of experimental allergic airway inflammation by selectively depleting the Treg population *in vivo*. DEpletion of REGulatory T cells (DEREG) mice were depleted of Treg by diphtheria toxin injection. Allergic airway inflammation was induced using OVA as a model allergen. Pathology was assessed by scoring for differential cellular infiltration in bronchoalveolar lavage, IgE and IgG1 levels in serum, cytokine secretion analysis of lymphocytes from lung draining lymph nodes and lung histology. Use of DEREG mice allowed us for the first time to track and specifically deplete both CD25⁺ and CD25[−] Foxp3⁺ Treg, and to analyze their significance in limiting pathology in allergic airway inflammation. We observed that depletion of Treg during the priming phase of an active immune response led to a dramatic exacerbation of allergic airway inflammation in mice, suggesting an essential role played by Treg in regulating immune responses against allergens as early as the sensitization phase *via* maintenance of functional tolerance.

Key words: Allergic airway inflammation · Depletion of regulatory T cells (DEREG) · Foxp3 · Treg

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Introduction

There has been a dramatic increase in prevalence of allergic disorders such as allergic rhinitis (hay fever), atopic dermatitis (eczema) and asthma in the past few decades, especially in developed countries [1, 2]. Given that allergic airway inflammation has been associated with dysregulated Th2 immune response [3], the increased prevalence of allergy has been explained by the revised hygiene hypothesis [4, 5]. This hypothesis suggests that early childhood infections prime immune-regulation and mediate protection from allergies by driving development of regulatory mechanisms rather than provoking aggressive immune responses [6, 7]. This definition, in an extension of the original hygiene hypothesis proposing a Th1–Th2 shift [8], encompasses the fact that Th1/Th17-mediated disorders such as type 1 diabetes and inflammatory bowel disorder also increased significantly during the past decades, while infectious diseases diminished [6].

Treg are accepted as the most potent immunomodulators of adaptive immunity [9], suppressing T effector cell responses. Treg have been implicated in allergic airway inflammation, where the ratio of Treg to Th2 cells has been reported to be a strong determinant of immunological outcome to common environmental antigens in humans [10]. Due to experimental limitations in specifically eliminating Treg *in vivo*, attempts to elucidate the role of Treg in controlling Th2 responses have yielded contradictory results.

In a mouse model lacking naturally occurring thymus-derived CD4⁺CD25⁺ Treg, antigen-specific induced Treg have been shown to effectively control airway inflammation [11, 12]. Other studies, on the other hand, demonstrated that while naturally occurring Treg suppress Th2-type effector functions, they were unable to curtail bronchial hyper-responsiveness [13]. The depletion of CD4⁺CD25⁺ T cells has been noted to exacerbate Th2-cell-mediated allergic inflammation [14], and intra-tracheal administration of naïve lung CD4⁺CD25⁺ T cells before immunization has been shown to reduce asthma features in an IL-10- and TGF- β -dependent manner [15]. However, a similar approach also employing CD25⁺ T-cell depletion produced conflicting results, demonstrating reduced Th2 cytokine production and no effect on eosinophilic inflammation [16]. Conversely, another study shows that mice pretransplanted with CD4⁺CD25⁺ T-cell-depleted splenocytes exhibited decreased antigen-induced eosinophil recruitment into the airways and reduced Th2 differentiation and cytokine production which were restored on supplementation with CD25⁺ T cell fraction [17]. Finally, mouse strain-specific differences have been observed in the course of CD25⁺ T-cell depletion, where depletion of Treg in an allergy resistant strain of mice C3H led to massive exacerbation of airway inflammation, while Treg depletion in the allergic sensitive mice strain A/J showed only mild differences [14].

