The Journal of Immunology

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J Immunol 2008; 180:5211-5221; ;
doi: 10.4049/jimmunol.180.8.5211
http://www.jimmunol.org/content/180/8/5211

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

1α,25-Dihydroxyvitamin D_3 Potentiates the Beneficial Effects of Allergen Immunotherapy in a Mouse Model of Allergic Asthma: Role for IL-10 and TGF-β[1]

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1α,25-Dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), a potent inhibitor of NF-κB expression, can prevent the maturation of dendritic cells in vitro leading to tolerogenic dendritic cells with increased potential to induce regulatory T cells. Herein, we investigated whether the combination of allergen immunotherapy with 1,25$(OH)_2D_3$ potentiates the suppressive effects of immunotherapy and whether the immunoregulatory cytokines IL-10 and TGF-β are involved in the effector phase. OVA-sensitized and challenged BALB/c mice displayed airway hyperresponsiveness (AHR) and increased serum OVA-specific IgE levels, bronchoalveolar lavage eosinophilia, and Th2 cytokine levels. In this model, the dose response of allergen immunotherapy 10 days before OVA inhalation challenge shows strong suppression of asthma manifestations at 1 mg of OVA, but partial suppression of bronchoalveolar lavage eosinophilia, IgE up-regulation, and no reduction of AHR at 100 μg. Interestingly, coadministration of 10 ng of 1,25$(OH)_2D_3$ with 100 μg of OVA immunotherapy significantly inhibited AHR and potentiated the reduction of serum OVA-specific IgE levels, airway eosinophilia, and Th2-related cytokines concomitant with increased IL-10 levels in lung tissues and TGF-β and OVA-specific IgA levels in serum. Similar effects on suboptimal immunotherapy were observed by inhibition of the NF-κB pathway using the selective IκB kinase 2 inhibitor PS-1145. The suppressive effects of this combined immunotherapy were partially reversed by treatment with mAb to either IL-10R or TGF-β before OVA inhalation challenge but completely abrogated when both Abs were given. These data demonstrate that 1,25$(OH)_2D_3$ potentiates the efficacy of immunotherapy and that the regulatory cytokines IL-10 and TGF-β play a crucial role in the effector phase of this mouse model. *The Journal of Immunology,* **2008, 180: 5211–5221.**

Allergen-specific immunotherapy (IT)[3] has proven to be beneficial and is recommended as an alternative for treating allergic disorders (1). Although administration of specific allergens is effective in rhinitis and insect venom allergy, the role of this intervention in allergic asthma remains controversial (2, 3). Furthermore, the major drawbacks of this treatment are the risk of eliciting rare but life-threatening systemic reactions in patients receiving build-up and maintenance doses of IT (3). Improvement of IT is needed to get better efficacy before widespread application in asthmatic patients can be considered. However, for rational improvement of IT elucidation of the precise underlying mechanisms is required. The efficacy of IT has been related to the induction of blocking IgG Abs (4), the induction of anergy in T cells (5), or a shift of cytokine expression from Th2 cytokines that are associated with an asthmatic phenotype toward Th1 cytokines (6, 7). Recently, induction of regulatory T (Treg) cells secreting the immunoregulatory cytokines IL-10 and TGF-β has been implicated in the down-regulation of allergen-specific T cell proliferation in vitro after IT (8–10).

Dendritic cells (DCs) are the most potent APCs and play a key role in the generation of adaptive T cell subsets (11). DCs are not only immunogenic but also tolerogenic, depending on their maturation state (12). Although several studies have suggested that mature DCs can induce $CD4^+$ T cell tolerance (13, 14), tolerogenic DCs generally are semimature with increased expression of MHC class II and B7-2, but low expression of CD40 and no production of the proinflammatory cytokines IL-6 and TNF-α (15). Likewise, incubation of T cells with semimature or immature DCs has been shown to induce Ag-specific Treg cells like $CD4^+$ Th3 cells and $CD4^+$ T regulatory 1 (Tr1) cells (16, 17). Although direct evidence is lacking, we assume that immature tolerogenic DCs can be instrumental in the generation of Treg cells during allergen IT.

Previously, we developed a mouse model of allergic asthma in which allergen IT suppressed allergen-induced airway manifestations of asthma (18). Moreover, allergen IT induces IL-10-dependent long-lasting tolerance to OVA-induced asthma manifestations, pointing to a role for Tr1 cells (19). Since many of the observed immunological changes following allergen IT may be mediated by the induction of Treg cells and since immature tolerogenic DCs play a critical role in Treg cell generation and peripheral tolerance, we were interested in whether allergen IT could be improved using a compound that inhibits DC maturation. The NF-κB protein Re1B is essential for DC differentiation and

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Received for publication February 28, 2007. Accepted for publication February 6, 2008.

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[1] This work was supported by Research Grant 1575.1370 from Al-Fateh Medical University, Tripoli-Libya (to Y.A.T.).

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[3] Abbreviations used in this paper: IT, immunotherapy; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; DRC, dose-response curve; Penh, enhanced pause; DC, dendritic cell; Treg, regulatory T; Tr1, T regulatory 1; mIgG, mouse IgG; rIgG, rat IgG; TLN, thoracic lymph node; IKK-2, IκB kinase 2; EU, experimental unit.

