IL-10 and Regulatory T Cells Cooperate in Allergen-Specific Immunotherapy To Ameliorate Allergic Asthma

Livia Böhm, Joachim Maxeiner, Helen Meyer-Martin, Sebastian Reuter, Susetta Finotto, Matthias Klein, Hansjörg Schild, Edgar Schmitt, Tobias Bopp and Christian Taube

*J Immunol* published online 19 December 2014
http://www.jimmunol.org/content/early/2014/12/19/jimmunol.1401612
IL-10 and Regulatory T Cells Cooperate in Allergen-Specific Immunotherapy To Ameliorate Allergic Asthma

Livia Böhm,* Joachim Maxeiner,* Helen Meyer-Martin,† Sebastian Reuter,‡ Susetta Finotto,‡ Matthias Klein,* Hansjörg Schild,* Edgar Schmitt,* Tobias Bopp,*,1 and Christian Taube6,1

Human studies demonstrated that allergen-specific immunotherapy (IT) represents an effective treatment for allergic diseases. IT involves repeated administration of the sensitizing allergen, indicating a crucial contribution of T cells to its medicinal benefit. However, the underlying mechanisms of IT, especially in a chronic disease, are far from being definitive. In the current study, we sought to elucidate the suppressive mechanisms of IT in a mouse model of chronic allergic asthma. OVA-sensitized mice were challenged with OVA or PBS for 4 wk. After development of chronic airway inflammation, mice received OVA-specific IT or placebo alternately to airway challenge for 3 wk. To analyze the T cell–mediated mechanisms underlying IT in vivo, we elaborated the role of T-bet–expressing Th1 cells, T cell–derived IL-10, and Ag-specific thymic as well as peripherally induced Foxp3+ regulatory T (Treg) cells. IT ameliorated airway hyperresponsiveness and airway inflammation in a chronic asthma model. Of note, IT even resulted in a regression of structural changes in the airways following chronic inhaled allergen exposure. Concomitantly, IT induced Th1 cells, Foxp3+, and IL-10–producing Treg cells. Detailed analyses revealed that thymic Treg cells crucially contribute to the effectiveness of IT by promoting IL-10 production in Foxp3-negative T cells. Together with the peripherally induced Ag-specific Foxp3+ Treg cells, thymic Foxp3+ Treg cells orchestrate the curative mechanisms of IT. Taken together, we demonstrate that IT is effective in a chronic allergic disease and dependent on IL-10 and thymic as well as peripherally induced Ag-specific Treg cells. The Journal of Immunology, 2015, 194: 000–000.

he prevalence of allergic diseases has dramatically increased in the developed world during the last decades (1, 2). Allergies are associated with the production of allergen-specific IgE and with the expansion of allergen-specific T cell populations. These T cells are mainly classified as Th2 cells and produce characteristic cytokines such as IL-4, IL-5, and IL-13 and promote eosinophilic inflammation (3, 4). Chronic allergic disorders such as allergic asthma are caused by persistent or repetitive exposure to allergens (5). Asthma is defined by reversible lung inflammation and bronchospasm, but is also accompanied by structural alterations. These pathological structural changes lead to thickening of the airway wall, goblet cell metaplasia, and subepithelial fibrosis (6).