Although most of the work has been focused on CD25⁺ Treg, CD25⁺Foxp3⁺ Treg can be found in various organs in mice [18, 19]. The CD4⁺CD25⁺ T-cell population has also been shown to efficiently prevent induction of diseases caused by exacerbated

immune responses, such as diabetes [20] and inflammatory bowel disease [21]. Previous studies addressing the role of Treg in allergic airway inflammation used depleting anti-CD25 antibodies or the transfer of *ex vivo* isolated and expanded cell populations. Thus, the existence of CD25⁺ Treg, especially within peripheral tissues [22, 23], as well as the expression of CD25 on activated conventional T cells, limits the interpretation of the above-mentioned data.

Forkhead box transcription factor Foxp3 has been identified as a key transcription factor required for Treg function and a specific marker for murine CD4⁺ Treg [24]. To address more specifically the *in vivo* role of Treg in allergic airway pathogenesis, we took advantage of bacterial artificial chromosome-transgenic mice – DEREg (DEpletion of REGulatory T cells) – expressing a diphtheria toxin (DT) receptor-eGFP fusion protein under the control of the Foxp3 locus. This allows both detection and inducible depletion of Foxp3⁺ Treg [18]. Thus, by using DEREg mice, we could specifically target and deplete Foxp3⁺ Treg including the CD25⁺ fraction, while sparing activated CD25⁺ conventional T cells. This depletion led to exacerbated allergic airway inflammation reflected by increased cellular infiltration in bronchoalveolar lavage (BAL) and cytokine production. Thereby, we specifically demonstrate the role of Foxp3⁺ Treg *in vivo* for the first time during the sensitization phase of allergic airway inflammation in mice.

Results

Treg depletion leads to enhanced eosinophil infiltration in BAL fluid in an inflammation model

To examine the effect of Treg depletion, an OVA-induced allergic airway inflammation model was used, in which mice were sensitized to OVA along with alum on days 0, 14 and 21 and challenged with OVA aerosols on days 31, 32 and 33. To achieve depletion of Foxp3⁺ Treg during sensitization, 1 μ g DT was administered i.p. to DEREg mice for three consecutive days following each sensitization. We observed a more than 96% reduction in the number of CD4⁺GFP⁺ Treg 1 day after the last DT injection in DEREg mice (data not shown). The Foxp3⁺ Treg numbers rebound to almost normal levels in DEREg mice 1 wk after withdrawal of DT treatment [18]. Hence, we rested the mice for 1 wk after the last DT injection (day 24 to day 31) before challenging the mice with OVA aerosols (Fig. 1A).

The total number of lung-infiltrating cells was counted in BAL samples by trypan blue exclusion. Treg-depleted and OVA-challenged DEREg mice showed a marked increase in total cellular infiltration in BAL compared with Treg non-depleted and OVA-challenged DEREg mice or DT-treated/non-treated WT mice (Fig. 1B). Differential count of leukocytes was performed by Diff-Quik staining and percentage of each cell type, namely eosinophils, lymphocytes, macrophages and neutrophils, was determined. The total number of each cell type was calculated on the basis of their percent infiltration and

