Allergen immunotherapy induces a suppressive memory response mediated by IL-10 in a mouse asthma model

Joost L. M. Vissers, PhD,[a] Betty C. A. M. van Esch, BSc,[a] Gerard A. Hofman, BSc,[a] Martien L. Kapsenberg, PhD,[b] Frank R. Weller, MD,[c] and Antoon J. M. van Oosterhout, PhD[a] *Utrecht, Amsterdam, and Hilversum, The Netherlands*

Background: Human studies have demonstrated that allergen immunotherapy induces memory suppressive responses and IL-10 production by allergen-specific T cells. Previously, we established a mouse model in which allergen immunotherapy was effective in the suppression of allergen-induced asthma manifestations.
Objective: In this study, we examined whether immunotherapy induces a long-lasting effect and investigated the role of IL-10 in successful immunotherapy.
Methods: Ovalbumin-sensitized BALB/c mice were treated with 3 injections of ovalbumin (1 mg, subcutaneous) on alternate days. After a short interval (1 week) and after a long interval (5 weeks), mice were challenged by ovalbumin inhalation, and subsequently, airway reactivity, airway eosinophilia, ovalbumin-specific IgE, and T_H2 cytokine profile were measured. Flow cytometry and blocking of IL-10 receptors in vivo were used to gain insight in the role of IL-10 in the beneficial effects of allergen immunotherapy.
Results: After a long interval between ovalbumin immunotherapy and ovalbumin challenge, the development of airway eosinophilia and hyperresponsiveness to methacholine were as strongly suppressed as after a short interval. These suppressive effects coincided with significantly reduced serum ovalbumin-specific IgE levels and T_H2 cytokine production. On immunotherapy, the IL-5:IL-10 ratio in the bronchoalveolar lavage fluid shifted toward IL-10. In ovalbumin-restimulated lung cell and thoracic lymph node cultures from these mice, IL-5 levels dramatically decreased, whereas the percentage of $IL\text{-}10^+CD4^+$ T cells was not affected. Finally, in mice treated with mAb against IL-10 receptors, the beneficial effects of immunotherapy were largely abrogated.
Conclusion: These data demonstrate that allergen immunotherapy induces a memory suppressive effect in which IL-10 is essential. (J Allergy Clin Immunol 2004;113:1204-10.)

Key words: *Asthma, allergy, immunotherapy, IL-10, IgE, hyperresponsiveness, eosinophilia, T_H2 lymphocytes, regulatory T cells, suppression*

Currently, allergen-specific immunotherapy is the only treatment available that can offer protection against allergen-induced complaints even for long periods after treatment is finished.[1,2] Although allergen immunotherapy is beneficial for treatment of rhinitis and insect venom allergy, it is less effective in allergic asthma and seldom results in complete alleviation of all symptoms.[3-5] To improve allergen immunotherapy in a way that is effective as a treatment for asthmatic patients, more insight into the underlying immunologic mechanisms is needed.

Successful allergen immunotherapy has been related to the induction of allergen-blocking IgG antibody, a reduced recruitment of eosinophils and T cells, a shift from a T_H2 to a T_H1 response, or the induction of T-cell anergy.[6,7] Recently, human studies suggested that allergen immunotherapy against bee venom, house dust mite, and grass pollen was associated with increased production of IL-10 by allergen-specific T cells.[8-11]

We developed a mouse model in which an allergen immunotherapy schedule, adopted from standard immunotherapy, suppressed allergen-induced airway inflammation and airway hyperreactivity.[12] Concomitant with these suppressive effects, upregulation of ovalbumin-specific IgE antibody in serum was inhibited, and T_H2 activity in T cells derived from local lymph nodes was downregulated. A shift toward T_H1 cytokine production was not observed; therefore, it was tempting to speculate that the mechanism underlying successful allergen immunotherapy was related to the induction of regulatory T (Treg) cells. These T cells play a pivotal role in peripheral tolerance to self-antigens as well as nonself-antigens. At least 3 different subpopulations of Treg cells haven been identified: naturally occurring $CD4^+CD25^+$ $CD45RB^{low}$ Treg cells, IL-10–secreting type 1 regulatory T (Tr1) cells, and TGF-β–secreting T_H3 cells.[13,14]

From [a]the Department of Pharmacology and Pathophysiology, Faculty of Pharmaceutical Sciences, Utrecht University, Utrecht; [b]the Department of Histology and Cell Biology and the Department of Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam; and [c]Asthmacenter Heideheuvel Netherlands and Asthmacenter Davos, Hilversum, The Netherlands.

Supported by research grant 3.2.00.48 from the Dutch Asthma Foundation (Dr Vissers) and by Stichting Astma Bestrijding (Mr van Esch).
Received for publication October 30, 2003; revised January 26, 2004; accepted for publication February 9, 2004.

Reprint requests: Antoon J. M. van Oosterhout, PhD, Department of Pharmacology and Pathophysiology, Faculty of Pharmaceutical Sciences, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands; E-mail: A.J.M.vanOosterhout@pharm.uu.nl.

