Indoleamine 2,3-dioxygenase–dependent tryptophan metabolites contribute to tolerance induction during allergen immunotherapy in a mouse model

Yousef A. Taher, MSc,[a,c] Benoit J. A. Piavaux, MSc,[c] Reneé Gras, BSc,[c] Betty C. A. M. van Esch, BSc,[a] Gerard A. Hofman, BSc,[a] Nanne Bloksma, PhD,[a,b] Paul A. J. Henricks, PhD,[a] and Antoon J. M. van Oosterhout, PhD[c] *Utrecht and Groningen, The Netherlands*

Background: The tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) has been implicated in immune suppression and tolerance induction.

Objective: We examined (1) whether IDO activity is required during tolerance induction by allergen immunotherapy or for the subsequent suppressive effects on asthma manifestations and (2) whether tryptophan depletion or generation of its downstream metabolites is involved.

Methods: Ovalbumin (OVA)–sensitized and OVA-challenged BALB/c mice that display increased airway responsiveness to methacholine, serum OVA-specific IgE levels, bronchoalveolar eosinophilia, and T_H2 cytokine levels were used as a model of allergic asthma. Sensitized mice received subcutaneous optimal (1 mg) or suboptimal (100 μg) OVA immunotherapy.

Results: Inhibition of IDO by 1-methyl-DL-tryptophan during immunotherapy, but not during inhalation challenge, partially reversed the suppressive effects of immunotherapy on airway eosinophilia and T_H2 cytokine levels, whereas airway hyperresponsiveness and serum OVA-specific IgE levels remained suppressed. Administration of tryptophan during immunotherapy failed to abrogate its beneficial effects toward allergic airway inflammation. Interestingly, administration of tryptophan or its metabolites, kynurenine, 3-hydroxykynurenine, and xanthurenic acid, but not 3-hydroxyanthranilinic acid, quinolinic acid, and kynurenic acid, during suboptimal immunotherapy potentiated the reduction of eosinophilia. These effects coincided with reduced T_H2 cytokine levels in bronchoalveolar lavage fluid, but no effects on IgE levels were detected.

Conclusion: During immunotherapy, the tryptophan metabolites kynurenine, 3-hydroxykynurenine, and xanthurenic acid generated through IDO contribute to tolerance induction regarding T_H2-dependent allergic airway inflammation.
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Key words: *Allergic asthma, immunotherapy, indoleamine 2,3-dioxygenase, tryptophan, kynurenine, dendritic cell, regulatory T cells, T_H2 lymphocytes, hyperresponsiveness, eosinophilia, IgE, IL-10, suppression*

Allergen immunotherapy conducted by means of subcutaneous administration of allergen extract is used for treating allergic diseases.[1] The therapy is allergen specific and is effective in the treatment of allergic rhinitis and insect venom allergy. Its efficacy in allergic asthma, however, remains controversial.[2] More insight into the underlying immunologic mechanisms of allergen immunotherapy is needed to improve efficacy, particularly in asthmatic patients. The beneficial effects of allergen immunotherapy are presumed to be mediated through reduction of allergen-induced inflammation. A variety of immunologic processes underlying these effects have been reported. Induction of blocking IgG antibodies, particularly of the IgG_4 isotype[3]; downregulation of T_H2 lymphocytes, upregulation of T_H1 lymphocytes, or both[4]; and induction of $CD8^+$ T cells[5] were claimed to be responsible for successful allergen immunotherapy. Recent data suggest an important role for IL-10–producing type 1 regulatory T (T_R1) cells and TGF-β–producing T_H3-type cells in immunotherapy against bee venom, house dust mite, grass pollen, and other airborne allergens.[6-8]

Exposure to antigen leads to its uptake, processing, and presentation by dendritic cells (DCs), which initiate and regulate T-cell responses.[9] In addition to skewing T cells toward T_H1 or T_H2, DCs have been shown to mediate the induction of adaptive regulatory T (Treg) cells, such as T_H3 and T_R1 cells.[9,10] DCs induce development of Treg cells through several mechanisms, including production of IL-10 or TGF-β[11,12] and expression of indoleamine 2,3-dioxygenase (IDO).[13,14] IDO is the rate-limiting enzyme that converts tryptophan into kynurenine and other downstream metabolites.[15] Several studies have demonstrated that IDO is expressed in DCs, inhibits T-cell proliferation, and promotes tolerance,[16-18] including maternal tolerance toward an allogeneic fetus.[19] Moreover, suppression of T-cell responses to MHC-mismatched allografts,[20] control of T cells in autoimmune disorders,[21] and suppression of immune response to tumors[22] have been attributed to IDO activation. IDO might mediate inhibition of T-cell proliferation by means of starvation caused by

From [a]the Department of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, and [b]the Department of Biology, Faculty of Sciences, Utrecht University, and [c]the Laboratory of Allergology and Pulmonary Diseases, University Medical Center Groningen, Groningen University.

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Reprint requests: Antoon J. M. van Oosterhout, PhD, Laboratory of Allergology and Pulmonary Diseases and Department of Pathology and Laboratory Medicine, UMCG, PO Box 30.001, 9700 RB, Groningen, The Netherlands. E-mail: a.j.m.van.oosterhout@path.umcg.nl.

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Abbreviations used

AHR: Airway hyperresponsiveness
BALF: Bronchoalveolar lavage fluid
DC: Dendritic cell
IDO: Indoleamine 2,3-dioxygenase
KA: Kynurenic acid
1MT: 1-Methyl-DL-tryptophan
3-OH-AA: 3-Hydroxyanthranilic acid
3-OH-KYN: 3-Hydroxykynurenine
OVA: Ovalbumin
QUINA: Quinolinic acid
Treg: Regulatory T
XA: Xanthurenic acid

tryptophan depletion and by the antiproliferative and proapoptotic effects of its downstream metabolites.[23,24]

In the present study the role of IDO in tolerance induction by experimental allergen immunotherapy was examined by using a mouse model of allergic airway inflammation.[25] In this model we demonstrated earlier that allergen immunotherapy by means of subcutaneous administration of ovalbumin (OVA) between sensitization and challenge inhibits the development of airway hyperresponsiveness (AHR) and eosinophilia.[25] Furthermore, we recently demonstrated that the beneficial effects of allergen immunotherapy were mediated by IL-10 because blocking of the IL-10 receptor completely negates the suppression of asthma manifestations.[26] Our present results clearly demonstrate that tryptophan metabolites generated by IDO during immunotherapy are crucial in the suppression of allergen-induced allergic airway eosinophilia and T_H2 cytokine levels in this mouse model.

METHODS

Animals

Specified pathogen-free male BALB/c mice (6-8 weeks old) were obtained from Charles River (Maastricht, The Netherlands). Animal care and use were conducted in accordance with the Animal Ethics Committee of Utrecht University, Utrecht, The Netherlands.

Sensitization, challenge, and immunotherapy protocol

The sensitization, challenge, and immunotherapy protocol was previously described (Fig 1, *A*).[26] In short, mice received 2 intraperitoneal injections of 10 μg of OVA adsorbed onto 2.25 mg alum in 100 μL of pyrogen-free saline on days 0 and 7. Two weeks after the second sensitization, mice were treated with 3 subcutaneous injections of 100 μg or 1 mg of OVA in 200 μL of pyrogen-free saline on alternate days. The control group was sham treated with 200 μL of saline. One week after OVA or sham treatment, mice were challenged by means of inhalation of OVA aerosols in pyrogen-free saline (1% wt/vol) for 20 minutes 3 times every third day.