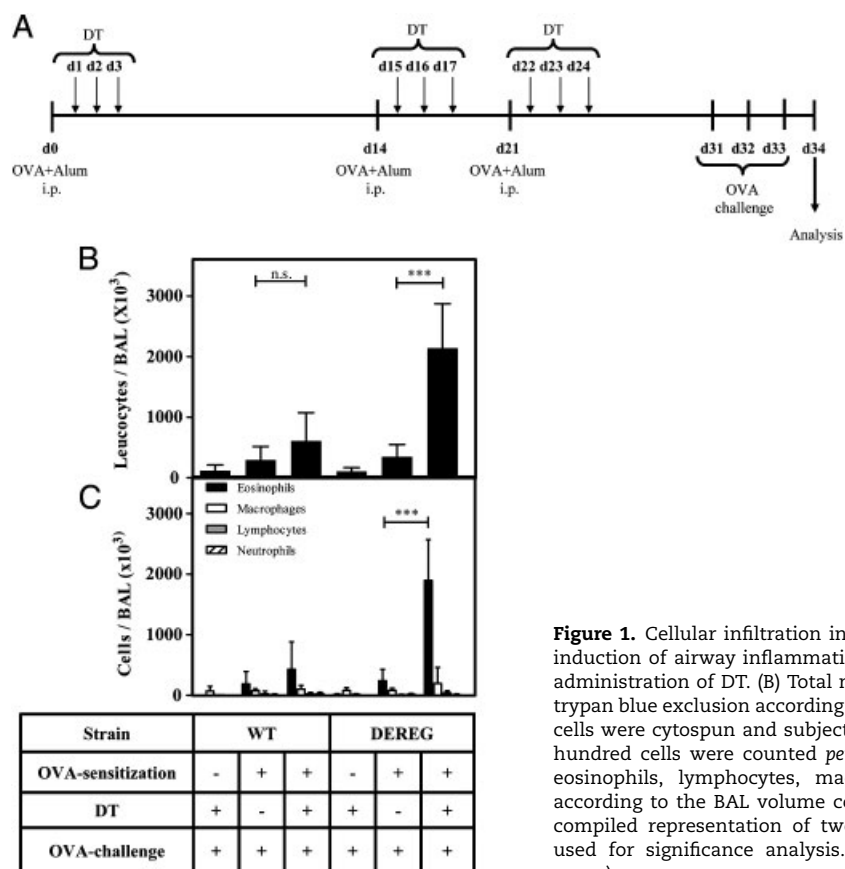


Figure 1. Cellular infiltration in BAL: (A) representation of the regimen followed for induction of airway inflammation using OVA and concomitant depletion of Treg via administration of DT. (B) Total number of cells infiltrating the BAL was calculated by trypan blue exclusion according to the BAL volume recovered *per mouse*. (C) $5\text{--}10 \times 10^4$ cells were cytospun and subjected to differential count after Diff-Quik staining. Two hundred cells were counted *per slide* from random fields and the total number of eosinophils, lymphocytes, macrophages and neutrophils estimated *per sample* according to the BAL volume collected. Data are expressed as mean $\pm$ SD and are a compiled representation of two separate experiments. Mann–Whitney U-test was used for significance analysis. *** $p < 0.001$, n.s. = not significant ($n = 4\text{--}6$ mice *per group*).

the total number of infiltrating cells in the BAL. Differential count of the infiltrating cells revealed an enhanced eosinophilia in BAL fluid from mice that were sensitized and challenged with OVA, when compared with control (sham sensitized) mice (Fig. 1C), in both WT and DERE groups. A further significant eosinophil infiltration was observed in Treg-depleted DERE mice (Fig. 1C) when compared with non-depleted and challenged DERE mice, indicating a heightened inflammatory response in these animals.

Increased serum Ig levels in Treg-depleted DERE mice in response to allergen challenge

OVA-specific serum immunoglobulin levels were determined by ELISA. OVA-specific IgG2a levels were below detection limits in all sera samples tested (data not shown). As expected, all the mice that were sensitized to OVA in the presence of alum had high titers of OVA-specific IgG1 in their sera, indicating a Th2-biased immune response (Fig. 2B). Interestingly, the OVA-specific IgG1 responses were equivalent in all the groups of mice that were sensitized irrespective of DT treatment; however, DERE mice depleted of Treg showed significantly higher levels of OVA-specific IgE in their serum when compared with their Treg non-depleted counterparts (Fig. 2A), indicating an enhanced allergic humoral response in the absence of Treg.