0091-6749/$30.00

doi:10.1016/j.jaci.2004.02.041

Abbreviations used
BALF: Bronchoalveolar lavage fluid
IL-10R: IL-10 receptor
TLN: Thoracic lymph nodes
Tr1: Type 1 regulatory T
Treg: Regulatory T

In this study, we examined whether allergen immunotherapy induces a suppressive memory response in our model of antigen-induced airway manifestations of asthma. Subsequently, to investigate in more detail the role of IL-10 in the beneficial effects of allergen immunotherapy, flow-cytometric analysis of $CD4^+$ T cells from thoracic lymph nodes (TLNs) and lung tissues was performed, and IL-10 receptors (IL-10Rs) were functionally blocked in vivo.

METHODS

Animals

Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. Specific pathogen-free (according to the Federation of European Laboratory Animal Science Associations)[15] male BALB/c mice (6 weeks old) were purchased from Charles River (Maastricht, The Netherlands). The mice were housed in macrolon cages in a laminar flow cabinet and provided with food and water ad libitum.

Antibodies

Anti–IL-10R mAb was purified from culture supernatant of hybridoma (HB-12538, American Type Culture Collection) by using a protein G column (Pharmacia, Peapack, NJ). Rat IgG was purchased from ICN Pharmaceuticals (Cosa Mesa, Calif). Both antibodies were treated with 10% (vol/vol) polymyxin B-agarose (Sigma-Aldrich, St Louis, Mo) for 1 hour at 4°C to remove LPS. After incubation, the agarose beads were removed by centrifugation, and the supernatant was sterilized by using a 0.22-μm filter (Omnilabo, Breda, The Netherlands).

Sensitization, treatment, and challenge

All mice were sensitized to ovalbumin (chicken egg albumin, crude grade V, Sigma-Aldrich) by 2 intraperitoneal injections (7 days apart) of 0.1 mL alum-precipitated antigen composed of 10 μg ovalbumin absorbed onto 2.25 mg alum (AlumImject; Pierce, Rockford, Ill). Two weeks after the last sensitization, the mice were divided in 2 groups. The ovalbumin-immunotherapy group was treated with 3 subcutaneous injections of 1 mg ovalbumin in 0.2 mL pyrogen-free saline (B. Braun, Melsungen, Germany) on alternate days. The other group was sham-treated with 0.2 mL saline. One week after treatment, mice were exposed to 3 ovalbumin inhalation challenges (10 mg/mL saline) for 20 minutes every third day. For long-term experiments, ovalbumin challenges were started 5 weeks after treatment. The aerosol was performed in a Plexiglas exposure chamber (5 L) coupled to a Pari L C Star nebulizer (particle size 2.5-3.1 μm; PARI Respiratory Equipment, Richmond, Va) driven by compressed air at a flow rate of 6 L/min. Aerosol was given in groups composed of maximally 8 mice.

To examine the involvement of IL-10 in the short-term immunotherapy protocol, half of the mice from each group were injected intraperitoneally with 0.5 mg anti–IL-10R mAb[16,17] together with both the first subcutaneous injection of ovalbumin and the first ovalbumin challenge. The other mice of each group were control-treated with 0.5 mg rat IgG.

Measurement of airway responsiveness in vivo

Airway responsiveness was measured in conscious, unrestrained mice using barometric whole-body plethysmography by recording respiratory pressure curves (Buxco; EMKA Technologies, Paris, France) in response to inhaled methacholine (acetyl-β-methylcholine chloride, Sigma-Aldrich). Airway responsiveness was expressed in enhanced pause, as described in detail previously.[18]

Determination of ovalbumin-specific immunoglobulin levels in serum

After measurement of in vivo airway responsiveness, mice were sacrificed and were bled by cardiac puncture. Subsequently, serum was collected and stored at −70°C until analysis. Ovalbumin-specific IgE, IgG1, and IgG2a in serum were measured as described.[12] For each isotype, a reference standard was obtained by intraperitoneal immunization of mice with ovalbumin and arbitrarily assigned a value of 1000 experimental units/mL. The detection levels of the ELISAs were 0.5 U/mL for IgE, 0.005 U/mL for IgG1, and 0.05 U/mL for IgG2a.

Analysis of the cellular composition in the bronchoalveolar lavage fluid

Bronchoalveolar lavage was performed immediately after bleeding of the mice by lavage of the airways through a tracheal cannula with 1 mL saline (37°C) containing 2 μg/mL aprotinine (Roche Diagnostics) and 5% BSA. Cytokines in the supernatant of this first milliliter of the bronchoalveolar lavage fluid (BALF) were determined by ELISA. Subsequently, mice were lavaged 4 times with 1 mL saline (37°C). Cells in the BALF were analyzed as described previously.[12]

Determination of cytokine production by ovalbumin-restimulated cells of the lung and thoracic lymph node in vitro

Cytokine production by ovalbumin-restimulated T cells in lung tissue and TLN was determined as described.[19] Cells of both the TLN (2×10^5 cells/well) and the lung (6×10^5 cells/well) were cultured in the presence of 10 μg/mL ovalbumin or were stimulated with plate-bound anti-CD3 mAb (clone 17A2). After 5 days of culture at 37°C, the supernatants were harvested and stored at −20°C until cytokine levels were determined by ELISA.