Currently, allergen-specific immunotherapy (IT) is the only causative treatment that can affect the natural course of allergy and reduces the risk to develop allergic asthma (7). The goal of IT is to change the immune response to specific Ags, which results in long-term benefits even following the cessation of therapy (8). To achieve this goal, peripheral T cell tolerance seems to be crucial (9). IT is proven to be highly effective for treatment of insect venom allergies and pollen-induced allergic rhinitis and conjunctivitis (10, 11). However, the effectiveness of IT in allergic asthma remains controversial, which is mainly due to small and methodological limited clinical studies (12). A better understanding of the underlying immunological effects of IT might help to find the effective mechanism of this treatment. Different immunological alterations following IT have been identified. IT causes the production of IgG Abs that are supposed to block mast cell degranulation and compete with IgE for the allergen (13). Furthermore, a shift from the pathogenic Th2 to Th1 cells was described that regulates the Th2-mediated inflammation via IFN-γ (14, 15). Furthermore, regulatory T (Treg) cells have been associated with the effectiveness of IT. Two general subsets are described as Treg cells, the natural Treg (nTreg) cells and the adaptive or induced Treg (iTreg) cells. nTreg cells are defined as thymic derived and express the transcription factor Foxp3 as well as the IL-2R α-chain (CD25) (16). iTreg cells include Foxp3+ cells that develop in the periphery and Foxp3− cells that exert their regulatory function by secretion of TGF-β and IL-10 (17). Those cells are often called regulatory Th3 or type 1 regulatory T (Tr1) cells. Both subsets, Foxp3+ and Foxp3− Treg cells, play a role in IT. Some studies identified IL-10 production (15, 18–20), and some studies discovered FOXP3+ Treg cells in IT-treated human subjects (21, 22) or both populations (23). Also, in murine models, IT has shown effects on the development of airway inflammation and airway hyperresponsiveness (AHR)
However, in most of the studies, IT was performed prior to first allergen challenge, and the effect of IT on already established airway disease is less well understood. Indeed, effects of IT on already established airway disease as well as structural changes in the airways, such as remodeling (28), are not well described. Therefore, we investigated whether IT is effective in the suppression of allergic airway inflammation in a chronic model of established allergic airway disease. We found that, in this chronic model, IT is effective in suppressing airway inflammation, AHR, and airway remodeling, independent from Th1 responses, but dependent on Treg cells and IL-10 signaling.

Materials and Methods

Animals

BALB/cJ mice (Janvier), BALB/c DO11.10, BALB/c DO11.10 Rag2−/−, and BALB/c T-het−/− mice were kept in the central animal facility of the University Medical Center of Johannes Gutenberg University in Mainz. All procedures were conducted in accordance with current federal, state, and institutional guidelines, and all experiments were approved by the local regulatory authorities. All mice entered the experimental protocol at the age of 6–10 wk.

Experimental protocols

To assess the effects of IT in a chronic model, female BALB/c mice received i.p. injections of 40 μg OVA (Sigma-Aldrich), complexed with Alum (Thermo Fisher Scientific) or PBS i.p. on days 0, 1, 4, and 21. Afterward, anesthetized (Ketamine/Rompun; Bayer) mice received intratracheal (i.t.) instillations of OVA in PBS (40 μg per injection) or PBS alone twice per week for 4 wk. On day 49, some mice were sacrificed to determine the status of AHR and airway inflammation. Therefore, airway plethysmography (Buxco Electronics) and bronchoalveolar lavage (BAL) were performed, and blood, thoracic lymph nodes, and lungs were isolated. From lymph nodes and the left lobe of the lung, single-cell suspensions were prepared, as described previously (29). Cells were investigated via FACS LSRII (BD Biosciences). Subsequently, remaining mice again received OVA in PBS i.t. twice per week. Mice received s.c. injections of OVA in PBS (1 mg per mouse) as IT or placebo (PBS) three times per week. On day 70, airway plethysmography was performed, followed by a BAL and isolation of lung, lymph nodes, and blood (see also Fig. 1A). For histopathology, lungs were fixed by inflation and immersion in 10% formalin and embedded in paraffin. Administration of Abs in the chronic model was done on days 57 and 64. Anti–IL-10R Ab (BD Biosciences) was injected in PBS i.p. 250 μg per mouse. Rat Ig (Sigma-Aldrich) was injected in the same dosage as control Ig.

To assess the effect of IT in DO11.10 transgenic mice, OVA in PBS (1 mg per mouse) or placebo (PBS) was injected s.c. on days 1, 4, and 7. On day 13, mice were bled via the tail vein. On days 15, 16, and 17, mice were aerosolized with OVA in PBS in a chamber connected to an aerosol generator (FMI). Forty-eight hours later, airway plethysmography was performed, followed by a BAL (Fig. 1B).

Intratracheal injection of OVA

Mice were anesthetized by i.p. injection with Ketamin/Xylazin mixture and fastened on a restraining device. The tongue was extended with forceps to open the mouth. With cold light illumination, a sterile crystal tip was inserted and placed between the vocal cords. Correct insertion was proven by movement of the liquid surface in the crystal tip. Then 20 μl PBS or OVA solution (2 mg/ml) was instilled per injection.

Assessment of the allergic airway disease

To analyze the allergic airway disease, AHR was measured and the cellular composition of the BAL was determined. Airway inflammation, goblet cell metaplasia, and airway remodeling were evaluated by appropriate stainings on fixed lung tissue sections. Titers of Ag-specific Ig in the sera were determined via ELISA.