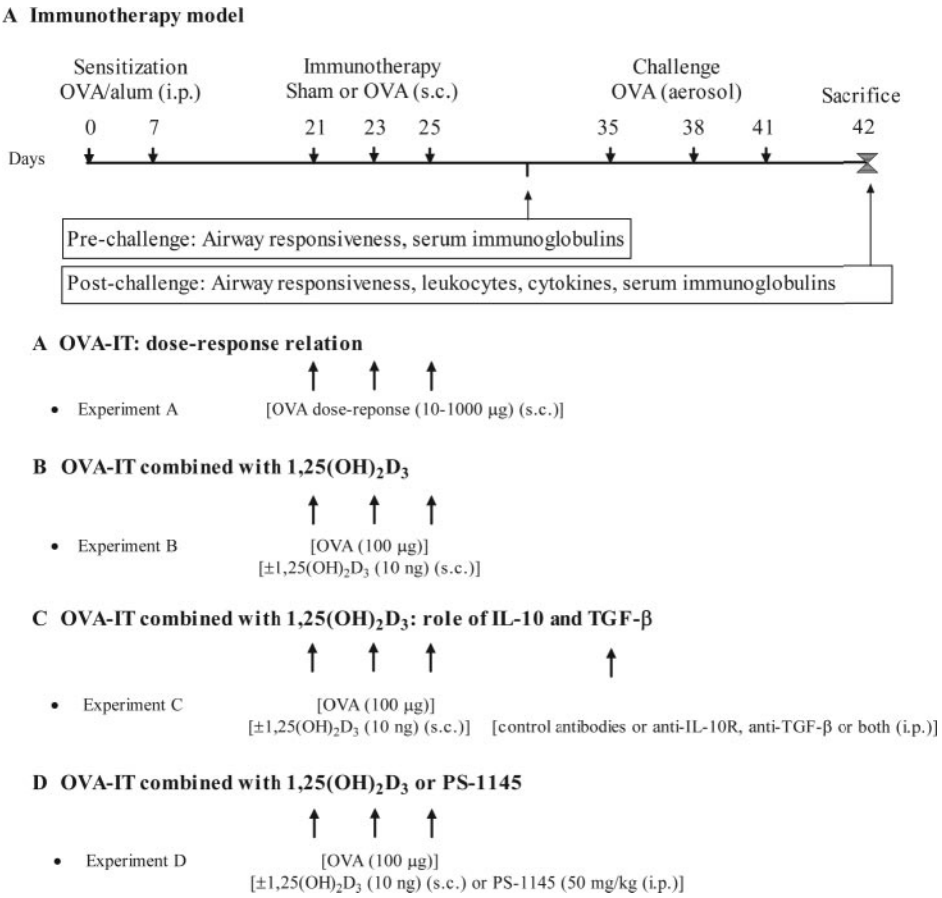

FIGURE 1. Outline of the IT protocol in a murine model of asthma and the intervention studies. OVA-sensitized mice received IT with 10–1000 μg of OVA on days 21, 23, and 25 (*A*), 100 μg of OVA combined with 10 ng of $1,25(OH)_2D_3$ s.c. on days 21, 23, and 25 (*B*), and IT with suboptimal (100 μg) OVA combined with 10 ng $1,25(OH)_2D_3$ s.c. on days 21, 23, and 25. Intervention with anti-IL-10R, anti-TGF-β, or both or control Abs (rIgG plus mIgG) was just before the first OVA aerosol challenge (day 35) (*C*), or 100 μg of OVA combined with 10 ng of $1,25(OH)_2D_3$ s.c. or 50 mg/kg PS-1145 i.p. on days 21, 23, and 25 (*D*). All mice were challenged with OVA aerosols on days 35, 38, and 41. Airway responsiveness to methacholine and serum levels of OVA-specific Igs were measured just before as well as 1 day after the last challenge and leukocyte numbers in BAL fluid and lung tissue and cytokine levels in BAL fluid, lung tissue, and TLN cell cultures 1 day after challenge.

maturation (20, 21). Inhibition of NF-κB signaling by various drugs has been shown to generate immature tolerogenic DCs (22–24). A number of studies have consistently shown that treatment of human DCs or murine bone marrow-derived DCs with 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) inhibits expression of Re1B, costimulatory molecules, and IL-12 secretion but enhances IL-10 production (20, 25, 26). Moreover, $1,25(OH)_2D_3$ appears to generate tolerogenic DCs in vivo, as demonstrated in models of transplantation and autoimmune diseases (27, 28).

In this study, we investigated whether coinjection of $1,25(OH)_2D_3$ potentiates the suppressive effects of IT in our mouse model. In addition, to investigate whether the regulatory cytokines IL-10 and TGF-β are involved in tolerance to OVA-induced airway manifestations of asthma, we functionally blocked IL-10 receptors and TGF-β in vivo.

Materials and Methods

Animals

Animal care and use were conducted in accordance with the Animal Ethics Committee of the Utrecht University. Specific pathogen-free (according to the Federation of European Laboratory Animal Science Associations) (29) male BALB/c mice (6–8 wk) were purchased from Charles River Laboratories.

Antibodies

Mouse IgG (mIgG) was purchased from Sigma-Aldrich. Rat IgG (rIgG) was purchased from Valeant Pharmaceuticals. Rat mAb to mouse IL-10 receptor (anti-IL-10R) and mouse mAb to mouse TGF-β (anti-TGF-β) were purified from culture supernatant of hybridomas (HB-12538 and HB-9849, respectively; American Type Culture Collection) by using a protein G column (Pharmacia). All Abs were treated with 10% (v/v) polymyxin B-agarose (washed twice with saline; Sigma-Aldrich) for 1 h at 4°C to remove LPS. After incubation, agarose beads were removed by centrifugation (1700 $\times$ *g*, 4°C, 15 min), and the supernatants were sterilized by using a 0.22-μm filter (Omnilabo). The endotoxin level in all treatment preparations was <1 endotoxin unit/mg.

Sensitization, challenge, and IT protocol

The protocol used for sensitization, IT, and inhalation challenge (Fig. 1) was the same as previously described (19). Mice were sensitized i.p. on days 0 and 7 with 10 μg of OVA (chicken egg albumin, crude grade V; Sigma-Aldrich) adsorbed onto 2.25-mg alum (ImjectAlum;, Pierce) in 0.1 ml of pyrogen-free saline (Braun). To study the efficacy of allergen IT, we performed a dose-response relation of OVA-IT (Fig. 1*A*). For that purpose, 0.5% (w/v) OVA in saline was prepared and freshly diluted before injection. In experiment A, mice were treated with 3 s.c. injections of OVA (10–1000 μg) in 0.2 ml of pyrogen-free saline on alternate days 2 wk after the second sensitization. The control group was sham treated with 0.2 ml of saline. Ten days after OVA-IT or sham treatment, mice were challenged with OVA aerosols in pyrogen-free saline (10 mg/ml) for 20 min three times every third day in a Plexiglas exposure chamber (5 L) coupled to a Pari LC Star nebulizer (particle size 2.5–3.1 μm; PARI Respiratory Equipment) driven by compressed air at a flow rate of 6 L/min. Aerosol was given in groups composed of no more than 12 mice.