Intervention studies

The IDO inhibitor 1-methyl-DL-tryptophan (1MT; Sigma-Aldrich, St Louis, Mo) was used to examine the role of IDO in immunotherapy (Fig 1, *B*). It was dissolved in a small volume of 1 N NaOH and further diluted with PBS. The pH was adjusted to 7.1 with 1 N HCl before injection. In experiment B1 mice of the intervention groups were daily (days 21-26) injected intraperitoneally with 1MT (10 mg per mouse per day in 1 mL of PBS; the dose was based on preliminary results and literature data),[21] starting 1 hour before the first subcutaneous injection of immunotherapy. Control animals received 1 mL of PBS intraperitoneally. In experiment B2, the intervention and control groups were treated with 1MT or PBS, respectively, during the OVA challenge period (days 35-41), starting 1 hour before the first OVA aerosol challenge. In both studies airway responsiveness to methacholine, OVA-specific IgE levels in serum, cellular infiltration, and T_H2 cytokine levels in the bronchoalveolar lavage fluid (BALF) were measured 24 hours after the last OVA challenge.

Because the studies above showed that IDO inhibition during immunotherapy reduced allergic airway inflammation, we next determined whether depletion of tryptophan or particular tryptophan metabolites mediated the effects of immunotherapy. Therefore in study B3 mice were treated intraperitoneally with either tryptophan (100 mg/kg),[27] kynurenine (900 mg/kg),[28] or saline during the entire period of immunotherapy or sham immunotherapy, starting 1 hour before (sham) immunotherapy.

The next series of experiments was aimed to analyze which tryptophan metabolite was involved in immunotherapy because kynurenine is further metabolized to kynurenines.[29] To this end, the effects of the following IDO-dependent tryptophan metabolites were tested (studies B4 and B5): kynurenic acid (KA; 300 mg/kg),[30] 3-hydroxykynurenine (3-OH-KYN; 50 mg/kg),[31] xanthurenic acid (XA; 300 mg/kg),[30] 3-hydroxyanthranilic acid (3-OH-AA; 50 mg/kg),[31] and quinolinic acid (QUINA; 300 mg/kg).[30] Compounds (all from Sigma-Aldrich) were dissolved in saline and daily injected intraperitoneally during immunotherapy, starting 1 hour before immunotherapy. Control mice received saline under the same conditions.

Airway resistance measurement

Before assessment, mice were anesthetized by means of intraperitoneal injection of ketamine (100 mg/kg; Pfizer, New York, NY) and medetomidine (1 mg/kg; Pfizer), tracheotomized (20-gauge intravenous cannula; Becton Dickinson, Alphen a/d Rijn, The Netherlands), and intravenously cannulated through the jugular vein. Thereafter, mice were attached to a computer-controlled small-animal ventilator (Flexivent; SCIREQ, Montreal, Quebec, Canada). Anesthesia was maintained by means of supplemental administration of 25% of the initial dose at 15-minute intervals. Mice were then ventilated at a breeding frequency of 300 breaths/min and a tidal volume of 10 mL/kg. Tidal volume was pressure limited at 300 mm H_2O. At the start of the measurement, oxygen saturation was 97% to 98%, as measured with a pulse oximeter (Nonin, The Netherlands) attached to the rear paw. Resistance in response to intravenous administration of increasing doses of methacholine (acetyl-β-methylcholine chloride, Sigma-Aldrich) was calculated from the pressure response to a 2-second pseudorandom pressure wave, as described previously.[32]

Determination of serum levels of OVA-specific IgE

Serum samples were taken 24 hours after the last challenge, and OVA-specific IgE levels were determined by means of ELISA.[25]

Analysis of the BALF and lung tissue

Bronchoalveolar lavage was performed immediately after bleeding of the mice by means of lavage of the airways through a tracheal cannula with 1 mL of saline (37°C) containing protease inhibitor (Complete mini tablet [Roche Diagnostics, Mannheim, Germany] and 5% BSA). The supernatant of this first milliliter of BALF was used to measure cytokine levels. Subsequently, mice were lavaged 4 times with 1 mL of saline (37°C). By using separate groups of mice, after lavage, single-cell suspensions from lung digests were prepared.[33] Briefly, lungs were gently minced; transferred to RPMI 1640 medium supplemented with 10% FCS, DNAse I (Roche Diagnostics), and collagenase I (Sigma-Aldrich); and incubated for 90 minutes at 37°C in 5% CO_2. The digested lung tissue was filtered through a 70-μm nylon cell strainer with 25 mL of PBS to obtain a single-cell suspension. Thereafter, cell suspensions were centrifuged (500*g* at 4°C for 5 minutes) and the cell pellets were resuspended in 5 mL of PBS. Cells in BALF and in lung digests were analyzed as described previously.[25]

Cytokine levels in BALF

IL-5, IL-10, and IL-13 levels in BALF were determined by means of ELISA (PharMingen, San Diego, Calif). The detection limits were 32 pg/mL for IL-5 and 15 pg/mL for IL-10 and IL-13.

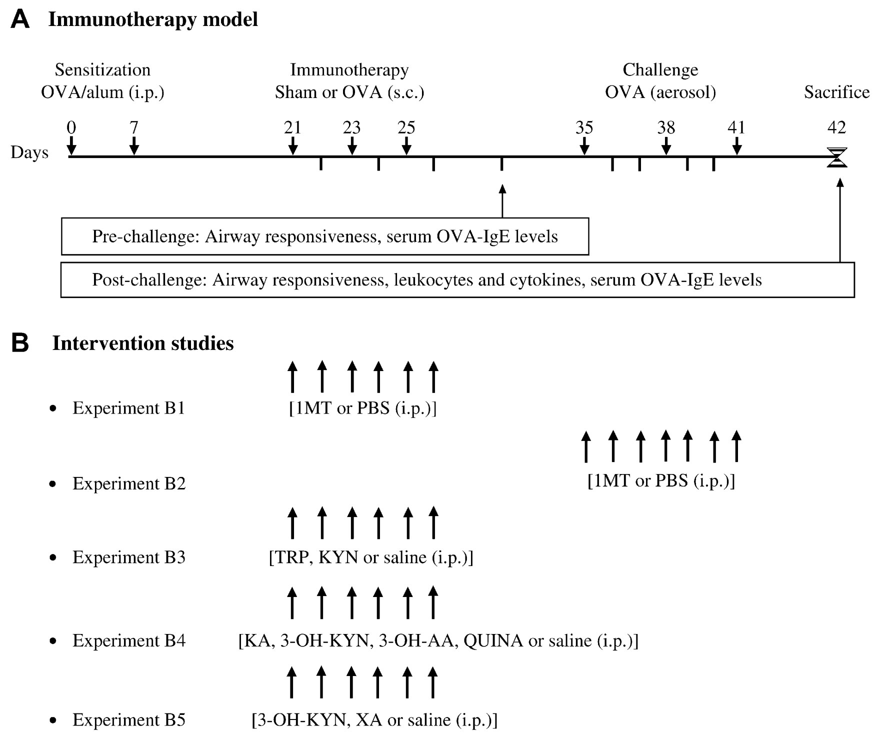

FIG 1. Outline of the immunotherapy protocol in a murine model of asthma and the immunotherapy intervention studies. **A**, OVA-sensitized mice received 1 mg or 100 μg of OVA immunotherapy subcutaneously *(s.c.)* and were challenged with OVA aerosols. **B**, Daily intervention with the IDO inhibitor 1MT or PBS during immunotherapy *(B1)* or during challenge *(B2)* or daily intervention with tryptophan *(TRP)*, kynurenine *(KYN)*, KA, 3-OH-KYN, 3-OH-AA, QUINA, XA, or saline *(B3-B5)*.