Measurement of AHR

Mice were anesthetized by injection with narcoren and intubated. Lung function test was performed with invasive plethysmograph (Buxco Electronics).

Bronchoalveolar lavage

Lungs were lavaged via the trachea with 1.0 ml PBS. BAL fluid was centrifuged, and cells were suspended in 200

FIGURE 1. Experimental designs of mouse models of allergen immunotherapy for chronic and acute experimental airway inflammation. (A) OVA-sensitized or nonsensitized BALB/c mice received airway challenge weekly by two i.t. injections of OVA solution or PBS. After day 49, mice received OVA-IT or placebo (PBS) three times per week. Before administration of OVA-IT (day 49) and after OVA-IT (day 70), analysis was performed. Intervention with anti–IL-10R Ab was achieved during OVA-IT. (B) DO11.10 mice or DO11.10 Rag−/− received OVA-IT or placebo by s.c. injections. Eight days after OVA-IT, mice were challenged with OVA aerosol on consecutive 3 d. Forty-eight hours later, mice were sacrificed and parameters were analyzed.
Lymph node and lung single-cell preparation

Lung lobes for flow cytometry and restimulation were mechanically disrupted and enzymatically digested with 280 U/ml collagenase type IA (Sigma-Aldrich) at 37°C. Single-cell suspensions were generated by resuspending digested tissue through a 0.9/40-mm cannula and pushed through a 70-μm nylon cell strainer. After centrifugation, RBCs were removed by osmotic lysis. After washing with PBS, cell counts were determined and adjusted. Lymph nodes were mechanically minced. After washing with HBSS, cell counts were determined.

FACS analysis

FACS analysis was used to identify Th cells and Treg populations in lung and draining lymph node ex vivo.

Single-cell suspensions of lymph nodes and lungs were made under sterile conditions according to the surface staining. To analyze intracellular cytokines, lung cells were treated as already described (30). Unspecific surface-binding areas were blocked by adding FcR blocking Abs (anti-CD16/CD32; BD Biosciences, Heidelberg, Germany). Cells were stained on the surfaces with FITC-labeled anti-CD3 (BD Biosciences), PE-labeled KJ1-26 Ab (BD Biosciences), and PE-Cy7-labeled anti-CD4 (clone RM4-5; BD Biosciences). For intracellular cytokine analysis, we used allophycocyanin- or FITC-labeled IFN-γ (XMG1.2; BD Biosciences), PE- or allophycocyanin-labeled IL-10 (JES5-16E3; BioLegend), and PE-labeled IL-5 (TRFK5; BD Biosciences). To analyze CD4+CD25+ Foxp3+ Treg cells, single-cell suspensions of tracheal lymph nodes, blood, or lung cells were adjusted and surface stained with PE-Cy7– or Pacific Blue–labeled anti-CD4 (clone RM4-5) and Alexa-488–labeled anti-CD25 (clone PC61; all BD Biosciences), as described. DO11.10 TCR (KJ1-26; BioLegend) was stained in experiments with DO11.10 TCR-expressing cells. After surface staining and washing in PBS, cells were resuspended in fixation buffer (eBioscience) twice, and subsequently, allopunchocyanin-labeled anti-Foxp3 (Miltenyi Biotec) was added.

FACS analysis was performed by gating for singlets (forward light scatter [FSC]-H versus FSC-A) and lymphocytes (side light scatter-A versus FSC-A). The lymphocyte gate is further analyzed for the expression of CD4 (and expression of DO11.10 TCR by detection of KJ1-16 Ab in DO11.10 transgenic mice). Expression of IFN-γ, IL-10 or Foxp3, and CD25 was then determined from CD4 (and KJ1-26+) population.

Measurement of OVA-specific Igs

Blood for assessment of serum Igs was collected during the readout 48 h after the last challenge. Serum was obtained, and OVA-specific IgG1 and IgG2a levels in serum were measured by ELISA, as previously described (31). Biotinylated Abs against IgG1 and IgG2a and streptavidin-connected HRP were obtained from BD Biosciences and used according to the manufacturer’s instructions. OVA-specific IgE was assessed using anti-IgE (clone EM-95.3, purified from hybridoma cultures) coated on microtiter plates (Thermo Fisher Scientific) and incubated with diluted sera. Afterward, biotinylated OVA was used and detected with streptavidin-conjugated HRP (BD Biosciences). HRP was conjugated and color development was performed by incubation with tetramethylbenzidine substrate solution (BD Biosciences) and stopped by adding H2SO4 (2 N). OD was detected at 450 nm. The Ab titer was defined as the reciprocal serum dilution yielding an absorbance reading of OD = 0.2 after linear regression analysis.