Since the dose-response relation of OVA-IT showed that IT with 100 μg of OVA partially suppressed asthma-like symptoms in this model, we next determined whether inhibition of NF-κB activation potentiates the suppressive effects of allergen IT (Fig. 1*B*). Therefore, the active form of vitamin D_3, $1,25(OH)_2D_3$ (Fluka) was dissolved in 96% (v/v) ethanol and freshly diluted in OVA solution before s.c. injection. Based on preliminary results (data not shown) and literature data (30), we chose the dose of 10 ng of $1,25(OH)_2D_3$ for our in vivo experiments. In experiment B, mice received 100 μg of OVA-IT alone or in combination with 10 ng of $1,25(OH)_2D_3$. Control mice were sham treated with saline alone or in combination with 10 ng of $1,25(OH)_2D_3$. In both experiments (A and B), airway responsiveness to methacholine, serum levels of Igs, cellular infiltration, and Th2-cytokine levels in the bronchoalveolar lavage (BAL) fluid were measured 24 h after the last OVA inhalation challenge.

The third series of experiments was aimed to analyze whether the immunoregulatory cytokines IL-10 and TGF-β are involved in the effector

phase of immunomodulation (Fig. 1*C*). In experiment C, mice were injected i.p. with 0.5 mg of anti-IL-10R mAb (19), 2.5 mg of anti-TGF-β-neutralizing mAb (31), or both 1 h before the first OVA aerosol challenge. Control mice were treated with 0.5 mg of rIgG plus 2.5 mg of mIgG.

The fourth series of experiments were aimed to inhibit the NF-κB signaling pathway at the time of IT with the selective IκB kinase 2 (IKK-2) inhibitor PS-1145 (50 mg/kg, i.p.; Sigma-Aldrich) (32) and to compare its effect on suboptimal IT to $1,25(OH)_2D_3$ (Fig. 1*D*).

Evaluation of airway responsiveness

Airway responsiveness to inhaled methacholine (acetyl-β-methylcholine chloride; Sigma-Aldrich) was measured twice (6 days before the first OVA aerosol challenge and at 24 h after the last challenge) in conscious, unrestrained mice using barometric whole-body plethysmography by recording respiratory pressure curves (Buxco; EMKA Technologies) as described in detail previously (33).

Determination of serum levels of OVA-specific Igs

Serum samples were taken 24 h after the last challenge, and OVA-specific IgE, IgG1, and IgG2a were determined by ELISA as described previously (18). For determination of OVA-specific IgA levels, wells were coated with 0.5 μg/ml anti-mouse IgA mAb (BD Pharmingen) in PBS. After blocking, diluted serum samples and duplicate dilution series of an IgA reference serum were added for 2 h. Next, after multiple washings, the wells were incubated for 1 h with 2 μg/ml biotinylated anti-mouse IgA, followed by another washing and incubation for 1 h with 1/10,000 diluted poly-HRP. For color development, 0.4 mg/ml *o*-phenylenediamine dichloride and 4 mM H_2O_2 in PBS were used, and the reaction was stopped by adding 75 μl of 4 M H_2SO_4. OD was read at 490 nm using a Benchmark microplate reader (Bio-Rad). Results were analyzed using Microplate Manager PC software (Bio-Rad).

Analyses of the BAL fluid and lung tissue

BAL was performed immediately after bleeding of the mice by lavage of the airways through a tracheal cannula with 1 ml of saline (37°C) containing protease inhibitor (complete mini tablet (Roche Diagnostics) and 5% BSA). The supernatant of this first milliliter of BAL fluid was used to measure cytokine levels. Subsequently, mice were lavaged four times with 1 ml of saline (37°C). Using separate groups of mice, single-cell suspensions from lung digest were prepared after lavage (34). 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 min 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. Hereafter, cells were pelleted (500 $\times$ *g* at 4°C for 5 min) and resuspended in 5 ml of PBS. Cells in BAL fluid and in lung digest were analyzed as described previously (18).

Determination of cytokines in lung tissue

The tissue of the smallest lobe of the right lung was used to measure cytokine levels. Briefly, lung tissue was placed in an Eppendorf microcentrifuge tube and homogenized in 20% (w/v) luminex buffer (50 mM Tris-HCl, 150 mM NaCl, 0.002% Tween 20, and protease inhibitor, pH 7.5) on ice. Subsequently, the lung tissue homogenates were centrifuged for 10 min at 12,000 $\times$ *g*. The supernatants were collected, kept at –70°C and cytokine levels were measured by ELISA.

Determination of cytokine production by OVA-restimulated thoracic lymph node cells in vitro

Cytokine production by Ag-restimulated T cells in thoracic lymph nodes (TLN) were determined as described previously (34). TLN cells derived from the paratracheal and parabronchial region were collected 24 h after the last OVA aerosol challenge. TLN cells were cultured in triplicate during polyclonal stimulation with 50 μg/ml immobilized anti-CD3 mAb (clone 17A2) or Ag-specific stimulation with OVA (10 μg/ml) to determine their capacity to produce cytokines. After 5 days of culture at 37°C in 5% CO_2, the supernatants were harvested, pooled, and stored at –70°C until determination of cytokine levels.

Measurement of cytokines

IL-5, IL-10, and IL-13 in the BAL fluid, lung tissue, and TLN cell cultures were determined by ELISA according to the manufacturer's instructions (BD Pharmingen). A commercially available ELISA kit was used to assess levels of TGF-β in BAL fluid, lung tissue, and serum (BioSource International). The detection limits were 32 pg/ml for IL-5 and 15 pg/ml for IL-10, IL-13, and TGF-β.