Statistical analysis

All data are expressed as means ± SEMs. Levels of cytokines and immunoglobulins were compared by using the Student *t* test (2-tailed, homoscedastic). Cell counts were compared by using the Mann-Whitney *U* test. The airway resistance curves to methacholine were statistically analyzed by using a general linear model of repeated measurements, followed by post-hoc comparison between groups. A *P* value of less than .05 was considered significant.

RESULTS

Effects of IDO inhibition on the efficacy of immunotherapy

Airway responsiveness. Sham-treated OVA-challenged mice exhibited significantly higher airway resistance to graded doses of methacholine than those of OVA-sensitized PBS-challenged mice (Fig 2, *A*). Compared with sham treatment, OVA immunotherapy significantly suppressed development of OVA challenge–induced airway resistance to methacholine (Fig 2, *A*). Inhibition of IDO by 1MT during immunotherapy did not change the suppressed airway resistance to methacholine by means of OVA immunotherapy compared with that seen in the OVA immunotherapy–treated group (Fig 2, *A*). Moreover, 1MT treatment did not change the AHR of sham immunotherapy–treated mice (data not shown).

OVA-specific IgE levels in serum. Sham-treated mice displayed significantly increased (96%, $P < .05$) OVA-specific IgE levels in serum after OVA challenge compared with prechallenge levels (Table I). Immunotherapy significantly suppressed the increase in IgE levels by 79% ($P < .05$) compared with that seen after sham immunotherapy. Neither inhibition of IDO during the time of immunotherapy (Table I) nor during the time of OVA challenge (data not shown) influenced the reduction in OVA-specific IgE levels by means of immunotherapy. OVA-specific IgE levels in sera of sham-treated mice were not affected by inhibition of IDO in either experiment (Table I and data not shown, respectively).

Eosinophils and cytokine levels in the lung. OVA challenge of sham-treated OVA-sensitized mice resulted in high numbers of eosinophils (Fig 2, *B* and *C*). OVA immunotherapy effectively suppressed the airway eosinophilia by 93% ($P < .01$) in BALF and by 74% ($P < .05$) in lung tissue compared with that seen in sham-treated mice (Fig 2, *B* and *C*, respectively). Importantly, inhibition of IDO during immunotherapy significantly antagonized the suppression in eosinophilia induced by immunotherapy (BALF: 56% reversal, $P < .001$; lung tissue: 34% reversal, $P < .05$) compared with that seen in mice treated only with immunotherapy (Fig 2, *B* and *C*, respectively). Treatment of mice with 1MT during the challenge period did not affect the immunotherapy-induced reduction in the number of eosinophils in the BALF (data not shown). Inhibition of IDO during sham immunotherapy (Fig 2, *B*) or during the subsequent challenge (data not shown) did not influence the number of eosinophils in the BALF.

Assessment of T_H2 cytokine levels in the BALF of sham-treated mice revealed high levels of IL-5 and IL-13 24 hours after challenge. Immunotherapy significantly reduced the levels of IL-5 by 92% ($P < .01$) and of IL-13 by 97% ($P < .001$) compared with those seen in sham-treated mice (Fig 2, *D*). Inhibition of IDO during immunotherapy considerably abrogated the suppression in IL-5 levels (51% reversal, $P < .01$) and IL-13 levels (40% reversal, $P < .05$). Immunotherapy also reduced the levels of IL-10 by 73%, but this reduction did not reach statistical significance ($P = .12$),