Cytokine ELISAs

IL-5 (detection limit, 32 pg/mL) and IL-10 (detection limit, 15 pg/mL) ELISAs (both BD PharMingen, San Diego, Calif) were performed according to the manufacturer's instructions.

Analysis of $CD4^+IL\text{-}10^+$ cells

Single-cell suspension from lungs and TLNs were restimulated with ovalbumin at 37°C and 5% carbon dioxide for 16 hours. Subsequently, a bispecific antibody to the transmembrane antigen CD45 and IL-10 was attached to all leukocytes, and the cells were incubated at 37°C for 45 minutes to allow cytokine secretion.[20] The secreted IL-10, bound by the bispecific antibody, was labeled with a second IL-10–specific antibody conjugated to phycoerythrin (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). In addition, cells were stained with Cy-Chrome–conjugated anti-CD4 (BD PharMingen) and analyzed with the FACScan flow cytometer by using CELLQuest software (both Becton Dickinson, San Jose, Calif).

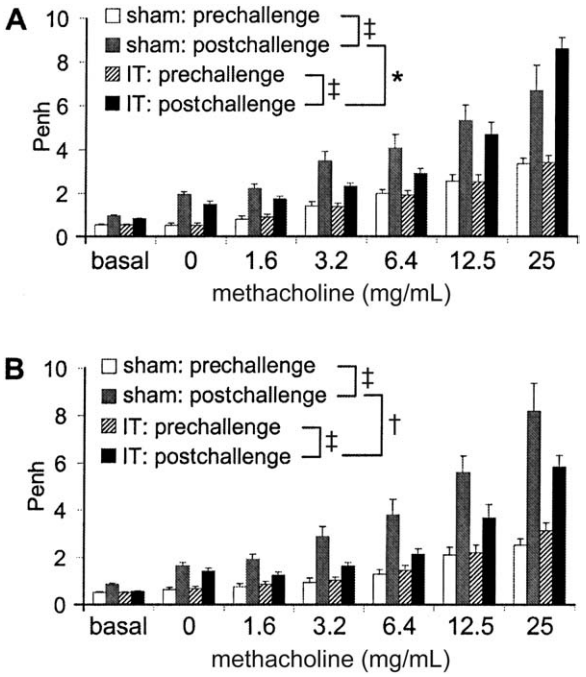

FIG 1. Development of airway hyperresponsiveness to methacholine after a short interval **(A)** and after a long interval **(B)** between ovalbumin immunotherapy (IT) and ovalbumin challenge. Values are expressed as the means ± SEMs (n = 6-8). *Penh,* Enhanced pause. **P* < .05 compared with sham-treated and ovalbumin-challenged mice (methacholine 0-12.5 mg/mL). †*P* < .05 compared with sham-treated and ovalbumin-challenged mice (methacholine 0-25 mg/mL). ‡*P* < .01 compared with the same mice before ovalbumin challenge (methacholine 0-25 mg/mL).

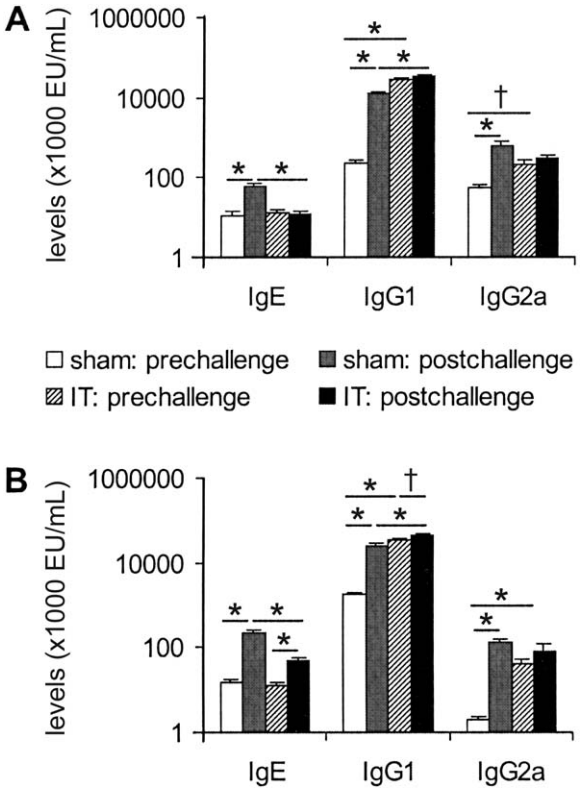

FIG 2. Ovalbumin-specific Ig levels in serum from saline-treated (sham) mice and mice that received immunotherapy (IT) were measured after a short interval **(A)** and after a long interval **(B)** between ovalbumin immunotherapy and ovalbumin challenge. Values are expressed as the means ± SEMs (n = 6-8). *EU,* Experimental units. **P* < .01. †*P* < .05.

Statistical analysis

All data are expressed as means ± SEMs. The airway dose-response curves to methacholine were statistically analyzed by a general linear model of repeated measurements followed by post hoc comparison between groups. Data were log-transformed before analysis to equalize variances in all groups. Statistical analysis on BALF cell counts was performed by using the nonparametric Mann-Whitney *U* test (2-tailed). For ELISA, results were statistically analyzed by using a Student *t* test (2-tailed, homoscedastic). Results were considered statistically significant at the $P < .05$ level.