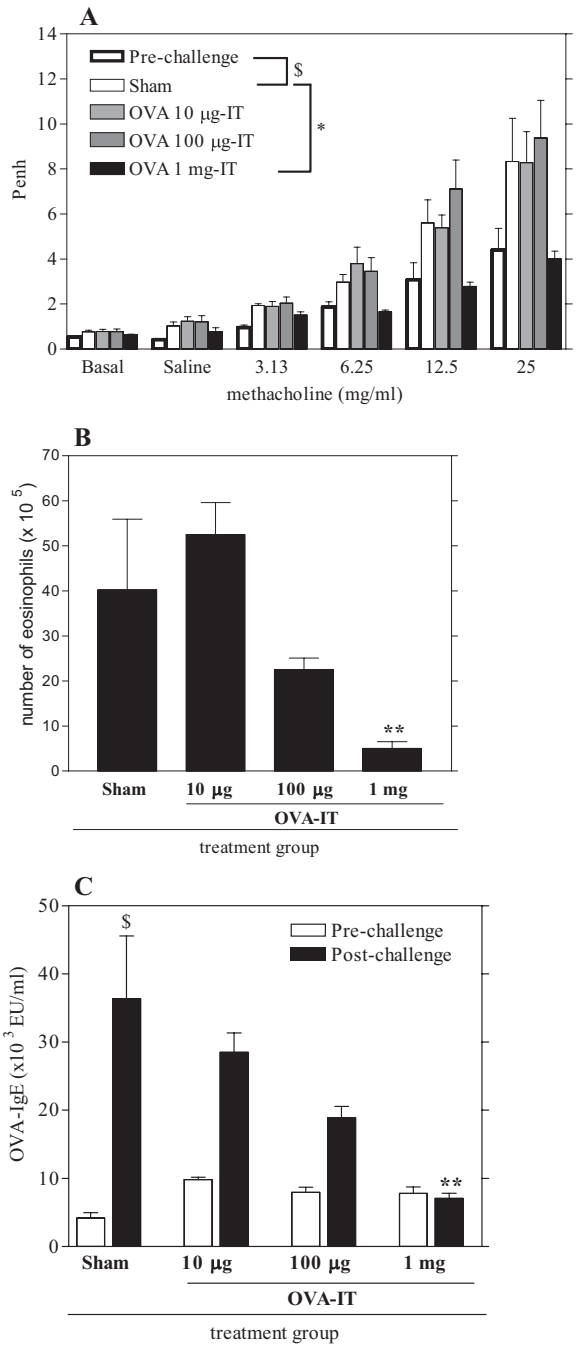

FIGURE 2. Dose-response relation of OVA-IT. OVA-sensitized mice received IT with 10 μg, 100 μg, or 1 mg of OVA. Airway responsiveness to methacholine just before (prechallenge) and 1 day after the last OVA aerosol challenge (*A*), eosinophil numbers in BAL fluid (*B*), and levels of OVA-specific IgE in serum just before (prechallenge) and 1 day after OVA aerosol challenge (*C*). Values are expressed as mean $\pm$ SEM ($n = 6$). \$, $p < 0.05$ compared with prechallenge; *, $p < 0.05$ and **, $p < 0.01$ compared with sham-treated mice.

Statistical analysis

All data are expressed as mean $\pm$ SEM. After ANOVA, levels of cytokines and Igs were compared by an unpaired and paired Student *t* test (two tailed, homosedastic), respectively. Cell counts were compared using the Man-Whitney *U* test. After log transformation, airway responsiveness to methacholine was statistically analyzed by a general linear model of repeated measurements followed by a post hoc comparison between groups using the Bonferroni method (19). A $p < 0.05$ was considered significant.