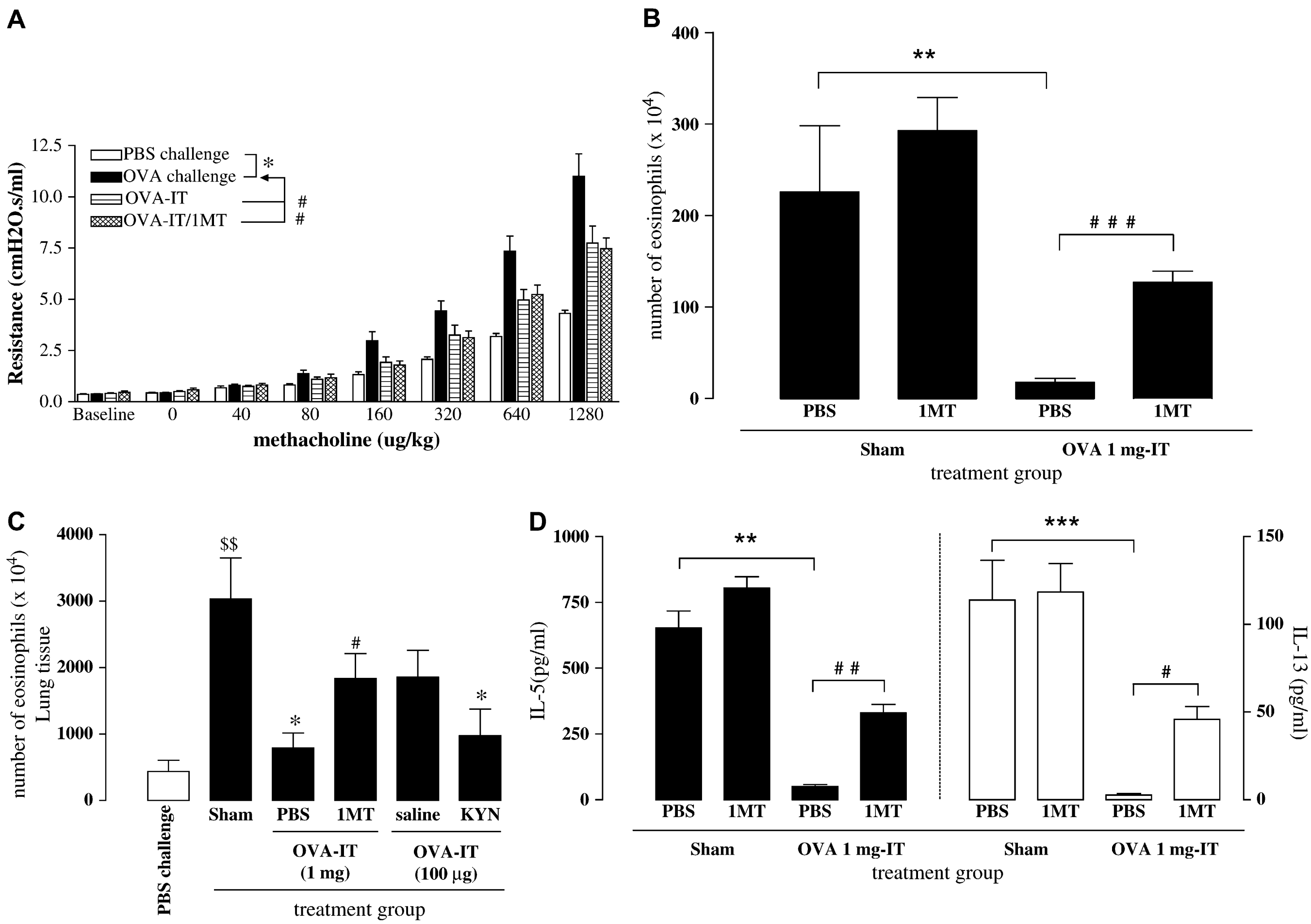

FIG 2. Effects of IDO inhibition on the efficacy of immunotherapy *(IT)*. **A**, Airway responsiveness 1 day after OVA aerosol challenge. **B**, Eosinophil numbers in the BALF. **C**, Eosinophil numbers in lung tissue. **D**, IL-5 and IL-13 levels in the BALF. Values are expressed as means ± SEMs (n = 7-8). *$P < .05$, **$P < .01$, and ***$P < .001$ compared with sham/PBS-treated mice. #$P < .05$, ##$P < .01$, and ###$P < .001$ compared with 1 mg of OVA immunotherapy/PBS–treated mice. $$$P < .01$ compared with PBS-challenged mice.

TABLE I. Serum levels of OVA-specific IgE

	OVA-specific IgE level (× 10^3 EU/mL [mean ± SEM])	
Treatment group	**Before challenge**	**After challenge**
Sham/PBS	1.36 ± 0.37	32.66 ± 10.12*
Sham/1MT	2.41 ± 0.55	49.17 ± 7.99†
OVA immunotherapy/PBS	7.93 ± 2.41	6.73 ± 1.11‡
OVA immunotherapy/1MT	4.80 ± 1.47	6.03 ± 0.43§

Values are expressed as means ± SEMs (n = 7-8).
*$P < .05$ and †$P < .001$ compared with prechallenge OVA-specific IgE levels.
‡$P < .05$ and §$P < .001$ compared with postchallenge OVA-specific IgE levels of sham-treated mice.

and the effect of immunotherapy was not influenced by inhibition of IDO (data not shown). The BALF from sham-treated mice showed no changes in IL-5 and IL-13 levels after inhibition of IDO (Fig 2, *D*). The T_H1 cytokine IFN-γ was not detectable in the BALF obtained from sham- or immunotherapy-treated mice (data not shown).

IDO inhibition during the challenge period did not affect the decrease in T_H2 cytokine levels caused by immunotherapy (data not shown).

Effects of tryptophan and kynurenine on immunotherapy

Mice were treated with tryptophan during optimal immunotherapy with 1 mg of OVA or with kynurenine during suboptimal immunotherapy with 100 μg of OVA to determine whether IDO affected the induction of tolerance toward eosinophilic airway inflammation by depleting tryptophan or by producing kynurenine or other downstream metabolites.

Eosinophils and cytokine levels in the BALF. Optimal immunotherapy with 1 mg of OVA significantly reduced BALF eosinophilia by 88% ($P < .01$), whereas suboptimal immunotherapy with 100 μg did not influence the number of BALF and lung tissue eosinophils (Figs 3, *A*, and 2, *C*, respectively). Administration of tryptophan did not affect the reduction in eosinophilia by means of optimal immunotherapy. However, administration of kynurenine during suboptimal immunotherapy successfully suppressed the influx of eosinophils in BALF by 68% ($P < .05$) compared with that seen in sham-treated mice and by 69% ($P < .05$) compared with that seen in mice that received suboptimal immunotherapy only (Fig 3, *A*). Moreover, administration of kynurenine during suboptimal immunotherapy also significantly potentiated the reduction of eosinophil numbers in lung tissue compared