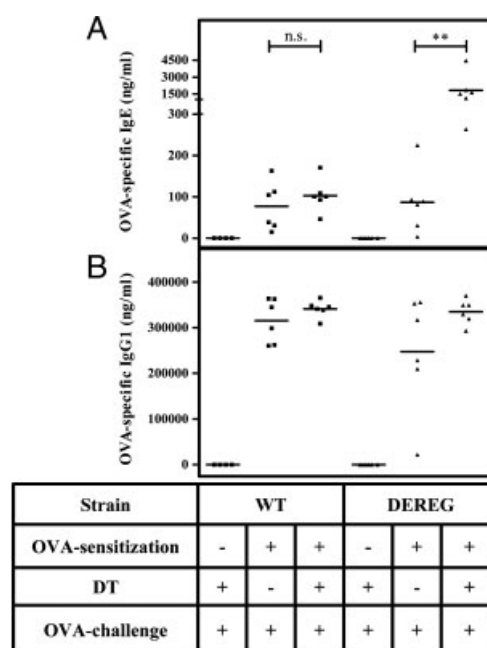


Figure 2. OVA-specific serum immunoglobulins (Ig). Using OVA as capture antigen and specific anti-isotype antibodies, (A) OVA-specific IgE and (B) IgG1 levels were determined in sera by ELISA. Data shown here are a representation of two separate experiments. Horizontal bars represent geometric means. Statistical significance was analyzed with Mann–Whitney U-test. ** $p < 0.01$, n.s. = not significant ($n = 4\text{--}6$ mice *per group*).

Enhanced cytokine production in DEREg mice depleted of Treg

Total single-cell suspensions obtained from mediastinal lymph nodes of mice were cultured in the presence of 100 µg/mL OVA for 72h. The supernatants from these cultures were analyzed for titers of IL-4, IL-5, IL-13 and IFN-γ by ELISA. There were no significant differences observed in cytokine levels produced by WT mice and Treg non-depleted DEREg mice; however, DEREg mice depleted of Treg showed significantly higher levels of IL-4 (Fig. 3A), IL-5 (Fig. 3B) and IL-13 (Fig. 3C). Very low levels of IFN-γ were detectable in samples from all groups of mice and there was no significant difference in the IFN-γ levels from Treg-depleted or non-depleted DEREg mice (data not shown).

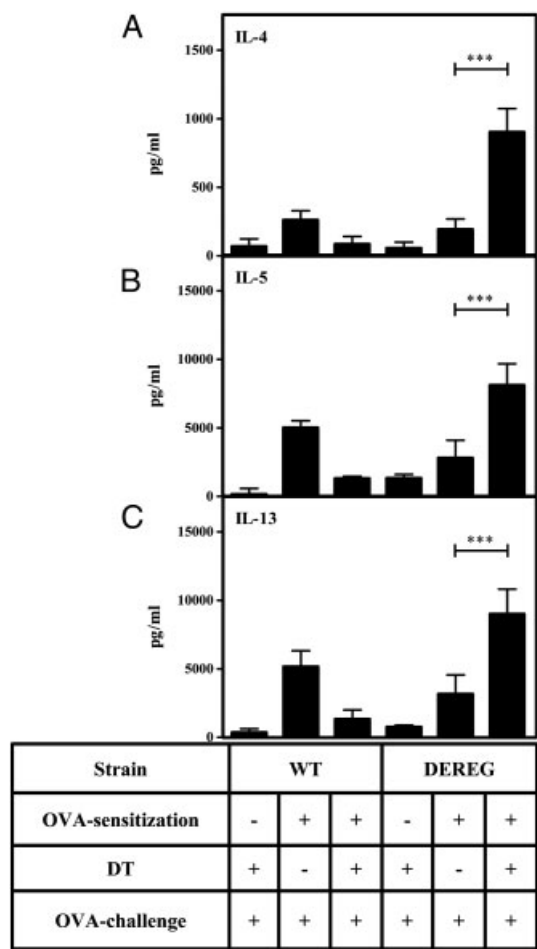


Figure 3. Cytokine production by re-stimulated mediastinal lymph node cells. Levels of IL-4 (A), IL-5 (B) and IL-13 (C) were measured from cell-free supernatant of *in vitro* re-stimulated lung draining lymph node cells by ELISA. Data are expressed as mean±SD and are a compiled representation of two separate experiments. Mann-Whitney U-test was used for significance analysis. ****p*<0.001, n.s. = not significant (*n* = 4–6 mice per group).