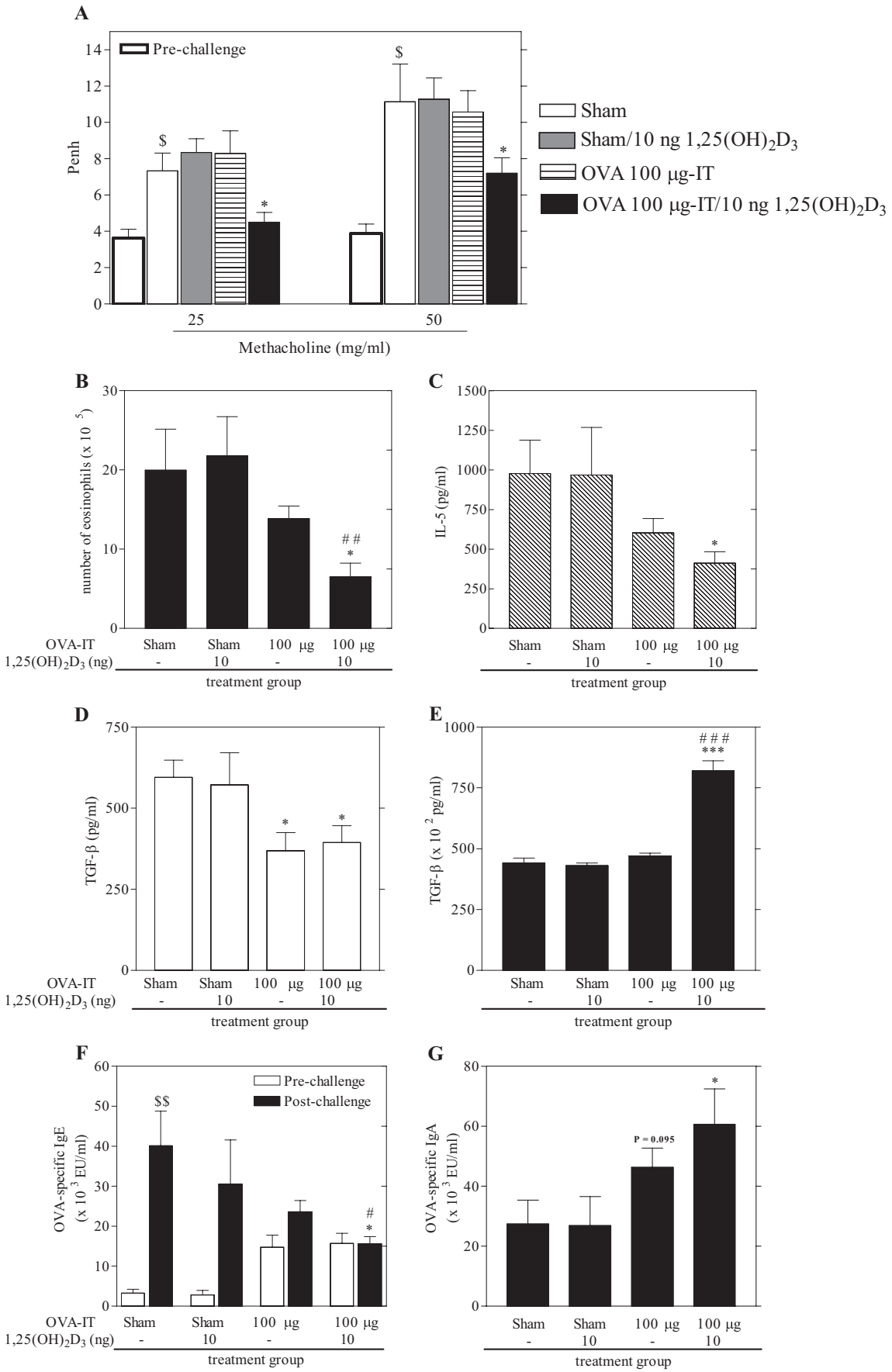

FIGURE 3. Effects of coadministration of 1,25(OH)$_2$D$_3$ on the efficacy of suboptimal IT. OVA-sensitized mice received IT with 100 μg of OVA combined with 10 ng of 1,25(OH)$_2$D$_3$. Airway responsiveness to methacholine just before (prechallenge) and 1 day after OVA aerosol challenge (*A*), eosinophil numbers in BAL fluid (*B*), IL-5 levels in BAL fluid (*C*), TGF-β levels in BAL fluid (*D*), TGF-β levels in serum (*E*), levels of OVA-specific

Results

OVA-IT: dose-response relation

To evaluate the efficacy of allergen IT, we first examined the dose-response relation of OVA-IT. OVA-sensitized mice were treated with 10 μg, 100 μg, or 1 mg of OVA.

Airway responsiveness

Airway responsiveness was measured in conscious, unrestrained mice by barometric whole-body plethysmography. At day 28, after sensitization and treatment but before challenge, OVA-sensitized mice demonstrated a dose-dependent increase in enhanced pause (Penh) values in response to methacholine. The complete methacholine dose-response curves (DRCs) did not differ between different groups. The DRC of the sham-treated group is depicted as representative of prechallenge measurements (Fig. 2*A*). Airway responsiveness in vivo was measured again at 24 h after the last OVA aerosol on day 42. After OVA aerosol challenge, sham-treated mice developed airway hyperresponsiveness (AHR) as the second methacholine DRC was significantly ($p < 0.05$) different from the DRC obtained before challenge (Fig. 2*A*). Mice treated with 1 mg of OVA-IT displayed significantly ($p < 0.05$) suppressed development of OVA-induced AHR in vivo compared with sham-treated mice (Fig. 2*A*). Neither 10 μg nor 100 μg of OVA-IT influenced AHR to methacholine (Fig. 2*A*).

BAL eosinophilia

To determine the efficacy of OVA-IT on airway eosinophilia, we examined the amount of inflammatory cells present in the airway lumen 24 h after the last OVA aerosol challenge. The BAL fluid of OVA-sensitized and -challenged mice contained high numbers of eosinophils (Fig. 2*B*) besides mononuclear cells and few neutrophils (data not shown). One milligram of OVA-IT effectively suppressed the airway eosinophilia as the number of eosinophils in the BAL fluid was significantly reduced by 87% ($p < 0.01$) compared with sham-treated mice (Fig. 2*B*). One hundred micrograms of OVA-IT partially suppressed the influx of airway eosinophilia but this reduction did not reach the level of significance (44%, $p = 0.93$; Fig. 2*B*). No reduction was found in the number of eosinophils in BAL fluid from mice treated with 10 μg of OVA-IT (Fig. 2*B*).

OVA-specific IgE levels in serum

We further investigated the efficacy of OVA-IT on the levels of OVA-specific IgE in serum. In sham-treated mice, OVA challenge displayed significantly increased (89%, $p < 0.05$) OVA-specific IgE levels in serum compared with prechallenge levels (Fig. 2*C*). One milligram of OVA-IT significantly suppressed the OVA-specific IgE levels in serum by 81% ($p < 0.01$) compared with sham-treated mice (Fig. 2*C*). IT with 100 μg of OVA also suppressed the levels of OVA-specific IgE in serum by 48%, but this reduction did not reach the level of significance ($p = 0.09$; Fig. 2*C*). The up-regulation of OVA-specific IgE levels in serum were slightly changed (22% decrease, not significant) by treatment of mice with 10 μg of OVA-IT (Fig. 2*C*).

Therefore, a low dose of OVA-IT (10 μg) failed to counteract the asthma-like symptoms after OVA inhalation challenge. In contrast, suboptimal OVA-IT (100 μg) partially suppressed the airway eosinophilia in the BAL fluid and OVA-specific IgE levels in serum but not the AHR. Treatment with optimal OVA-IT (1 mg) effectively suppressed AHR, airway eosinophilia, and OVA-specific IgE levels in serum.

Table I. *Number of neutrophils in BAL fluid and lung tissue*[a]

Treatment Group	No. of Neutrophils ($\times 10^4$)	
	BAL Fluid	Lung Tissue
Sham	2.90 ± 1.08	42.88 ± 19.86
Sham/1,25$(OH)_2D_3$ (10 ng)	1.53 ± 0.73	ND
OVA (100 μg)-IT	1.21 ± 0.78	39.46 ± 25.21
OVA (100 μg)-IT/1,25$(OH)_2D_3$ (10 ng)	1.02 ± 0.43	49.88 ± 19.75

[a] OVA-sensitized mice received IT with 100 μg of OVA combined with 10 ng of 1,25$(OH)_2D_3$ prior to OVA inhalation challenges. Values are expressed as mean ± SEM ($n = 6$).

Effects of coadministration of 10 ng of 1,25$(OH)_2D_3$ on suboptimal OVA-IT

Subsequently, we aimed to determine whether coadministration of 10 ng of 1,25$(OH)_2D_3$ could potentiate suboptimal IT. For this purpose, mice were treated with 100 μg of OVA-IT combined with 10 ng of 1,25$(OH)_2D_3$.