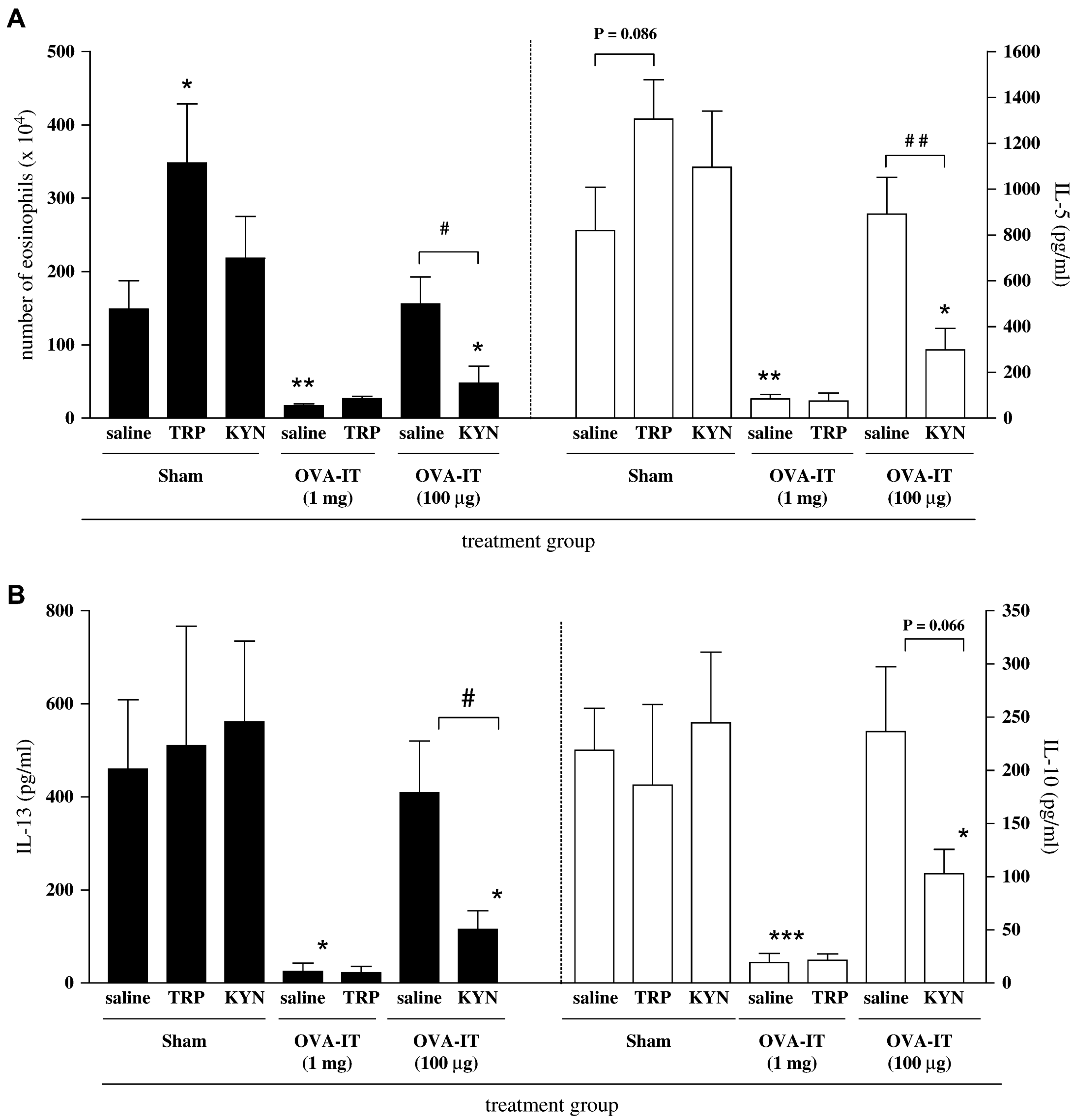

FIG 3. Effects of tryptophan *(TRP)* or its metabolite, kynurenine *(KYN)*, on the efficacy of optimal or suboptimal immunotherapy *(IT)*. **A**, Eosinophil numbers and IL-5 levels. **B**, IL-13 and IL-10 levels in the BALF. Values are expressed as means ± SEMs (n = 6). *$P < .05$, **$P < .01$, and ***$P < .001$ compared with sham/saline-treated mice. #$P < .05$ and ##$P < .01$ compared with 100 μg of OVA immunotherapy/saline–treated mice.

with that seen in sham-treated mice (68% reduction, $P < .05$; Fig 2, *C*). Significant suppression of BALF eosinophilia was also obtained after coadministration of tryptophan at 300 mg/kg with suboptimal immunotherapy (see Fig E1 in the Online Repository at www.jacionline.org). Remarkably, in sham-treated mice tryptophan administration caused a significant further increase in eosinophil numbers in the BALF by 57% ($P < .05$; Fig 3, *A*), but numbers of neutrophils and mononuclear cells were not changed (data not shown). Administration of tryptophan during optimal immunotherapy did not influence the immunotherapy-induced reduction of IL-5, IL-13, and IL-10 levels in the BALF (Fig 3). In contrast, administration of kynurenine during suboptimal immunotherapy significantly decreased the levels of IL-5 by 64% ($P < .05$), IL-13 by 75% ($P < .05$), and IL-10 by 53% ($P < .05$) compared with levels seen in sham-treated mice and decreased the levels of IL-5 by 67% ($P < .01$), IL-13 by 72% ($P < .05$), and IL-10 by 57% ($P = .066$) compared with levels seen in mice treated only with suboptimal immunotherapy (Fig 3).

OVA-specific IgE levels in serum. The downregulation of OVA-specific IgE levels in serum induced by optimal immunotherapy was not changed by treatment with tryptophan (data not shown). Also, kynurenine administration did not affect the OVA-specific IgE levels in sera from sensitized mice receiving suboptimal immunotherapy or sham immunotherapy (data not shown).

Effects of kynurenine metabolites on the efficacy of immunotherapy

Because *in vivo* kynurenine is quickly degraded to kynurenines, we aimed to determine whether kynurenine itself or one of its metabolites that are physiologically generated downstream of the initial and rate-limiting step mediated by IDO in tryptophan degradation mediated the beneficial effects of immunotherapy on the

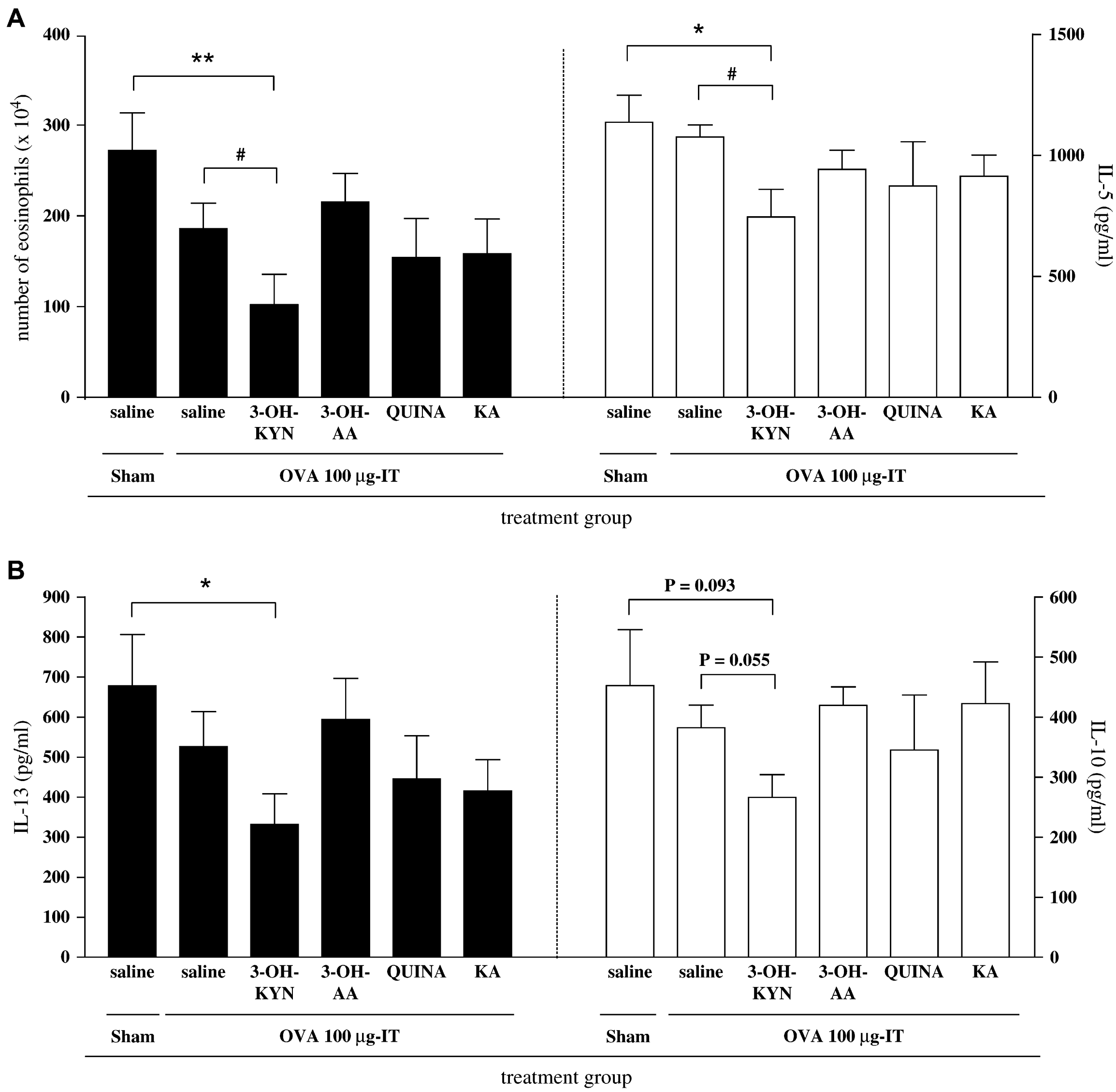

FIG 4. Effects of kynurenines on the efficacy of suboptimal immunotherapy *(IT)*. **A**, Eosinophil numbers and IL-5 levels. **B**, IL-13 and IL-10 levels in the BALF 1 day after airway challenge with OVA aerosols. Values are expressed as means ± SEMs (n = 6). **P* < .05 and ***P* < .01 compared with sham/saline-treated mice. #*P* < .05 and *P* < .01 compared with 100 μg of OVA immunotherapy/saline–treated mice.

reduction of eosinophilic airway inflammation. Mice were treated with suboptimal immunotherapy and one of the metabolites (ie, KA, 3-OH-KYN, 3-OH-AA, or QUINA) or saline.