FIGURE 2. Reduction of AHR, IgE, and allergic airway inflammation by OVA-IT in a chronic asthma model. (A) Airway resistance following methacholine (MCh) challenge in PBS/PBS (n = 6), PBS/OVA (n = 7), OVA/OVA (n = 15), and OVA-sensitized, challenged, and OVA-IT–treated mice (OVA IT, n = 15). (B) Enumeration of eosinophils in BAL. (C) Frequencies of IL-5–producing CD4+ T cells in tracheal lymph nodes (tLN) and lung of IT-treated mice, calculated as percentage of CD4+ T cells. In the right panel, dot plots of cells gated on CD4+ are shown. Cells were analyzed for expression of IL-5 and CD3. Thereby, CD3 is used as a marker to confirm the T-lymphocyte character of those cells. (D) Levels of OVA-specific IgE and IgG1. Data from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
Pathology
Paraffin-embedded lung sections (cut at 5 μm) were stained with H&E to evaluate general morphology. The degree of bronchial inflammation was scaled by using a scoring system on five randomly chosen areas on each slide (0, no inflammation, to 4, maximal inflammation), as previously described (32, 33).
Periodic acid-Schiff staining was performed to visualize mucus-producing goblet cells, as described before (34). Collagen deposition in chronic airway inflammation was assessed on Sirius red–stained sections. Therefore, three medium-sized conducting airways measuring between 150 and 250 μm in diameter were analyzed per slide, and 10 measurements of collagen thickness of each bronchiole were performed. Areas near blood vessels were excluded. Thickness of the airway smooth muscle layer around medium-sized conducting airways measuring between 150 and 250 μm in diameter was measured on α-smooth muscle actin (Abcam)–stained slides.

Statistical analysis
Data were analyzed using Prism for Mac (GraphPad Software). Data are reported as mean and SEM. The significance of categorical and numerical differences was calculated by two-tailed Student t test. Relationships between different variables were assessed by bivariate analysis (Pearson’s correlation coefficient). A p value <0.05 was considered significant.

Results
Allergen immunotherapy ameliorates development of airway inflammation and AHR in a chronic mouse model of allergic airway disease
We investigated the immunosuppressive effects of IT in a mouse model of chronic allergic airway inflammation. Thereby, IT was given during repeated allergen challenges, as described in Fig. 1A. To determine the development of allergic airway disease in this model, airway inflammation and AHR were assessed at day 49. Indeed, OVA sensitization and challenge (OVA/OVA) resulted in significantly increased airway response to methacholine and increased number of eosinophils in BAL fluid compared with the PBS-treated and also with the challenged-only animals (Fig. 2A, 2B). Consistently, analysis of lung and tracheal lymph node cells by intracellular flow cytometry staining showed increased frequencies of CD4+ T cells producing the Th2 cytokine IL-5 in the sensitized and challenged animals (Fig. 2C). Furthermore, serum levels of OVA-specific Abs IgE, IgG1 (Fig. 2D), and IgG2a (data not shown) were increased following sensitization and challenge, but not increased in animals challenged alone. These results demonstrate that airway disease develops in this chronic model. To assess whether s.c. IT is effective in mice with already established allergic airway disease, mice were treated with OVA (OVA-IT) or PBS and were challenged in parallel by i.t. instillations twice per week. After the last challenge, on day 70, measurement of AHR showed reduced airway resistance in OVA-IT–treated (OVA/OVA IT), sensitized, and challenged animals compared with untreated sensitized and challenged mice (OVA/OVA sham) (Fig. 2A). In accordance, OVA-IT also significantly reduced numbers of eosinophils (Fig. 2B) and numbers of total cells in BAL (mean ± SEM, OVA/OVA, 574 × 10³ ± 86 × 10³; OVA-IT, 380 × 10³ ± 48 × 10³). In contrast, OVA-IT had no significant effect on numbers of neutrophils, macrophages, and lymphocytes in BAL fluid compared with sensitized and challenged animals (data not shown).
Yet, OVA-IT resulted in reduced frequencies of IL-5–producing T cells solely in draining lymph nodes of OVA-IT–treated animals (Fig. 2C). A decline of allergen-specific IgE and induction of allergen-specific IgG responses have been demonstrated in human subjects after IT (35, 36). Accordingly, also levels of OVA-specific IgE were reduced in the present model in mice with OVA-IT, whereas levels of OVA-specific IgG1 were increased in comparison with sensitized and challenged animals (Fig. 2D).