Treg depletion leads to enhanced cellular infiltration but does not influence mucus production

DEREG mice depleted of Treg showed increased cellular infiltration in their lungs when compared with non-depleted DEREg mice and WT controls as observed by hematoxylin–eosin staining (Fig. 4A). Quantitative analysis of lung sections stained with periodic acid–schiff (PAS) stain demonstrated a tendency toward higher mucus production by Treg-depleted DEREg mice when compared with their non-depleted counterpart (Fig. 4B and C). Interestingly, there was significantly higher amount of stored mucus seen in the lungs of sham-sensitized DT-treated and OVA-challenged DEREg mice. We also noted that the amount of mucus quantified in lungs of Treg-depleted DEREg mice was similar to that measured in the WT mice sensitized to OVA and challenged (Fig. 4C).

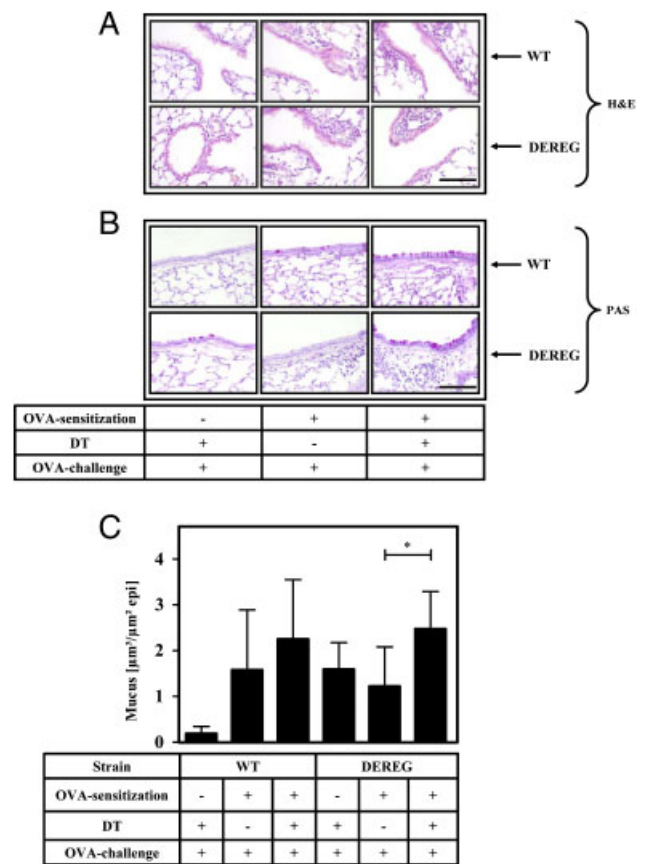


Figure 4. Lung histology: 3-µm sections of paraffin embedded lungs were stained with H&E (A) or PAS (B) stains (scale bar = 100 µm). (C) Mucus deposition was quantified and is expressed as volume of mucus deposited per µm² epithelial basement membrane area. Data are expressed as mean±SD and are a compiled representation of two separate experiments. Mann-Whitney U-test was used for significance analysis. **p*<0.05 (*n* = 4–6 mice per group).

Discussion

In this study, we demonstrate the critical involvement of all Foxp3⁺ (CD25⁺ and CD25[−]) Treg in maintaining a tolerant environment in the lung using a mouse model of allergic airway inflammation. Unlike other similar models [25], adult DERE mice do not show any kind of lung pathology merely as a result of Treg depletion [26]. This was also confirmed in our current study using a group of sham-sensitized and OVA-challenged DERE mice, which received DT treatment for Treg depletion. This group of mice did not develop features of allergic airway inflammation.