Eosinophils and cytokine levels in BALF. Suboptimal immunotherapy suppressed the airway eosinophilia, although not significantly (Fig 4, *A*), and this was not changed by administration of 3-OH-AA, KA, or QUINA during the immunotherapy. Interestingly, administration of 3-OH-KYN effectively suppressed the number of eosinophils in the BALF by 62% (*P* < .01) compared with that seen in sham-treated mice and by 45% (*P* < .05) compared with that seen in mice receiving suboptimal immunotherapy only. The suppression was paralleled by a reduction in BALF levels of IL-5 by 34% (*P* < .05), IL-13 by 51% (*P* < .05), and IL-10 by 41% (*P* = .09) compared with those seen in sham-treated mice and by 31% (*P* < .05), 37% (*P* = .12), and 30% (*P* = .055) for IL-5, IL-13, and IL-10, respectively, compared with levels seen in mice that received suboptimal immunotherapy only (Fig 4). Levels of these cytokines were not changed by administration of KA, 3-OH-AA, or QUINA during suboptimal immunotherapy (Fig 4).

OVA-specific IgE levels in serum. Suboptimal immunotherapy alone or in combination with the kynurenine metabolites did not affect OVA-specific IgE levels in sera compared with those on sham immunotherapy (data not shown).

Effects of 3-OH-KYN and XA on the efficacy of immunotherapy

Herein we wanted to determine the effects of combination of 3-OH-KYN and the direct downstream metabolite of kynurenine aminotransferase, XA, on the beneficial effects of immunotherapy responses because under physiologic conditions tryptophan metabolites probably do not act as single substance and to answer the interesting question of whether the combination of active metabolites is more effective than a single substance. Mice were treated with 3-OH-KYN, XA, or both during suboptimal immunotherapy.

Eosinophils and cytokine levels in the BALF. Suboptimal immunotherapy did not significantly affect OVA challenge–induced eosinophilia. Administration of 3-OH-KYN, XA, or the combination during the therapy significantly reduced eosinophilia compared with that seen in sham-treated mice (Fig 5), but when compared with mice receiving suboptimal immunotherapy, only 3-OH-KYN significantly suppressed eosinophilia.

Suboptimal immunotherapy caused no significant changes of cytokine levels in BALF, but when combined with 3-OH-KYN, XA, or the combination, levels of IL-5, IL-10, and IL-13 were significantly less than in sham-treated mice. Compared with mice merely receiving suboptimal immunotherapy, levels of IL-5 and IL-13 were significantly less on cotreatment with 3-OH-KYN or XA and IL-13 levels also on cotreatment with the combination (data not shown).

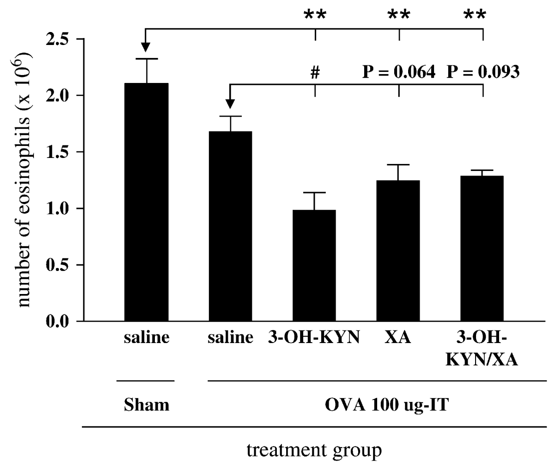

FIG 5. Effects of 3-OH-KYN and XA alone or in combination on the efficacy of suboptimal immunotherapy *(IT)* on eosinophil numbers in the BALF. Values are expressed as means ± SEMs (n = 6). $^{**}P < .01$ compared with sham/saline-treated mice. $\#P < .05$ compared with 100 μg of OVA immunotherapy/saline–treated mice.

DISCUSSION

The present study demonstrates that IDO plays a role in the efficacy of allergen immunotherapy with respect to reduction of airway eosinophilia and T_H2 cytokine levels. Our observation that inhibition of IDO with 1MT interfered with immunotherapy when it was administered during immunotherapy, but not during challenge, demonstrates that tolerance induction is partially mediated by IDO activation during immunotherapy and that activity of IDO is irrelevant thereafter. This and the observation that tryptophan, when administered during immunotherapy, appeared not to inhibit the efficacy of immunotherapy indicate that formation of tryptophan metabolites, rather than tryptophan depletion, is a mechanism by which immunotherapy induces tolerance to the induction of allergic airway inflammation. This conclusion was supported by our findings that tryptophan itself (see the Online Repository at www.jacionline.org), as well as the tryptophan metabolites kynurenine, 3-OH-KYN, and XA, potentiated the efficacy of suboptimal immunotherapy. Although it cannot be excluded that the effects of 1MT are related to the recently described interference with Toll-like receptor signaling in DCs,[34] this appears rather unlikely considering the effects of tryptophan metabolites on immunotherapy.

Until now, IDO was shown to be involved in maternal tolerance during pregnancy,[19] control of allograft rejection,[20] and protection against autoimmunity,[35] nasal tolerance,[18] and experimental colitis.[36] This is, to the best of our knowledge, the first study showing that IDO plays at least a partial role in tolerance induction by allergen immunotherapy in a mouse model of T_H2-dependent allergic airway inflammation. IDO-dependent tryptophan metabolites appeared involved in tolerance induction by immunotherapy as to airway eosinophilia and T_H2 cytokines. The immunotherapy-induced reductions in allergen-specific IgE and AHR, however, appeared not to be mediated by an IDO-dependent mechanism because the efficacy of optimal immunotherapy to reduce IgE levels and AHR was not affected by 1MT. In addition, tryptophan metabolites did not potentiate reduction of IgE levels by means of suboptimal immunotherapy. These data demonstrate that immunotherapy differentially regulates the pathways leading to allergen-induced airway inflammation and those increasing serum IgE levels and airway responsiveness, indicating that multiple mechanisms are at play. In addition, these data support earlier observations that production of allergen-specific IgE and the development of AHR can be dissociated from the induction of eosinophilic airway inflammation in mouse models.[37] Moreover, data are in agreement with the observation that B cells, unlike T cells, are insensitive to the cytotoxic action of tryptophan metabolites[23] and with studies showing that tolerizing B cells is T cell independent.[38] The mechanisms by which IDO-dependent tryptophan metabolites mediate the immunotherapy-induced suppression of eosinophilia and T_H2 cytokine levels are not known at present. Because IFN-γ levels remained less than the detection limit after immunotherapy, a shift from a T_H2 to a T_H1 response is probably not at play. Therefore it is not unlikely that one or more subsets of regulatory T cells are implicated because a role for IDO in the generation of Treg cells has been suggested.[14] Particularly T_R1 cells might be involved because efficacy of immunotherapy in our model was earlier shown to involve IL-10[26] and because T_R1 cells are potent producers of this immunoregulatory cytokine.[39] Moreover, in human studies it was clearly demonstrated that allergen immunotherapy against bee venom, house dust mite, and grass pollen is associated with increased numbers of IL-10–producing regulatory T cells, TGF-β–producing regulatory T cells, or both.[6-8] However, the IDO-dependent effect of immunotherapy is selective for allergic inflammatory responses, but not for IgE and AHR, which indicates that multiple regulatory mechanisms are at play. Another explanation for the differential regulation of asthma manifestations by immunotherapy might be that each requires a different level of immunosuppression or is less dependent on a memory T-cell response.