**FIGURE 3.** Effect of OVA-IT on airway inflammation, mucus production, and remodeling in a chronic model of experimental asthma and allergen immunotherapy. Pulmonary tissue sections of PBS/PBS (n = 5), PBS/OVA (n = 6), OVA/OVA (n = 11), and OVA-IT (n = 12)–treated mice stained with H&E (A), periodic acid-Schiff (PAS) (B), anti-α-smooth muscle actin (αSMA) Ab (C), and Sirius Red (D). Data from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. BM, basal membrane.
**Allergen immunotherapy reduces airway remodeling and goblet cell metaplasia in the lung**

As sustained airway disease may result in structural changes in the airways, we assessed the histopathologic changes in the lung of these sensitized and challenged animals. Lung tissue analysis revealed inflammatory infiltrates in sensitized and challenged animals. OVA-IT resulted in a reduction of inflammation (Fig. 3A). In addition, evaluation of mucus-producing cells revealed a decreased goblet cell metaplasia in comparison with sensitized and challenged mice after OVA-IT (Fig. 3B). Further evaluation of collagen deposition and airway smooth muscle via Sirius red and anti-α smooth muscle actin staining showed evidence for reduced airway remodeling following OVA-IT in comparison with sensitized and challenged animals (Fig. 3C, 3D).

**Allergen immunotherapy induces CD4⁺Foxp3⁺ Treg cells and promotes IL-10 production by CD4⁺ T cells**

To further assess the effect of IT on the T cell response, we analyzed cells from lung and thoracic lymph nodes by flow cytometry. IT has been related to a shift in T cell responses from Th2 to Th1 (15). In our animal model, we confirmed that frequencies of IFN-γ-producing CD4⁺ T cells are increased in lung and draining lymph nodes following IT indicative of a Th1 shift present also in this model (Fig. 4A). However, to further assess whether the OVA-IT–induced Th1 response accounts for the protective effect of IT, we used mice that cannot develop Th1 responses due to genetic deficiency in the Tbox21 gene encoding the lineage-specific transcription factor T-bet (37). Genetic ablation of Tbox21 results in a higher susceptibility to develop spontaneous asthmatic symptoms (38). Thus, it was hypothesized that Th1 cells are able to counterbalance Th2 response by IFN-γ production in vivo (39). However, IT was similarly effective in reducing AHR and airway inflammation in Tbox21-deficient mice and respective wild-type control mice (data not shown), demonstrating that the induction of Th1 responses is not critical for the effectiveness of IT. In addition, the number of Tregs was not different following IT between Tbox21-deficient and respective wild-type animals (data not shown).

Another mechanism that has been associated with the effectiveness of IT is the induction of Treg cells in the periphery. We therefore analyzed the frequency of CD4⁺CD25⁺Foxp3⁺ cells among peripheral CD4⁺ T cells (Fig. 4B). Compared with sensitized and challenged mice, OVA-IT–treated mice showed an increased frequency of CD4⁺CD25⁺Foxp3⁺ cells specifically in the tracheal lymph node. Notably, this increase in the frequency of Treg cells was accompanied by an increase in the numbers of IL-10–producing CD4⁺ T cells in lung (Fig. 4C). To further elucidate the role of IL-10–producing T cells and suppression of airway inflammation, we assessed the relation of these cells and the number of eosinophils in the BAL fluid. Indeed, when analyzing the association in untreated and treated animals, we found a negative correlation between number of eosinophils and IL-10–producing cells \((r = -0.39)\), which was not statistically significant \((p = 0.086)\).
Allergen immunotherapy induces Foxp3-negative IL-10–producing CD4+ T cells