Airway responsiveness

In Fig. 3*A*, only the responses to 25 and 50 mg/ml methacholine of the complete DRCs are shown. Compared with sham treatment, IT with suboptimal (100 μg) OVA failed to reduce development of OVA-induced AHR to methacholine (Fig. 3*A*). Interestingly, coadministration of 10 ng of 1,25$(OH)_2D_3$ with 100 μg of OVA-IT significantly suppressed AHR to methacholine compared with sham-treated mice ($p < 0.05$; Fig. 3*A*). Ten nanograms of 1,25$(OH)_2D_3$ by itself did not change AHR in sham-treated mice (Fig. 3*A*).

Eosinophils and cytokine levels in the BAL fluid and lung tissue

One hundred micrograms of OVA-IT partially suppressed (~30%, not significant) the influx of eosinophils in the BAL fluid and lung tissue (Figs. 3*B* and 6*A*, respectively). Importantly, coadministration of 10 ng of 1,25$(OH)_2D_3$ with 100 μg of OVA-IT effectively reduced airway eosinophilia in the BAL fluid and lung tissue by ~70% ($p < 0.05$) compared with sham-treated mice and by ~55% ($p < 0.01$) compared with mice merely receiving 100 μg of OVA-IT (Figs. 3*B* and Fig. 6*A*). The BAL fluid from sham-treated mice showed no changes in numbers of eosinophils after treatment with 10 ng of 1,25$(OH)_2D_3$ (Fig. 3*B*). Compared with sham-treated mice, coadministration of 10 ng of 1,25$(OH)_2D_3$ with OVA-IT did not significantly change the number of neutrophils in BAL fluid nor in lung tissue (Table I).

In parallel with the reduction in airway eosinophilia, there were significant falls in IL-5 levels by 58% ($p < 0.05$; Fig. 3*C*) and by 64% ($p < 0.01$; Fig. 6*C*) and IL-13 levels by 76% ($p < 0.05$; data not shown) and 64% ($p < 0.001$; Fig. 6*B*) in BAL fluid and lung tissue, respectively, after coadministration of 10 ng of 1,25$(OH)_2D_3$ with 100 μg of OVA-IT compared with sham-treated mice. One hundred micrograms of OVA-IT partially reduced (37%, not significant) the levels of IL-10 in BAL fluid compared with sham-treated mice and this effect was not influenced by

IgE in serum just before (prechallenge) and 1 day after OVA aerosol challenge (*F*), and levels of OVA-specific IgA in serum 1 day after OVA aerosol challenge (*G*). Values are expressed as mean ± SEM ($n = 6$). \$, $p < 0.05$ and \$\$, $p < 0.01$ compared with prechallenge. *, $p < 0.05$ and ***, $p < 0.001$ compared with sham-treated mice. #, $p < 0.05$; ##, $p < 0.01$; and ###, $p < 0.001$ compared with OVA (100 μg)-IT-treated mice.

Table II. *Serum OVA-specific Ab levels*[a]

Treatment Group	OVA-Specific IgG1 Isotype ($\times 10^6$ EU/ml)		OVA-Specific IgG2a Isotype ($\times 10^5$ EU/ml)	
	Prechallenge	Postchallenge	Prechallenge	Postchallenge
Sham	0.38 ± 0.18	26.49 ± 9.56*	0.17 ± 0.13	6.44 ± 2.07*
Sham/$1,25(OH)_2D_3$ (10 ng)	0.26 ± 0.13	21.48 ± 6.31†	0.56 ± 0.29	7.16 ± 3.91
OVA (100 μg)-IT	48.26 ± 17.67*	39.12 ± 6.99	11.12 ± 2.91‡	8.27 ± 1.36
OVA (100 μg)-IT/$1,25(OH)_2D_3$ (10 ng)	53.73 ± 13.87‡	61.73 ± 11.85§	34.18 ± 9.81‡¶	22.92 ± 6.83§

[a] OVA-sensitized mice received IT with 100 μg of OVA combined with 10 ng of $1,25(OH)_2D_3$. Levels of OVA-specific IgG1 and IgG2a in serum were measured before (prechallenge) and after (postchallenge) OVA aerosol challenge. Values are expressed as mean ± SEM ($n = 6$).

*, $p < 0.05$ and ‡, $p < 0.01$ compared to prechallenge levels of sham-treated mice. †, $p < 0.05$ compared to prechallenge levels. ¶, $p < 0.05$ compared to prechallenge levels of OVA (100 μg)-IT-treated mice. §, $p < 0.05$ compared to postchallenge levels of sham-treated mice.

coadministration of 10 ng of $1,25(OH)_2D_3$ (data not shown). Interestingly, coadministration of 10 ng of $1,25(OH)_2D_3$ with 100 μg of OVA-IT significantly increased IL-10 levels in lung tissue by 85% compared with sham-treated mice and with mice treated only with 100 μg of OVA-IT ($p < 0.05$; Fig. 6*D*). No changes were found in IL-5, IL-13, and IL-10 levels in BAL fluid from sham-treated and 10 ng of $1,25(OH)_2D_3$-treated mice (Fig. 3*C* and data not shown).

TGF-β levels in BAL fluid, lung tissue, and serum

We next examined whether coadministration of 10 ng of $1,25(OH)_2D_3$ influenced the levels of the regulatory cytokine TGF-β. High levels of TGF-β were present in BAL fluid and lung tissue of sham-treated mice. One hundred micrograms of OVA-IT significantly reduced the levels of TGF-β by 38% ($p < 0.05$; Fig. 3*D*) in BAL fluid and by 44% ($p < 0.05$; data not shown) in lung tissue compared with sham-treated mice. Coadministration of 10 ng of $1,25(OH)_2D_3$ with suboptimal OVA-IT did not influence the reduction in TGF-β levels in BAL fluid (Fig. 3*D*) and lung tissues (data not shown). Interestingly, we found that coadministration of 10 ng of $1,25(OH)_2D_3$ with 100 μg of OVA-IT displayed markedly increased serum levels of TGF-β compared with sham-treated mice and with mice treated only with 100 μg of OVA-IT ($p < 0.001$; Fig. 3*E*). In addition, treatment with 10 ng of $1,25(OH)_2D_3$ did not influence the levels of TGF-β in the BAL fluid and serum obtained from sham-treated mice (Fig. 3, *D* and *E*, respectively).