RESULTS

Ovalbumin immunotherapy induces a long-term immunosuppressive effect

We compared the immunosuppressive effects of ovalbumin immunotherapy in mice that were challenged 1 week after treatment (short-term protocol) with those that were challenged 5 weeks after treatment (long-term protocol).

Airway responsiveness. Mice were sensitized with ovalbumin/alum and were treated with allergen immunotherapy or were sham-treated with saline. Of each mouse, the baseline of the airway responsiveness to the bronchoconstrictive stimulus methacholine was determined 1 day before ovalbumin challenge (Fig 1, *A* and *B*). Subsequently, the mice were challenged by ovalbumin inhalation, and the effect of allergen immunotherapy on airway hyperresponsiveness was examined. As Fig 1, *A*, shows, after a short interval between ovalbumin immunotherapy and challenge, allergen immunotherapy significantly suppressed (as much as 33%, $P < .05$) the airway responsiveness to methacholine (0-12.5 mg/mL) compared with sham-treated mice. Interestingly, after the long-term protocol, ovalbumin immunotherapy significantly suppressed ($P < .05$) the airway hyperresponsiveness, also at 25 mg/mL methacholine (Fig 1, *B*).

Ovalbumin-specific immunoglobulin levels in serum. In sham-treated mice, ovalbumin challenge induced an increase of serum ovalbumin-specific IgE (Fig 2). After the long-term protocol, ovalbumin immunotherapy suppressed the serum ovalbumin-specific IgE levels to the same extent as after the short-term protocol (both about 80% compared with sham-treated mice, $P < .01$; Fig 2).

In sham-treated mice, ovalbumin challenge induced a significant increase of IgG1 and IgG2a after both the short-term and the long-term protocols (Fig 2). Immunotherapy strongly increased the serum levels of IgG1 and IgG2a in ovalbumin-sensitized mice compared with sham-treated mice. These levels did not increase further on ovalbumin challenge, except for the IgG1 serum levels after a long-term interval (Fig 2, *B*).

Eosinophils and cytokines in bronchoalveolar lavage fluid. Ovalbumin challenge induced high numbers of eosinophils in the BALF of sham-treated mice (Fig 3, *A* and *B*). After a short interval between immunotherapy and challenge, the influx of eosinophils into the BALF was suppressed for 80% ($P < .01$) by ovalbumin immunotherapy compared with sham-treated mice (Fig 3, *A*). The influx of eosinophils in the BALF of mice treated with the long-term protocol was reduced for 95% ($P < .01$) by ovalbumin immunotherapy (Fig 3, *B*).

T_H2 cytokine IL-5 is essential for the development and chemoattraction of eosinophils.[21,22] On ovalbumin immunotherapy, the IL-5 levels in the BALF of mice treated with the short-term protocol were significantly decreased ($P < .01$) compared with sham-treated mice (0.4 ng/mL and 2.1 ng/mL, respectively; Fig 3, *C*). As Fig 3, *D*, shows, after the long-term protocol, ovalbumin immunotherapy also reduced the IL-5 levels in the BALF compared with sham-treated mice (0.1 ng/mL and 1.9 ng/mL, respectively; $P < .01$). The T_H1 cytokine IFN-γ was not detectable in the BALF from sham-treated mice or immunotherapy-treated mice (data not shown).

In contrast with IL-5, for the pleiotropic cytokine IL-10, which has anti-inflammatory and immunomodulatory properties, levels did not diminish on ovalbumin immunotherapy (Fig 3, *C* and *D*). After a short interval as well as after a long interval between ovalbumin immunotherapy and ovalbumin challenge, the IL-10 levels in sham-treated and immunotherapy-treated mice were ~0.2 ng/mL. Importantly, the IL-5:IL-10 ratio in the BALF shifted toward IL-10 on immunotherapy in both the short-term and the long-term protocols (from 12.8 to 2.2 and from 8.2 to 0.7, respectively).

By using an IL-10 secretion assay, we examined in more detail the percentage of $CD4^+$ T cells that excreted IL-10 in lung and TLN cultures from sham-treated and immunotherapy-treated mice. After a 16-hour period of restimulation with ovalbumin, we found that the percentage of $CD4^+$ T cells that produced IL-10 in both lung (5%) and TLN (1%) was equal in sham-treated and immunotherapy-treated mice (Fig 4).

IL-10 is crucial in the beneficial effects of allergen immunotherapy

Because we observed, on allergen immunotherapy, a shift toward IL-10 in the ratio between IL-5 and IL-10 and others demonstrated that immunotherapy induces IL-10 positive (T) cells in human beings,[8-11] we examined the role of IL-10 in allergen immunotherapy in vivo by treating mice with anti−IL-10R mAb[16,17] on the first day of immunotherapy and ovalbumin challenge.