To further investigate the role of Ag-specific IL-10–producing CD4+ T cells and Foxp3+ Treg cells in IT, we established an acute T cell–dependent model using mice transgenic for the MHC-II–restricted TCR specific for OVA323–339 peptide Ag (DO11.10 transgenic BALB/c mice). Allergen challenge of DO11.10 transgenic BALB/c mice results in airway inflammation, evidenced by an influx of neutrophilic granulocytes (40). In the current study, DO11.10 transgenic BALB/c mice were initially treated with high-dosed OVA s.c. (OVA-IT) for IT or PBS as control. Eight days later, the mice were challenged with nebulized OVA. PBS-pretreated mice that were challenged with OVA showed AHR and airway inflammation (Fig. 5A, 5B). In contrast, OVA-IT–treated mice showed no induction of AHR and significantly less neutrophilic airway inflammation (Fig. 5A, 5B), demonstrating the effectiveness of OVA-IT in this model. FACS analysis of blood cells revealed increased frequencies of DO11.10 TCR+ Foxp3+ Treg cells in blood, lung regional lymph nodes (Fig. 5C), and spleen (data not shown) of mice treated with OVA-IT indicative of a de novo induction of Foxp3+ Treg cells by OVA-IT. Intracellular analysis demonstrated an increase in IL-10– and IFN-γ–producing CD4+ T cells following OVA-IT treatment. Interestingly, expression of IL-10 and Foxp3 was mutually exclusive (Fig. 5D).

Treg cells of thymic origin are important for the induction of IL-10–producing CD4+ T cells by allergen immunotherapy

Discrimination of Treg cells from thymic and peripheral origin is a challenging task. To analyze whether OVA-IT results in the peripheral induction of Ag-specific Treg cells or induces the expansion of Treg cells from thymic origin, we made use of DO11.10 transgenic BALB/c mice genetically deficient in Rag2 (DO11.10 Rag2−/−). The advantage of this system is the Ag specificity of analysis of blood cells revealed increased frequencies of DO11.10 TCR+ Foxp3+ Treg cells in blood, lung regional lymph nodes (Fig. 5C), and spleen (data not shown) of mice treated with OVA-IT indicative of a de novo induction of Foxp3+ Treg cells by OVA-IT. Intracellular analysis demonstrated an increase in IL-10– and IFN-γ–producing CD4+ T cells following OVA-IT treatment. Interestingly, expression of IL-10 and Foxp3 was mutually exclusive (Fig. 5D).

FIGURE 5. Preventive effects of OVA-IT on AHR and airway inflammation in DO11.10 TCR transgenic BALB/c mice. (A) Airway resistance in response to methacholine (MCh) of PBS-treated (PBS, n = 4), only OVA-challenged (OVA, n = 12), and OVA-IT–treated and OVA-challenged (OVA-IT, n = 12) DO11.10 mice. (B) Enumeration of neutrophilic granulocytes. (C) Frequencies of KJ1-26+ CD4+CD25+ Foxp3+ T cells in blood, lung, and draining lymph nodes after OVA challenge. KJ1-26+ T cells are OVA-specific T cells that carry the DO11.10 TCR. (D) FACS analysis of IL-10 or IFN-γ production and Foxp3 expression of lung CD4+ T cells. Data from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
CD4+ T cells and a complete lack of Treg cells from thymic origin (41). To analyze the role of peripherally induced Ag-specific Treg cells, DO11.10 RAG−/− mice were exposed to nebulized OVA that resulted in development of AHR and airway inflammation. Again, treatment with OVA-IT ameliorated AHR with a concomitant slight, but not significant reduction of neutrophils in lung (Fig. 6A, 6B). However, similar to the previous model also, strong increased frequencies of CD4+CD25+ Foxp3+ cells were detected in blood following IT, demonstrating the de novo induction of Ag-specific Foxp3+ Treg cells by OVA-IT (Fig. 6C).

Notably, the induction of IL-10–producing CD4+ T cells was reduced in the absence of Treg cells from thymic origin compared with DO11.10 transgenic mice (Fig. 6D). An increase in IL-10– and IFN-γ–producing CD4+ T cells following OVA-IT treatment was still detected (Fig. 6E). Compared with DO11.10 transgenic mice, IFN-γ production was highly increased in the absence of Treg cells from thymic origin.