Serum levels of Igs

We further examined whether coadministration of 10 ng of $1,25(OH)_2D_3$ with 100 μg of OVA-IT could influence the levels of Igs in serum. In sham-treated mice, OVA challenge displayed significantly increased (92%, $p < 0.01$) OVA-specific IgE levels in serum compared with prechallenge levels (Fig. 3*F*). IT with 100 μg of OVA partially down-regulated (41%, not significant) the

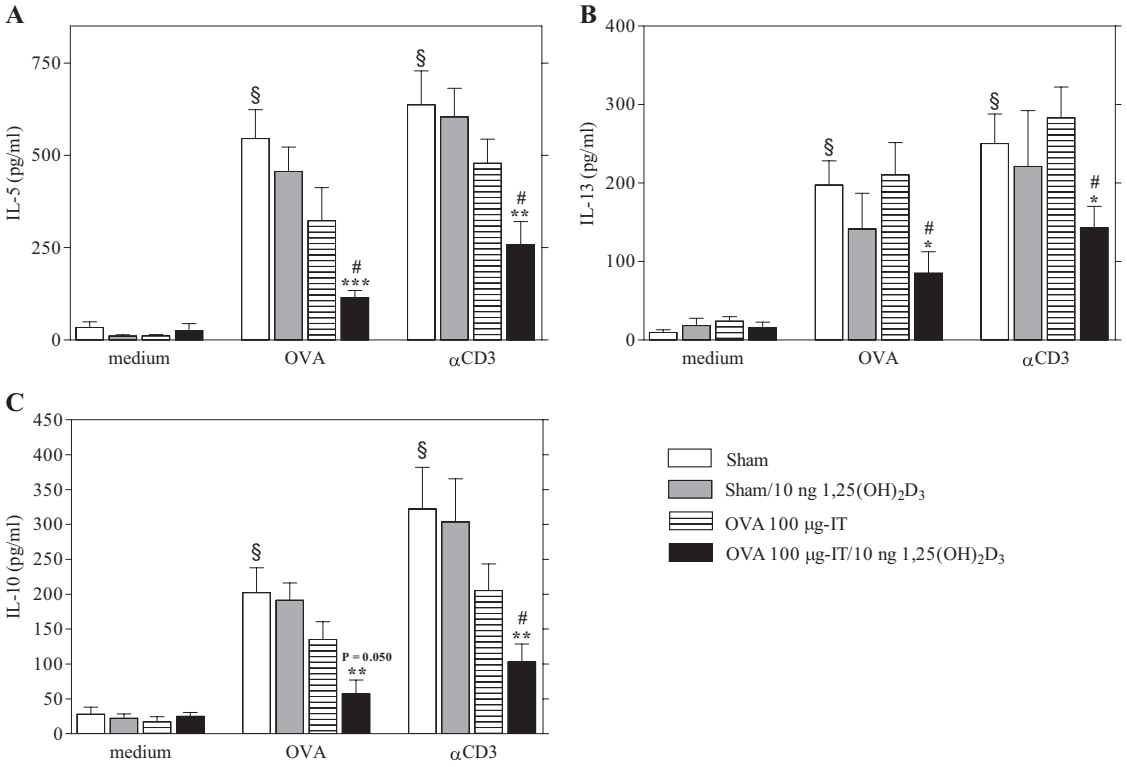

FIGURE 4. Effects of coadministration of $1,25(OH)_2D_3$ on the efficacy of suboptimal IT. OVA-sensitized mice received IT with 100 μg of OVA combined with 10 ng of $1,25(OH)_2D_3$. IL-5 (*A*), IL-13 (*B*), and IL-10 (*C*) levels in OVA-restimulated TLN cell cultures 1 day after OVA aerosol challenge. Values are expressed as mean ± SEM ($n = 6$). §, $p < 0.001$ compared with nonstimulated (medium) sham-treated mice. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with sham-treated mice. #, $p < 0.05$ compared with OVA (100 μg)-IT-treated mice.

OVA-specific IgE levels in serum (Fig. 3*F*). Interestingly, coadministration of 10 ng of $1,25(OH)_2D_3$ with 100 μg of OVA-IT displayed significantly suppressed OVA IgE levels in serum by 61% ($p < 0.05$) compared with sham-treated mice and by 34% ($p < 0.05$) compared with mice treated with 100 μg of OVA-IT alone (Fig. 3*F*). In contrast, we found that the combination of 10 ng of $1,25(OH)_2D_3$ with 100 μg of OVA-IT caused significantly increased OVA-specific IgA (55%, $p < 0.05$) levels in serum compared with sham-treated mice (Fig. 3*G*). In addition, 10 ng of $1,25(OH)_2D_3$ by itself had no significant effect on OVA-specific IgE and IgA levels in serum of sham-treated mice (Fig. 3, *F* and *G*, respectively). Furthermore, in sham-treated mice, OVA challenge induced an increase of OVA-specific IgG1 (99%, $p < 0.05$) and IgG2a (97%, $p < 0.05$) levels in serum compared with prechallenge levels (Table II). In OVA-sensitized mice, suboptimal OVA-IT markedly increased the serum OVA-specific IgG1 (99%, $p < 0.05$) and IgG2a (98%, $p < 0.01$) levels compared with prechallenge levels of sham-treated mice (Table II). Coadministration of 10 ng of $1,25(OH)_2D_3$ with 100 μg of OVA-IT caused a further increase in levels of OVA-specific IgG1 (10%, $p = 0.81$) and of IgG2a (68%, $p < 0.05$) in serum compared with mice treated with 100 μg of OVA-IT alone (Table II). These levels did not increase further on OVA challenge, except for the IgG1 serum levels (Table II).