Airway responsiveness. One day after the final ovalbumin challenge, the airway responsiveness to methacholine was examined. In mice that received ovalbumin immunotherapy and were injected with control antibody, the airway hyperresponsiveness to increased concentrations of methacholine (1.6-25 mg/mL) was significantly suppressed ($P < .05$) compared with mice without immunotherapy. As an example, the airway hyperresponsiveness to 25 mg/mL methacholine is depicted in Fig 5, *A*. In mice that received ovalbumin immunotherapy and in which the IL-10Rs were blocked with anti−IL-10R mAb, the airway hyperresponsiveness to the same range of methacholine was no longer significantly suppressed (Fig 5, *A*).

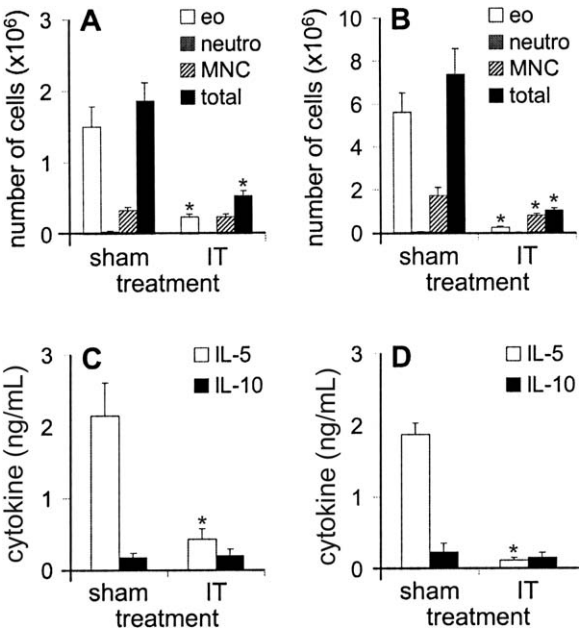

FIG 3. The number of eosinophils *(eo)*, neutrophils *(neutro)*, and mononuclear cells *(MNC)* in the BALF after 1 week **(A)** and after 5 weeks **(B)** between ovalbumin immunotherapy *(IT)* and ovalbumin inhalation challenge. IL-5 and IL-10 levels were determined in the BALF of mice after 1 week **(C)** and after 5 weeks **(D)**. Values are expressed as the means ± SEMs (n = 6-8). **P* < .01 compared with sham-treated mice.

Serum ovalbumin-specific IgE. Analysis of ovalbumin-specific IgE in serum of mice sensitized and challenged with ovalbumin demonstrated that the levels of ovalbumin-specific IgE were higher in mice treated with anti−IL-10R mAb than in mice that received control antibody (Fig 5, *B*). However, Fig 5, *B*, demonstrates clearly that blocking of the IL-10R in mice sensitized and challenged with ovalbumin completely abrogated the suppression of serum ovalbumin-specific IgE induced by ovalbumin immunotherapy.

Eosinophils and IL-5 in bronchoalveolar lavage fluid. In mice that received control antibody, ovalbumin immunotherapy readily suppressed (90%; $P < .01$) the ovalbumin-induced influx of eosinophils into the BALF compared with untreated mice (Fig 5, *C*). In contrast, in mice in which the IL-10Rs were blocked, ovalbumin immunotherapy failed to suppress the ovalbumin-induced influx of eosinophils into the BALF (Fig 5, *C*). In addition, the decreased levels of IL-5 in the BALF of ovalbumin-treated mice were partly abrogated by blocking the IL-10R (Fig 5, *D*).

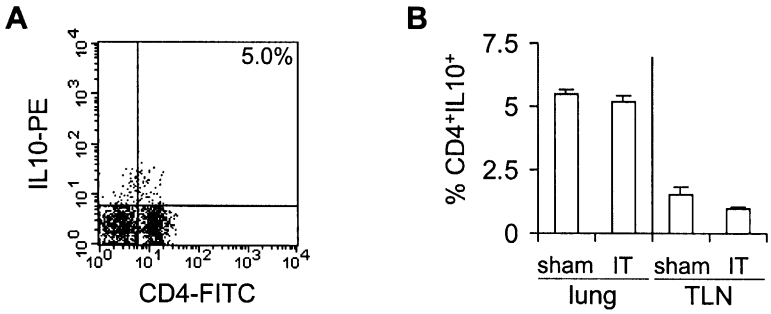

FIG 4. The percentage of $CD4^+$ T cells that express IL-10 in the lung and TLN on immunotherapy (IT). **A,** Representative dot blot of lymphocytes from ovalbumin-restimulated lung cells isolated from mice treated with ovalbumin immunotherapy. **B,** Quantitative analysis of the percentage of $CD4^+$ T cells that express IL-10. Values are expressed as the means ± SEMs (n = 4). *FITC,* Fluorescein isothiocyanate; *PE,* phycoerythrin.