Depletion of Foxp3⁺ Treg in mice during the sensitization phase led to augmented features of allergic response, such as increased levels of cellular infiltration (mostly due to an increase in eosinophils in the BAL), increased levels of serum IgE. Higher levels of cytokines IL-4, IL-5 and IL-13 secreted by *in vitro* re-stimulated DERE lung draining lymph node cells further indicate an enhancement of inflammatory response. Our results indicate a highly exacerbated inflammation seen in DERE mice depleted of Treg when compared with WT or Treg non-depleted DERE mice.

Many studies have attempted *in vivo* Treg depletion in mouse models of allergic airway inflammation using anti-CD25 antibodies. The outcomes, however, have been contradictory. Depletion of CD25⁺ cells led to enhanced lung pathology in some studies [14, 16], while others found diminished pathology on transplantation with CD25⁺ T-cell-depleted splenocytes [17]. The existing CD25[−] Treg population and anti-CD25-mediated depletion of effector T cells could be the cause of these discrepancies observed [22, 23]. Lewkowich *et al.* have shown that depletion of CD25⁺ cells in a susceptible mouse strain (A/J) leads to a moderate increase in airway eosinophilia without affecting IgE levels, airway hyperactivity or cytokine expression. In a comparatively resistant strain (C3H), by contrast, a highly aggravated allergic pathology developed upon depletion of CD25⁺ cells [14]. This could be explained by either a putatively enhanced Th-cell response and higher CD25 expression on effector T cells in allergy susceptible strains, or by a generally weaker Treg activity in these mice. Both would result in milder differences between CD25⁺ T-cell-depleted and undepleted mice. Use of DERE mice allows these issues to be addressed for the first time by permitting selective depletion of Foxp3⁺ Treg including CD25[−] Treg population without affecting the CD25⁺ effector T-cell population. The data presented in this study pertain to DERE mice on a C57BL/6 background strain that is comparatively resistant to allergic airway inflammation. The results are consistent with the Lewkowich *et al.* data [14]. We have now generated DERE mice on the susceptible BALB/c background, and preliminary results in these animals suggest that Treg indeed play a major role in controlling allergic airway inflammation also in this strain (unpublished data).

An interesting observation in this study was the enhanced mucus production observed in sham-sensitized and Treg-depleted DERE mice. Although not sensitized to OVA, these mice did receive alum as a strong Th2-driving adjuvant. We believe that the enhanced mucus production seen in these mice is due to a non-specific provocation that becomes synergistically augmented in the absence of Treg. This phenomenon was not seen in WT mice even upon administration of

DT, confirming that this is a direct effect of Treg depletion in DERE mice and not due to any side-effects caused by DT itself.

There was a tendency toward higher mucus production in Treg-depleted DERE mice when compared with their non-depleted counterpart. Although other parameters of allergic inflammation, such as eosinophil recruitment to airways, were significantly augmented in Treg-depleted DERE mice, there was no statistically significant difference observed in mucus production across groups of WT or DERE mice that were sensitized and challenged with OVA, whether in the presence or absence of Treg in the latter groups. By transfer of *in vitro* differentiated Th1 and Th2 cells, Cohn *et al.* have shown that mucus production by epithelial cells and recruitment of eosinophils to the airways are controlled by different mechanisms [27]. Hence, there is a possibility that Treg do not influence the pathway involved in mucus production under conditions employed in our study regimen. It has also been shown that mucus production is a two-step process involving EGFR phosphorylation in epithelial cells followed by IL-13-induced conversion of these cells into mucus-producing goblet cells [28]. EGFR can be promiscuously activated by multiple ligands (TNF- α , EGF or reactive oxygen species), which are induced upon airway damage [29]. We speculate that the regimen followed in this study (*i.e.* three sensitizations and three challenges) induces considerable damage to the airway epithelial layer and the system is already pushed to its threshold in terms of activating primary signal involved in mucus production in both WT and DERE mice. Additionally, even the low titers of IL-13 produced in WT mice could strongly aid epithelial cell conversion to goblet cells, resulting in no observable difference between mucus production between WT and Treg-depleted DERE mice. Furthermore, the extent to which Treg control EGFR activation on epithelial cells and their conversion to goblet cells remains largely unknown.