The fact that not all kynurenines are active and no additive or synergistic effects between 3-OH-KYN and XA were found when administered together during suboptimal immunotherapy might suggest that one of these 2 substances is responsible for the induction of immune tolerance mediated by IDO. Interestingly, 3-OH-KYN was recently found to inhibit proliferation, to increase IL-10 production by murine splenocytes stimulated with a T_H1 response–inducing peptide antigen, and to stimulate IL-10 production *in vivo*.[40] It can be questioned whether the suppressive action of 3-OH-KYN in our *in vivo* model is mediated through this mechanism because 3-OH-AA that was inactive in our model acted similarly on the antigen-stimulated splenocytes.[40] However, this might merely be a matter of different pharmacokinetic profiles *in vivo*. Therefore although 3-OH-AA, QUINA, and KA were not active in potentiating the effect of immunotherapy, we cannot completely exclude that they do play a role in immunotherapy.

Even though in the current study the identity of cells expressing IDO is not yet known, several mouse and human studies showed that macrophages and DCs are the cells with the most prominent IDO activity.[16,23,41] Because human macrophages can generate 3-OH-AA, but not 3-OH-KYN, on stimulation with IFN-γ[42] and because 3-OH-KYN, but not 3-OH-AA, was active in our study, immunotherapy-induced IDO expression by DCs rather than macrophages might be involved in this model. More studies are needed to address the antigen-presenting cell type or types that express IDO and generate kynurenines during immunotherapy.

Our data are not completely in line with the hypothesis that the combined effects of tryptophan depletion and kynurenine production are required for the generation of IL-10– and TGF-β–producing regulatory T cells.[13,14] Although we observed potentiation of immune tolerance toward eosinophilic airway inflammation and T_H2 cytokines by using the specific kynurenines 3-OH-KYN and XA, tryptophan administration did not reverse this potentiation. Moreover, tryptophan administration during suboptimal immunotherapy potentiated the suppression of airway eosinophilia (see the Online Repository at www.jacionline.org). One likely explanation for this discrepancy might be that Belladonna et al[13] and Fallarino et al[14] used *in vitro* T-cell activation, whereas we used an *in vivo* model.

In the present study we observed that inhibition of IDO during the effector phase did not antagonize the beneficial effects of allergen immunotherapy after allergen inhalation. In agreement with the latter, neither Hessel et al[43] nor Hayashi et al[44] observed an effect of IDO inhibition by 1MT during allergen inhalation challenge in previously sensitized sham-treated mice. However, Hayashi et al[44] did observe a role for IDO during allergen inhalation challenge in mice treated systemically with immunostimulatory oligodeoxynucleotide sequences. This indicates that the mechanism of suppression after allergen immunotherapy is different from that after immunostimulatory oligodeoxynucleotide sequence treatment.

In summary, we clearly demonstrated that IDO activity contributes to tolerance induction during allergen immunotherapy toward eosinophilic airway inflammation and T_H2 cytokine levels and that generation of tryptophan metabolites rather than tryptophan depletion is involved in promoting this type of tolerance. These findings provide further understanding of the complex mechanisms that might contribute to immunotherapy intervention and might be helpful to enhance the prospects for successful immunotherapy in allergic airway inflammation.

Clinical implications: Indoleamine 2,3-dioxygenase activity might be of therapeutic utility in allergen immunotherapy.

REFERENCES

1. Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. J Allergy Clin Immunol 1998;102: 558-62.
2. 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.
3. Wachholz PA, Durham SR. Induction of "blocking" IgG antibodies during immunotherapy. Clin Exp Allergy 2003;33:1171-4.
4. Durham SR, Ying S, Varney VA, Jacobson MR, Sudderick RM, Mackay IS, et al. Grass pollen immunotherapy inhibits allergen-induced infiltration of CD4+ T lymphocytes and eosinophils in the nasal mucosa and increases the number of cells expressing messenger RNA for interferon-gamma. J Allergy Clin Immunol 1996;97:1356-65.
5. Rocklin RE, Sheffer AL, Greineder DK, Melmon KL. Generation of antigen-specific suppressor cells during allergy desensitization. N Engl J Med 1980;302: 1213-9.
6. Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. J Clin Invest 1998;102:98-106.
7. 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.
8. 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.
9. Kapsenberg ML. Dendritic-cell control of pathogen-driven T-cell polarization. Nat Rev Immunol 2003;3:984-93.
10. Lambrecht BN, Hammad H. Taking our breath away: dendritic cells in the pathogenesis of asthma. Nat Rev Immunol 2003;3:994-1003.
11. Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. Nat Immunol 2001;2:725-31.
12. Weiner HL. The mucosal milieu creates tolerogenic dendritic cells and T(R)1 and T(H)3 regulatory cells. Nat Immunol 2001;2:671-2.
13. Belladonna ML, Grohmann U, Guidetti P, Volpi C, Bianchi R, Fioretti MC, et al. Kynurenine pathway enzymes in dendritic cells initiate tolerogenesis in the absence of functional IDO. J Immunol 2006;177:130-7.
14. Fallarino F, Grohmann U, You S, McGrath BC, Cavener DR, Vacca C, et al. The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells. J Immunol 2006;176:6752-61.
15. Takikawa O, Yoshida R, Kido R, Hayaishi O. Tryptophan degradation in mice initiated by indoleamine 2,3-dioxygenase. J Biol Chem 1986;261:3648-53.
16. Munn DH, Sharma MD, Lee JR, Jhaver KG, Johnson TS, Keskin DB, et al. Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. Science 2002;297:1867-70.
17. Grohmann U, Fallarino F, Bianchi R, Belladonna ML, Vacca C, Orabona C, et al. IL-6 inhibits the tolerogenic function of CD8 alpha+ dendritic cells expressing indoleamine 2,3-dioxygenase. J Immunol 2001;167:708-14.
18. van der Marel AP, Samsom JN, Greuter M, van Berkel LA, O'Toole T, Kraal G, et al. Blockade of IDO inhibits nasal tolerance induction. J Immunol 2007;179: 894-900.
19. Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B, et al. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science 1998;281: 1191-3.
20. Grohmann U, Orabona C, Fallarino F, Vacca C, Calcinaro F, Falorni A, et al. CTLA-4-Ig regulates tryptophan catabolism in vivo. Nat Immunol 2002;3: 1097-101.
21. Sakurai K, Zou JP, Tschetter JR, Ward JM, Shearer GM. Effect of indoleamine 2,3-dioxygenase on induction of experimental autoimmune encephalomyelitis. J Neuroimmunol 2002;129:186-96.
22. Friberg M, Jennings R, Alsarraj M, Dessureault S, Cantor A, Extermann M, et al. Indoleamine 2,3-dioxygenase contributes to tumor cell evasion of T cell-mediated rejection. Int J Cancer 2002;101:151-5.
23. Frumento G, Rotondo R, Tonetti M, Damonte G, Benatti U, Ferrara GB. Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase. J Exp Med 2002;196: 459-68.
24. Terness P, Bauer TM, Rose L, Dufter C, Watzlik A, Simon H, et al. Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. J Exp Med 2002;196:447-57.
25. Van Oosterhout AJ, 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.
26. Vissers JL, van Esch BC, Hofman GA, Kapsenberg ML, Weller FR, van Oosterhout AJ. Allergen immunotherapy induces a suppressive memory response