Cytokine production by ovalbumin-restimulated cells of the lung and thoracic lymph nodes in vitro. To examine T-cell responses on antigen-specific restimulation, single-cell suspensions of both lung tissue and TLN of each mouse were prepared 24 hours after the final aerosol. In these cultures, the typical T_H1 cytokine IFN-γ was undetectable, even after restimulation with ovalbumin (data not shown). However, as Fig 5, *E*, shows, on restimulation with ovalbumin, the amount of IL-5 in lung cell cultures from mice that received immunotherapy and control antibody was readily ($P < .01$) reduced compared with lung cell cultures from mice that were sham-treated and control-antibody–treated (0.6 ng/mL and 8.2 ng/mL, respectively). This reduction of IL-5 was also observed when the lung cell cultures were restimulated with anti-CD3 antibody (Fig 5, *E*). Lung cell cultures from mice treated with anti–IL-10R antibody in vivo all contained high levels of IL-5 (Fig 5, *E*).

Similar to IL-5 in lung cell cultures from mice sensitized and challenged with ovalbumin, the levels of IL-10 and IL-13 were significantly reduced on immunotherapy. In addition, high levels of IL-10 and IL-13 were measured in lung cell cultures from anti–IL-10R antibody-treated mice that were not stimulated, restimulated with ovalbumin or anti-CD3 antibody (data not shown).

In mice that received control antibody, ovalbumin-restimulated TLN cell cultures from sham-treated mice produced significantly ($P < .05$) higher levels of IL-5 than those from mice treated with ovalbumin immunotherapy (4.1 ng/mL and 1.4 ng/mL, respectively; Fig 5, *F*). Moreover, TLN cells from mice treated with anti–IL-10R antibody in vivo produced high levels of IL-5 (Fig 5, *F*). These phenomena were also observed when IL-10 and IL-13 levels were measured in the same TLN cell cultures (data not shown).

DISCUSSION

Recently, we developed a mouse model in which allergen immunotherapy suppressed antigen-induced airway manifestations of asthma.[12] In this study, we further explored the immune deviation induced by allergen immunotherapy. By increasing the period between allergen immunotherapy and allergen challenge from 1 week (short-term protocol) to 5 weeks (long-term protocol), we demonstrated that allergen immunotherapy induces long-term immunosuppressive effects. Moreover, in mice that received anti–IL-10R mAb at the time of immunotherapy and challenge, the beneficial effects of allergen immunotherapy were abrogated. These data indicate that suppressive memory T cells in combination with IL-10 play a pivotal role in successful allergen immunotherapy.

After a prolonged time between ovalbumin immunotherapy and ovalbumin challenge, immunotherapy significantly suppressed the airway hyperresponsiveness to methacholine, eosinophilia, and IL-5 levels in the BALF. These manifestations were more suppressed than after a short interval between ovalbumin immunotherapy and ovalbumin challenge, most likely because of a more mature suppressive memory response. These data show that in our mouse model, allergen immunotherapy induces long-lasting protection against allergen-induced airway manifestations of asthma. Similar results have been observed in human studies.[1]

As with the short interval between ovalbumin immunotherapy and ovalbumin challenge, serum ovalbumin-specific IgE levels were suppressed and ovalbumin-specific IgG1 as well as ovalbumin-specific IgG2a levels remained high by ovalbumin immunotherapy in mice treated with the long-term protocol. High serum IgG2a levels together with a decrease of IgE levels suggest that on immunotherapy, the antigen-specific T_H2 response is downmodulated.[23] This downmodulation was not reflected in the levels of the T_H2-associated IgG1 antibody; however, a dissociation between IgG1 and IgE has often been observed.[24] A similar dissociation between IgE and IgG4 (the equivalent of mouse IgG1) occurs in human beings after allergen immunotherapy.[7] Though on the cytokine level, immunotherapy significantly reduced the levels of T_H2-type cytokines, such as IL-5 and IL-13, in ovalbumin-restimulated lung cell and TLN cultures. Because we were not able to detect IFN-γ in any of these cultures, these data suggest that immunotherapy affects the cytokines produced by antigen-specific T_H2 cells but does not induce a shift toward a T_H1 response.

Because no shift toward T_H1 was observed, we investigated whether Tr1 cells play a role in successful allergen immunotherapy. Tr1 cells are antigen-inducible $CD4^+$ Treg cells that suppress immune responses by secretion of IL-10.[13,14] A role for IL-10 in the beneficial effects of allergen immunotherapy has been suggested.[8-11] These reports showed that allergen immunotherapy increased the production of IL-10 in $CD4^+CD25^+$ T cells present in the blood of allergic patients. However, allergen immuotherapy also potentiated IL-10 production by monocytes and B cells.[8] By using our mouse model, we demonstrated that IL-10 undeniably has a pivotal role in the beneficial effects of allergen immunotherapy. The finding that allergen immunotherapy is mediated by IL-10, together with its antigen specificity as well as its suppressive memory response, indicates that IL-10–producing lymphocytes are involved in successful immunotherapy.