Using mice lacking natural Treg, Curotto de Lafaille *et al.* have demonstrated that induced Treg can indeed confer functional tolerance in a mouse model of acute allergic asthma [12]. This study has been performed in an allergen biased T- and B-cell repertoire transgenic mouse model. In our study, using DERE mice harboring unbiased T- and B-cell repertoire, we have been able to deplete all Foxp3⁺ Treg and have demonstrated their role in maintaining tolerance, as one would expect in a more natural setting of an asthmatic flare. Hay fever has been considered as a risk factor for asthma and up to 78% of asthma patients have coexisting allergic rhinitis [30, 31]. Based on our experimental data, one could speculate that the sensitization phase of allergic airway inflammation may represent the critical phase when a lack of or dysfunction of Treg may have dramatic consequences for chronic airway disease. DERE mice have provided a more specific tool, and our data demonstrate the pivotal role played by Treg during the sensitization phase of allergic airway inflammation signifying their importance. In the future, we intend to investigate the role of Treg during the challenge phase and observe whether Treg depletion could augment an established immune response generated in the presence of Treg (during the sensitization phase) against a given antigen. Addressing the role of Treg during different phases of allergic diseases for maintenance of functional tolerance and deciphering the mechanisms of anti-allergic immune regulation will provide novel custo-

mized therapeutic avenues and prophylactic approaches in clinical settings.

Materials and methods

Animals

DEREG mice and C57BL/6 littermate controls were bred at the animal facility of the Institute for Medical Microbiology, Immunology and Hygiene (TU Munich, Germany) and at Helmholtz Centre for Infection Research (HZI, Braunschweig, Germany). Six- to 12-wk-old sex-matched mice were used for all experiments. All animals were housed under specific pathogen-free conditions and experiments were performed according to guidelines approved by institutional, state and federal committees.

OVA-induced allergic airway inflammation

Mice were sensitized to OVA (Sigma, Deisenhofen, Germany) by injection of 10 µg OVA i.p. (grade VI) adsorbed on 1.5 mg aluminum hydroxide (Sigma) in a total volume of 200 µL PBS on days 0, 14 and 21. A group of OVA-sensitized DEREG and WT mice received 1 µg DT injection i.p. on three consecutive days after every sensitization and were later challenged by exposure to OVA aerosol with 1% OVA (grade V) in PBS delivered by Pari-Master nebulizer (PARI GmbH, Starnberg, Germany) for 20 min on three consecutive days from day 31 onward. Another group of OVA-sensitized DEREG and WT mice were treated with PBS instead of DT and were challenged as mentioned above. Sham-sensitized (PBS+Alum) WT and DEREG mice, which were DT-treated and challenged with OVA, were used as negative controls. Animals were sacrificed and analyzed 24 h after the last challenge.

Bronchioalveolar lavage (BAL)

Animals were sacrificed by anesthesia (lethal dose of injection of Ketamine and Rompun i.p.) and their tracheae cannulated. Airways were flushed twice with 1 mL ice-cold PBS. BAL fluid was centrifuged at 1500 rpm (at 4°C for 5 min) and total cell number was calculated using trypan blue exclusion dye. Approximately $5\text{--}10 \times 10^4$ cells in 100 µL PBS were used for cytospin (700 rpm, 5 min; Cytospin3; Shandon). Slides were stained with Diff-Quik staining kit (Medion Diagnostics, Germany) according to the manufacturers' protocol for estimation of infiltrating leucocytes. Two hundred leucocytes were counted *per slide* from random fields.