mediated by IL-10 in a mouse asthma model. J Allergy Clin Immunol 2004; 113:1204-10.
27. Hilakivi-Clarke LA. Effects of tryptophan on depression and aggression in STZ-D mice. Diabetes 1991;40:1598-602.
28. Vecsei L, Miller J, MacGarvey U, Beal MF. Kynurenine and probenecid inhibit pentylenetetrazol- and NMDLA-induced seizures and increase kynurenic acid concentrations in the brain. Brain Res Bull 1992;28:233-8.
29. Stone TW. Inhibitors of the kynurenine pathway. Eur J Med Chem 2000;35: 179-86.
30. Heyliger SO, Goodman CB, Ngong JM, Soliman KF. The analgesic effects of tryptophan and its metabolites in the rat. Pharmacol Res 1998;38:243-50.
31. Lapin IP, Prakh'e IB, Khaunina RA. [Effect of kynurenine and its metabolites on the concentration of 11-hydroxycorticosteroids in rat plasma]. Vopr Med Khim 1976;22:600-2.
32. Kang HS, Blink SE, Chin RK, Lee Y, Kim O, Weinstock J, et al. Lymphotoxin is required for maintaining physiological levels of serum IgE that minimizes Th1-mediated airway inflammation. J Exp Med 2003;198:1643-52.
33. Hylkema MN, Hoekstra MO, Luinge M, Timens W. The strength of the OVA-induced airway inflammation in rats is strain dependent. Clin Exp Immunol 2002;129:390-6.
34. Agaugue S, Perrin-Cocon L, Coutant F, Andre P, Lotteau V. 1-Methyl-tryptophan can interfere with TLR signaling in dendritic cells independently of IDO activity. J Immunol 2006;177:2061-71.
35. Grohmann U, Fallarino F, Bianchi R, Orabona C, Vacca C, Fioretti MC, et al. A defect in tryptophan catabolism impairs tolerance in nonobese diabetic mice. J Exp Med 2003;198:153-60.
36. Gurtner GJ, Newberry RD, Schloemann SR, McDonald KG, Stenson WF. Inhibition of indoleamine 2,3-dioxygenase augments trinitrobenzene sulfonic acid colitis in mice. Gastroenterology 2003;125:1762-73.
37. Mehlhop PD, van de Rijn M, Goldberg AB, Brewer JP, Kurup VP, Martin TR, et al. Allergen-induced bronchial hyperreactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma. Proc Natl Acad Sci U S A 1997; 94:1344-9.
38. Kouskoff V, Lacaud G, Nemazee D. T cell-independent rescue of B lymphocytes from peripheral immune tolerance. Science 2000;287:2501-3.
39. Roncarolo MG, Bacchetta R, Bordignon C, Narula S, Levings MK. Type 1 T regulatory cells. Immunol Rev 2001;182:68-79.
40. Platten M, Ho PP, Youssef S, Fontoura P, Garren H, Hur EM, et al. Treatment of autoimmune neuroinflammation with a synthetic tryptophan metabolite. Science 2005;310:850-5.
41. Mellor AL, Baban B, Chandler P, Marshall B, Jhaver K, Hansen A, et al. Cutting edge: induced indoleamine 2,3 dioxygenase expression in dendritic cell subsets suppresses T cell clonal expansion. J Immunol 2003;171:1652-5.
42. Werner ER, Hirsch-Kauffmann M, Fuchs D, Hausen A, Reibnegger G, Schweiger M, et al. Interferon-gamma-induced degradation of tryptophan by human cells in vitro. Biol Chem Hoppe Seyler 1987;368:1407-12.
43. Hessel EM, Chu M, Lizcano JO, Chang B, Herman N, Kell SA, et al. Immunostimulatory oligonucleotides block allergic airway inflammation by inhibiting Th2 cell activation and IgE-mediated cytokine induction. J Exp Med 2005;202:1563-73.
44. Hayashi T, Beck L, Rossetto C, Gong X, Takikawa O, Takabayashi K, et al. Inhibition of experimental asthma by indoleamine 2,3-dioxygenase. J Clin Invest 2004; 114:270-9.

METHODS

Mice were daily treated intraperitoneally with tryptophan at doses of 30, 100, and 300 mg/kg during the entire period of immunotherapy, starting 1 hour before 100 μg of OVA immunotherapy.

RESULTS

Eosinophils in the BALF

On allergen inhalation challenge, the BALF contained high numbers of inflammatory cells consisting predominantly of eosinophils (Fig E1) in addition to mononuclear cells and a few neutrophils (data not shown). Optimal OVA immunotherapy (1 mg) effectively suppressed the airway eosinophilia because the number of eosinophils in the BALF was significantly reduced by 97% ($P < .05$) compared with that seen in sham-treated mice (Fig E1). One hundred micrograms of OVA immunotherapy partially suppressed (41%, not significant) the influx of eosinophils in the BALF (Fig E1). Coadministration of tryptophan at 30 and 100 mg/kg slightly potentiated (not significant) the suppressive effects of 100 μg of OVA immunotherapy on the number of eosinophils in the BALF (Fig E1). Administration of tryptophan at 300 mg/kg significantly potentiated the reduction of eosinophil number in the BALF by 100 μg of OVA immunotherapy compared with that seen in mice receiving sham treatment (79% reduction, $P < .05$; Fig E1).

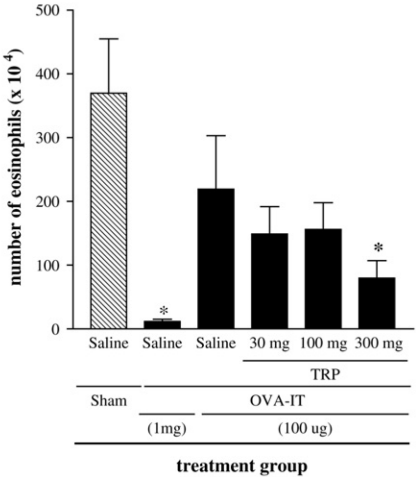

FIG E1. Effect of tryptophan *(TRP)* administration on the efficacy of suboptimal OVA immunotherapy (100 μg) on eosinophil numbers in the BALF 1 day after OVA aerosol challenge. Values are expressed as means ± SEMs (n = 6). $^*P < .05$ compared with sham-treated mice.