To investigate in more detail the presence of Tr1 cells in the effector organs, we analyzed the levels of IL-10 in BALF and supernatants of ovalbumin-restimulated lung cell cultures as well as TLN cultures. Interestingly, on allergen immunotherapy, the IL-5:IL-10 ratio shifted toward IL-10 in the BALF. In contrast, in supernatants of ovalbumin-restimulated lung cell and TLN cultures, both IL-5 levels and IL-10 levels decreased on allergen immunotherapy. However, a more detailed investigation using flow cytometry demonstrated that the percentage of $CD4^+IL\text{-}10^+$ T cells in these cultures was not affected on immunotherapy. These results could be a first indication that, in these mice, the T_H2 response is downregulated and, at the same time, IL-10–producing $CD4^+$ suppressor cells are induced on allergen immunotherapy. In human studies, it was clearly demonstrated that $CD4^+CD25^+$ cells express IL-10 on immunotherapy.[8-11] Because we studied asthma manifestations 24 hours after the last challenge, the T-cell population in the lung consisted of activated T_H2 cells and potential Tr1 cells. Activated T_H2 cells have the same phenotype ($CD4^+CD25^+$) as Tr1 cells, and both produce IL-10.[13] Because the IL-10 levels and $CD4^+IL\text{-}10^+$ T cells were not increased in the lung, it is difficult to substantiate further whether Tr1 cells are indeed involved in the beneficial effects of allergen immunotherapy during the effector phase of asthma. It remains possible that at another time point or site, the Tr1 cells produce transient and locally high IL-10 levels. Moreover, while measuring IL-10 secretion by T cells, we found that also a $CD4^-$ cell subset expressed IL-10 in the lung (data not shown), as found in human beings.[8] Experiments to elucidate which IL-10–producing suppressor cells are induced on allergen immunotherapy and where they actively suppress the antigen-induced airway manifestations are currently in progress.

In agreement with previous studies, we observed that blocking the IL-10 signaling pathway by anti–IL-10R mAb increased serum ovalbumin-specific IgE levels, the number of eosinophils, and the production of T_H2-type cytokines in our model of allergic asthma. Recently, Oh et al[25] also demonstrated that neutralization of IL-10

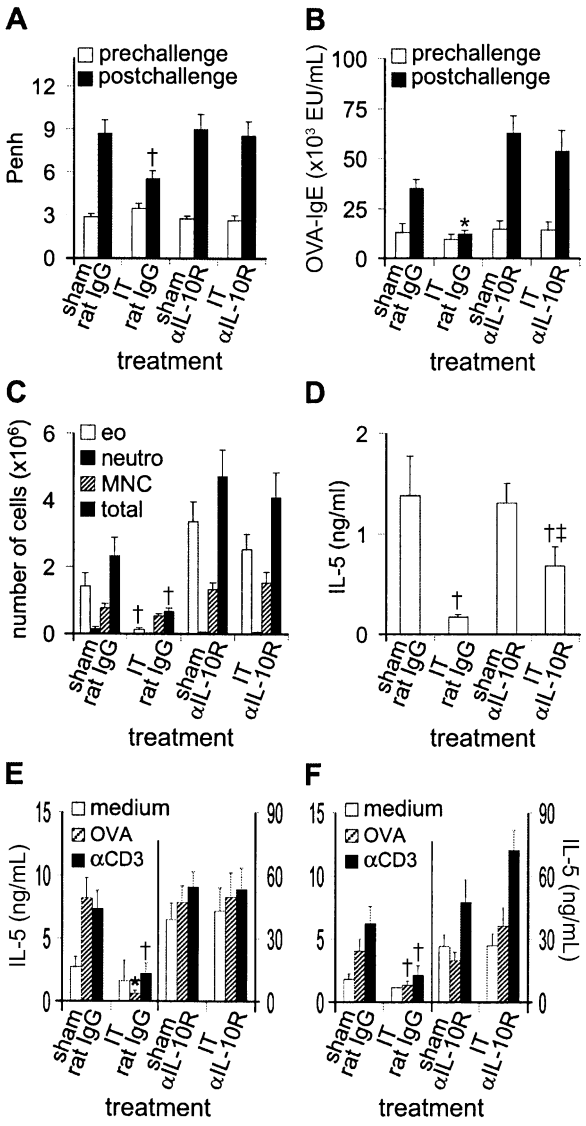

FIG 5. The immunosuppressive effects of allergen immunotherapy *(IT)* in the presence of anti–IL-10R were examined. **A,** The airway reactivity to 25 mg/mL methacholine. **B,** Ovalbumin (OVA)-specific IgE levels in serum before and after challenge. **C,** The number of eosinophils *(eo)*, neutrophils *(neutro)*, and mononuclear cells *(MNC)* in BALF. IL-5 levels in BALF **(D)**, in ovalbumin-restimulated lung cultures **(E)**, and in ovalbumin-restimulated TLN cultures **(F)**. Values are expressed as the means ± SEMs (n = 6-8). *Penh*, Enhanced pause. *$P < .01$ and †$P < .05$ compared with sham-treated and ovalbumin-challenged mice. ‡$P < .05$ compared with mice treated with ovalbumin immunotherapy and control rat IgG antibody.

augmented antigen-induced airway inflammation and IL-4 production by T cells. In addition, increased levels of IL-10 in this model of allergic asthma showed opposite effects.[25] These observations indicate that IL-10 plays a crucial role in the suppression of airway manifestations of asthma.

In conclusion, we demonstrated in a mouse model of airway manifestations of asthma that allergen immunotherapy induces a suppressive memory response and that

IL-10 plays a crucial role in the beneficial effects of immunotherapy. Our preclinical mouse model of allergic asthma will be helpful to search for novel strategies to potentiate IL-10 production further during allergen immunotherapy and thereby improve this therapy for management of allergic diseases.