**Effective immunotherapy requires the induction of IL-10–producing CD4+ T cells**

To further elucidate the role of IL-10 in the chronic model of allergic airway inflammation (see Fig. 1A), lung cells were analyzed for expression of IL-10, IFN-γ, and Foxp3 by flow cytometry. Expression of Foxp3 and IL-10 was mutually exclusive in BALB/c after OVA-IT in the chronic asthma model (Fig. 7A).

![Figure 6](http://www.jimmunol.org/)
Furthermore, we administered a blocking mAb against IL-10R or rat IgG control Ab to OVA-IT–treated mice. Notably, neutralization of IL-10 signaling completely abrogated the immunosuppressive effect of IT, evidenced by a restored high AHR, high numbers of eosinophilic granulocytes, and high OVA-specific IgE titers (Fig. 7B, 7C), as well as strong airway inflammation and airway remodeling (Fig. 8).

Discussion
The present study demonstrates that s.c. IT can suppress cardinal features of allergic airway disease in a chronic asthma model. Notably, this treatment was even effective to reduce the development of airway remodeling. Moreover, these data show that IT results in the de novo induction of peripheral Treg cells, but that its effectiveness most probably relies on the induction of IL-10–producing CD4+ T cells in the presence of Treg cells from thymic origin. In humans, IT can suppress allergic symptoms, reduces Th2 cytokines (14, 42) and IgE levels (43), and can lead to long-term remission of symptoms (10). In our model of IT for chronic asthma, s.c. injections of allergen (OVA) were given during OVA challenge phase and suppressed main features of allergic asthma, which were AHR, eosinophilic inflammation, and reduced levels of OVA-specific IgE. Also, IT led to a reduction of pathological changes in the lung tissue; thus, decreased mucus production, collagen deposition, and thickness of airway musculature were seen after OVA-IT. Airway remodeling and fixed airway obstruction are features that can be found in patients with long-standing severe asthma. To date, prevention of airway remodeling has been demonstrated in experimental models following administration of budesonide (44) or after transfer of Treg cells (45). In the current study, we additionally demonstrate that the suppressive effects of IT are also effective to ameliorate airway remodeling.

Despite the effectiveness of IT in experimental models (24) and human patients, the underlying mechanisms are not clearly defined. The first assumption about the suppressive mechanism of IT was described as a blocking Ab hypothesis (13). Indeed, in the current study, an allergen-specific IgG1 (murine equivalent to IgG4) response was induced. Still, recent work by Shirinbak et al. (46) demonstrated that allergen-specific IgG and IgA responses, induced by IT in sensitized mice, are not critically required for the suppression of Th2 cell–driven allergen-specific IgE responses. Even B cell–deficient mice (μMT mice) benefit from the suppressive effects of IT, suggesting that mechanisms independent from Abs mediate the protective effect of IT (46). Another potential mechanism that has been postulated for the effectiveness of IT is the induction of Th1 responses. To further elucidate the role of Th1 cells in mediating the effects of IT, we used Tbx21-deficient mice that cannot develop Th1 responses, and previous studies have shown that these animals show different features of asthma (38, 47, 48). Interestingly, in the current study, cardinal features of the allergen-driven allergic response are suppressed by OVA-IT independently of transcription factor T-bet, and thus independent of Th1 cells. Together, these data imply that other mechanisms than Th1 induction contribute to the IT-induced suppression of allergic inflammation. Indeed, potential mechanisms
include induction of Treg cells or local IL-10 production. Indeed, in the present analysis, we found a negative association in the number of IL-10–producing T cells and eosinophils, suggesting a relationship between the number of IL-10–producing cells and suppression of airway inflammation. However, based on the variability of results, it is not possible to use this parameter as an individual predictor for the response to IT.