Measurement of OVA-specific Ig in serum

To measure OVA-specific IgE, IgG1 and IgG2a, 0.5 µg OVA (Grade V, Sigma) was coated overnight onto Maxisorp microtiter plates

(Nunc, Roskilde, Denmark) in PBS at 4°C. After blocking with 1% BSA in PBS, samples and standards (IgG1- clone OVA-14, Sigma-Aldrich; IgE, clone-2C6, Abd Serotec; IgG2a, clone-HYB 094-07, AntibodyShop) were added and incubated overnight at 4°C. Detection limit was 3.125 ng/mL for IgE and IgG1 and 1.56 ng/mL for IgG2a. After washing with PBS containing 0.05% Tween-20, specific bound antibodies were probed with biotinylated anti-mouse IgG1 (A85-1, Pharmingen) or anti-mouse IgE (R35-92, Pharmingen) or anti-mouse IgG2a (R19-15, Pharmingen) for 1 h at 37°C. For detection of biotinylated antibodies, streptavidin-conjugated alkaline phosphatase (Strep-AKP; Pharmingen) was added after washing the plates and incubated for 1 h at 37°C. Phosphatase substrate (Sigma) was added after final washings and plates were allowed to develop at room temperature. The reaction was stopped after about 20 min and absorbance was measured at 450 nm with a 570-nm filter as reference.

Lung histology and quantification

The lungs were fixed by intra-tracheal instillation of 4% buffered paraformaldehyde, ligated and stored in fixative until further use. As the airway tree is anisotropic, lung orientation was randomized using an orientator [32]. Uniform systematic random samples were embedded into paraffin, and 3 µm sections were stained with hematoxylin–eosin stain or PAS stain to estimate cellular infiltration and mucus production.

Systematic uniform random samples of lung tissue were taken according to standard methods. Airway epithelial mucus was identified by means of PAS staining. The surface area of mucus-containing goblet cells (S_{gc}) *per* total surface area of airway epithelial basal membrane (S_{ep}) and the volume of PAS-stained epithelial mucus (V_{mucus}) *per* S_{ep} were determined using a computer-assisted stereology tool (newCAST®; Hiersholm, Visiopharm, Denmark) according to the formulas [33].

$$\frac{S_{gc}}{S_{ep}} = \frac{\sum I_{gc}}{\sum I_{ep}}$$

$$\frac{V_{mucus}}{S_{ep}} = LP \times \frac{\sum P_{mucus}}{2 \times \sum I_{ep}}$$

where $\sum I_{gc}$ is the sum of intersections of test lines with goblet cells; $\sum I_{ep}$ the sum of all intersections of test lines with epithelial basal membrane; $\sum P_{mucus}$ the sum of all points hitting mucus; and LP the test line length at final magnification.

Re-stimulation of T cells from lung draining lymph nodes and cytokine analysis from supernatants

The cytokine secretion profile of T cells from lung draining lymph nodes was determined using an *in vitro* re-stimulation assay. Single-cell suspensions from mediastinal lymph nodes were obtained and $1\text{--}2 \times 10^6$ total cells were seeded in 96-well round-bottom plates in 200 µL cRPMI medium containing

100 µg/mL OVA. Culture supernatants were harvested after 72 h and frozen at -80°C until further use. IL-4, IL-5, IL-13 and IFN- γ were measured in cell-free supernatants by ELISA using matched antibody pairs purchased from R&D systems. ELISA assays were performed according to the manufacturer's directions.

Statistical analysis

Data represented as mean $\pm$ SD. Comparative groups were tested for statistical significance using non-parametric Mann–Whitney U-test in GraphPad Prism software (Prism 5). *p*-Values less than 0.05 were considered statistically significant. n.s. is non-significant, **p* ≤ 0.05; ***p* ≤ 0.01 and ****p* ≤ 0.001.

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Abbreviations: BAL: bronchioalveolar lavage · DEREg: DEpletion of REGulatory T cells · DT: diphtheria toxin · PAS: periodic acid–schiff

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