We thank Dr S. McKay and Dr I. J. M. de Vries for critical reading of the manuscript.

REFERENCES

1. Durham SR, Walker SM, Varga EM, Jacobson MR, O'Brien F, Noble W, et al. Long-term clinical efficacy of grass-pollen immunotherapy. N Engl J Med 1999;341:468-75.
2. Valenta R. The future of antigen-specific immunotherapy of allergy. Nat Rev Immunol 2002;2:446-53.
3. Abramson MJ, Puy RM, Weiner JM. Is allergen immunotherapy effective in asthma? a meta-analysis of randomized controlled trials. Am J Respir Crit Care Med 1995;151:969-74.
4. Bousquet J, Michel FB. Specific immunotherapy in asthma: is it effective? J Allergy Clin Immunol 1994;94:1-11.
5. Barnes PJ. Is immunotherapy for asthma worthwhile? N Engl J Med 1996;334:531-2.
6. Wachholz P, Durham S. Induction of "blocking" IgG antibodies during immunotherapy. Clin Exp Allergy 2003;33:1171-4.
7. Frew AJ. Immunotherapy of allergic disease. J Allergy Clin Immunol 2003;111:S712-9.
8. Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. J Clin Invest 1998;102: 98-106.
9. Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszcz M, Blaser K, et al. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. Eur J Immunol 2003;33:1205-14.
10. Francis JN, Till SJ, Durham SR. Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. J Allergy Clin Immunol 2003;111: 1255-61.
11. Fellrath JM, Kettner A, Dufour N, Frigerio C, Schneeberger D, Leimgruber A, et al. Allergen-specific T-cell tolerance induction with allergen-derived long synthetic peptides: results of a phase I trial. J Allergy Clin Immunol 2003;111:854-61.
12. Van Oosterhout AJM, Van Esch B, Hofman G, Hofstra CL, Van Ark I, Nijkamp FP, et al. Allergen immunotherapy inhibits airway eosinophilia and hyperresponsiveness associated with decreased IL-4 production by lymphocytes in a murine model of allergic asthma. Am J Respir Cell Mol Biol 1998;19:622-8.
13. Maloy KJ, Powrie F. Regulatory T cells in the control of immune pathology. Nat Immunol 2001;2:816-22.
14. Shevach EM. Certified professionals: CD4(+)CD25(+) suppressor T cells. J Exp Med 2001;193:F41-6.
15. Nicklas W, Baneux P, Boot R, Decelle T, Deeny AA, Fumanelli M, et al. Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. Lab Anim 2002;36:20-42.
16. O'Farrell AM, Liu Y, Moore KW, Mui AL. IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms: evidence for Stat3-dependent and -independent pathways. EMBO J 1998;17:1006-18.
17. Castro AG, Neighbors M, Hurst SD, Zonin F, Silva RA, Murphy E, et al. Anti-interleukin 10 receptor monoclonal antibody is an adjuvant for T helper cell type 1 responses to soluble antigen only in the presence of lipopolysaccharide. J Exp Med 2000;192:1529-34.
18. De Bie JJ, Kneepkens M, Kraneveld AD, Jonker EH, Henricks PA, Nijkamp FP, et al. Absence of late airway response despite increased airway responsiveness and eosinophilia in a murine model of asthma. Exp Lung Res 2000;26:491-507.
19. Hofstra CL, Van Ark I, Nijkamkp FP, Van Oosterhout AJM. Antigen-stimulated lung CD4+ cells produce IL-5, whereas lymph node CD4+ cells produce Th2 cytokines concomitant with airway eosinophilia and hyperresponsiveness. Inflamm Res 1999;48:602-12.
20. Becker C, Pohla H, Frankenberger B, Schuler T, Assenmacher M, Schendel DJ, et al. Adoptive tumor therapy with T lymphocytes enriched through an IFN-gamma capture assay. Nat Med 2001;7:1159-62.
21. Hessel EM, Van Oosterhout AJM, Van Ark I, Van Esch B, Hofman G, Van Loveren H, et al. Development of airway hyperresponsiveness is dependent on interferon-gamma and independent of eosinophil infiltration. Am J Respir Cell Mol Biol 1997;16:325-34.
22. Schweizer RC, Welmers BA, Raaijmakers JA, Zanen P, Lammers JW, Koenderman L. RANTES- and interleukin-8-induced responses in normal human eosinophils: effects of priming with interleukin-5. Blood 1994;83:3697-704.
23. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989;7:145-73.
24. Finkelman FD, Holmes J, Urban JF Jr, Paul WE, Katona IM. T help requirements for the generation of an in vivo IgE response: a late acting form of T cell help other than IL-4 is required for IgE but not for IgG1 production. J Immunol 1989;142:403-8.
25. Oh JW, Seroogy CM, Meyer EH, Akbari O, Berry G, Fathman CG, et al. CD4 T-helper cells engineered to produce IL-10 prevent allergen-induced airway hyperreactivity and inflammation. J Allergy Clin Immunol 2002;110:460-8.