To further elucidate the role of peripherally induced Treg cells in IT, we used genetic modified animals that allow a more detailed approach in defining the role of certain cell populations in mediating the observed suppressive effects of OVA-IT. Indeed, in these models, we found an increase in the frequency of de novo induced peripheral Treg cells following OVA-IT similarly to other reports in different models of airway disease (27). However, when investigating DO11.10 RAG \(^{-/-}\) mice, which lack naturally occurring Treg cells, we still found that OVA-IT was effective in this model. Also, in this setting, we found an increase in CD4\(^+\)/CD25\(^+\)/FOXP3\(^+\) cells, suggesting an induction of Treg cells in the recipients of OVA-IT. Indeed, also in humans, an increased frequency of FOXP3\(^+\) Treg cells was found in patients treated with IT (22). Interestingly, local presence of FOXP3\(^+\) Treg cells in the effector organ has been associated with clinical efficacy of IT (21), and this has been linked to the activation and selective homing of circulating Treg cells (22). However, the underlying mechanisms mediating the homing of the cells to the lung are not well elucidated. In addition, the effects of IT can only be partially explained by Treg cells (22). However, the underlying mechanisms mediating the homing of the cells to the lung are not well elucidated. Furthermore, absence of Treg cells from thymic origin is accompanied with an overwhelming production of IFN-\(\gamma\) by CD4 \(^+\) T cells. Thus, Treg cells are involved in controlling the undesirable Th1 induction by IT (27). Indeed, previous studies have demonstrated that in the airways Treg cells more effectively block Th1-induced inflammation compared with Th2-induced airway disease (49).

When further defining Treg cell populations that may play a role for the suppressive effects of IT, also IL-10–producing Tr1 that do not express Foxp3 induce tolerance in beekeepers during beekeeping season (50) and after IT treatment (20). Indeed, in our chronic model, we observed elevated frequencies of IL-10–producing Foxp3-negative CD4\(^+\) T cells. Similar results were detected in DO11.10 TCR transgenic mice after OVA-IT. Furthermore, when analyzing DO11.10 RAG \(^{-/-}\) mice, we found that IT is still effective, and induction of Foxp3\(^+\) Treg cells is not dependent on thymic-derived nTreg cells. Indeed, DO11.10 RAG \(^{-/-}\) mice showed a de novo induction of peripheral Foxp3\(^+\) Treg cells following OVA-IT detectable in lung, draining lymph nodes, and blood. We further analyzed the induced Treg cells in DO11.10 mice, confirming that IL-10–producing Tr1 cells were Foxp3\(^-\). Interestingly, those cells were predominantly positive for intracellular IFN-\(\gamma\), suggesting that these cells resemble already described effector-like Tr1 cells (51).

Different previous studies have demonstrated that depletion of IL-10 signaling in a mouse model of IT leads to abrogation of IT-mediated suppression (26, 52). In the current study, we could extend these results in a chronic model of allergic airway disease that resembles more closely the human situation when compared with prophylactic models mostly used. After anti–IL-10R treatment, AHR, eosinophilia, and specific IgE were restored in OVA-IT–treated animals comparable to animals treated with control Ig. Moreover, when analyzing DO11.10 RAG \(^{-/-}\) mice, we found that IT is still effective, and induction of Foxp3\(^+\) Treg cells is not dependent on thymic-derived nTreg cells. Indeed, DO11.10 RAG \(^{-/-}\) mice showed a de novo induction of peripheral Foxp3\(^+\) Treg cells following OVA-IT detectable in lung, draining lymph nodes, and blood. We further analyzed the induced Treg cells in DO11.10 mice, confirming that IL-10–producing Tr1 cells were Foxp3\(^-\). Interestingly, those cells were predominantly positive for intracellular IFN-\(\gamma\), suggesting that these cells resemble already described effector-like Tr1 cells (51).

Taken together, the current study provides evidence that IT induces tolerogenic effects, leading to amelioration of allergic symptoms even in a chronic disease model. Especially, OVA-IT was able to prevent the occurrence of airway remodeling in this setting. Experimental IT was accompanied by immune deviations as Th1 shift, increased levels of blocking Abs, IL-10
immunotherapy and the treatment of T cell responses. Suppressive effects were not dependent on Th1 cells, but rather on the induction of IL-10–producing Foxp3-negative CD4+ T cells. Thus, our data suggest that mainly IL-10–producing CD4+ T cells, but not peripherally induced Foxp3+ Treg cells, are pivotal for the effectiveness of IT.

Acknowledgments

We thank Michael Stassen for critical reading of the manuscript. We are grateful to Anke Heinze, Christina Belz, and Petra Schuster for excellent technical help and Claudia Braun (Histology Core Facility, Forschungszentrum Immunologie Mainz) for help with the histological staining.

The disclosures

The authors have no financial conflicts of interest.

References

24. Lou, W., C. Wang, Y. Wang, D. Han, and L. Zhang. 2012. Responses of CD4+ CD25+ Foxp3+ and IL-10-secreting type 1 regulatory cells to clust-