Determination of cytokine production by OVA-restimulated TLN cells in vitro

To examine T cell responses on Ag-specific restimulation, single-cell suspensions of TLN of each mouse were prepared 24 h after the last OVA aerosol challenge. OVA- restimulated TLN cell cultures derived from sham-treated mice produced significantly ($p < 0.001$) higher amounts of IL-5, IL-13, and IL-10 (Fig. 4) than those from nonrestimulated TLN cell cultures. TLN cell cultures derived from mice treated only with 100 μg of OVA-IT or only with 10 ng of $1,25(OH)_2D_3$ produced similar levels of these cytokines after OVA restimulation in vitro as those of sham-treated mice. However, the amount of IL-5 in TLN cell cultures from mice that received the combination of 100 μg of OVA-IT with 10 ng of $1,25(OH)_2D_3$ was reduced (79%, $p < 0.001$) compared with those from sham-treated mice and by 65% ($p < 0.05$) compared with those from mice treated only with 100 μg of OVA-IT (Fig. 4*A*). Also, the levels of IL-13 and IL-10 were significantly reduced in the OVA-restimulated TLN cell cultures from mice treated with 100 μg of OVA-IT combined with 10 ng of $1,25(OH)_2D_3$ compared with those from sham-treated mice (57%, $p < 0.05$, and 72%, $p < 0.01$, Fig. 4, *B* and *C*, respectively) and by 60% ($p < 0.05$) and by 58% ($p < 0.05$) for IL-13 and IL-10, respectively, compared with those from mice that merely received 100 μg of OVA-IT (Fig. 4, *B* and *C*). These reductions of IL-5, IL-13, and IL-10 levels were also observed when TLN cell cultures were restimulated with anti-CD3 Ab (Fig. 4).

Therefore, coadministration of 10 ng of $1,25(OH)_2D_3$ potentiated the immunosuppressive effects of suboptimal IT with 100 μg of OVA on AHR, OVA-specific IgE levels in serum, airway eosinophilia, and Th2 cytokine levels in BAL fluid and lung tissue and on in vitro allergen-restimulated TLN cell cultures. Furthermore, these immunosuppressive effects are associated with increased levels of IL-10 in lung tissue and TGF-β in serum.

IL-10 and TGF-β are crucial in tolerance induction by coadministration of 10 ng of $1,25(OH)_2D_3$ with suboptimal IT

We next investigated the role of IL-10 and TGF-β in the observed immunosuppressive effects induced by the combination of 100 μg of OVA-IT with 10 ng of $1,25(OH)_2D_3$. OVA-sensitized mice were treated with 100 μg of OVA-IT combined with 10 ng of $1,25(OH)_2D_3$ and IL-10R and TGF-β were functionally blocked using mAbs in vivo on the first day of OVA aerosol challenge.

Eosinophils and cytokine levels

We determined the number of eosinophils in BAL fluid 24 h after the last OVA aerosol challenge. In mice that received control Abs, 100 μg of OVA-IT combined with 10 ng of $1,25(OH)_2D_3$ readily suppressed OVA-induced influx of eosinophils into the BAL fluid by 74% ($p < 0.05$) compared with sham-treated mice (Fig. 5*A*). In contrast, in mice treated with anti-IL-10R mAb, 100 μg of OVA-IT combined with 10 ng of $1,25(OH)_2D_3$ failed to suppress OVA-induced influx of eosinophils into the BAL fluid (Fig. 5*A*). Similar results were obtained with anti-TGF-β (Fig. 5*A*). However, blocking of IL-10R along with anti-TGF-β showed a further increase in the number of eosinophils in BAL fluid compared with blocking of IL-10R or anti-TGF-β alone, implying an additive effect between both cytokines IL-10 and TGF-β (not significant; Fig. 5*A*).

In parallel with airway eosinophilia, treatment with anti-IL-10R or anti-TGF-β alone partially reversed the suppression in the levels of the Th2-associated asthmatogenic cytokines IL-5 (Fig. 5*B*) and IL-13 (Fig. 5*C*) in BAL fluid induced by 100 μg of OVA-IT combined with 10 ng of $1,25(OH)_2D_3$. IL-5 and IL-13 levels were completely reversed when both IL-10R and TGF-β were functionally blocked in vivo (Fig. 5, *B* and *C*, respectively). In addition, IL-10 levels were completely reversed in BAL fluid obtained from mice treated with either anti-IL-10R, anti-TGF-β, or both (Fig. 5*D*). Furthermore, the increased TGF-β levels in serum of 100 μg of OVA-IT combined with 10 ng of $1,25(OH)_2D_3$-treated mice were not affected by treatment with anti-IL-10R or anti-TGF-β (Fig. 5*E*), while it was significantly suppressed in mice treated with both anti-IL-10R and anti-TGF-β ($p < 0.05$; Fig. 5*E*).

Serum levels of Igs

The serum Ab levels of OVA-specific IgE from mice that received 100 μg of OVA-IT combined with 10 ng of $1,25(OH)_2D_3$ and control Abs were significantly lower ($p < 0.05$) than those from sham and control Ab-treated mice (Fig. 5*F*). A partial recovery in OVA-specific IgE levels in serum was observed after treatment with either anti-IL-10R or anti-TGF-β alone. Treatment of mice with both anti-IL-10R and anti-TGF-β completely reversed the suppression of OVA-specific IgE levels in serum (Fig. 5*F*). Interestingly, the increased OVA-specific IgA levels in serum from mice treated with 100 μg of OVA-IT combined with 10 ng of $1,25(OH)_2D_3$ were remarkably down-regulated after treatment with anti-IL-10R (57% suppression, $p = 0.08$), anti-TGF-β (71% suppression, $p < 0.05$), or both (70% suppression, $p < 0.05$; Fig. 5*G*). Furthermore, treatment with anti-IL-10R, anti-TGF-β, or both did not change the IgG1 levels in serum of 100 μg of OVA-IT combined with 10 ng of $1,25(OH)_2D_3$ and control Ab-treated mice (Table III). Compared with mice merely receiving 100 μg of OVA-IT combined with 10 ng of $1,25(OH)_2D_3$ and control Abs, IgG2a levels were partially lower (not significant) after treatment with anti-IL-10, anti-TGF-β, or both (Table III).

Effects of coadministration of the IKK-2 inhibitor PS-1145 on suboptimal OVA-IT

To strengthen the importance to inhibit the NF-κB signaling pathway at the time of IT, the effect of the selective IKK-2 inhibitor PS-1145 (50 mg/kg, i.p.) on the efficacy of suboptimal OVA-IT was determined in comparison with the effect of $1,25(OH)_2D_3$. In this series of experiments, the numbers of eosinophils and cytokine levels in lung tissue were analyzed.

Suboptimal OVA-IT did not significantly change the number of eosinophils nor the levels of IL-5, IL-10, and IL-13 in lung tissue

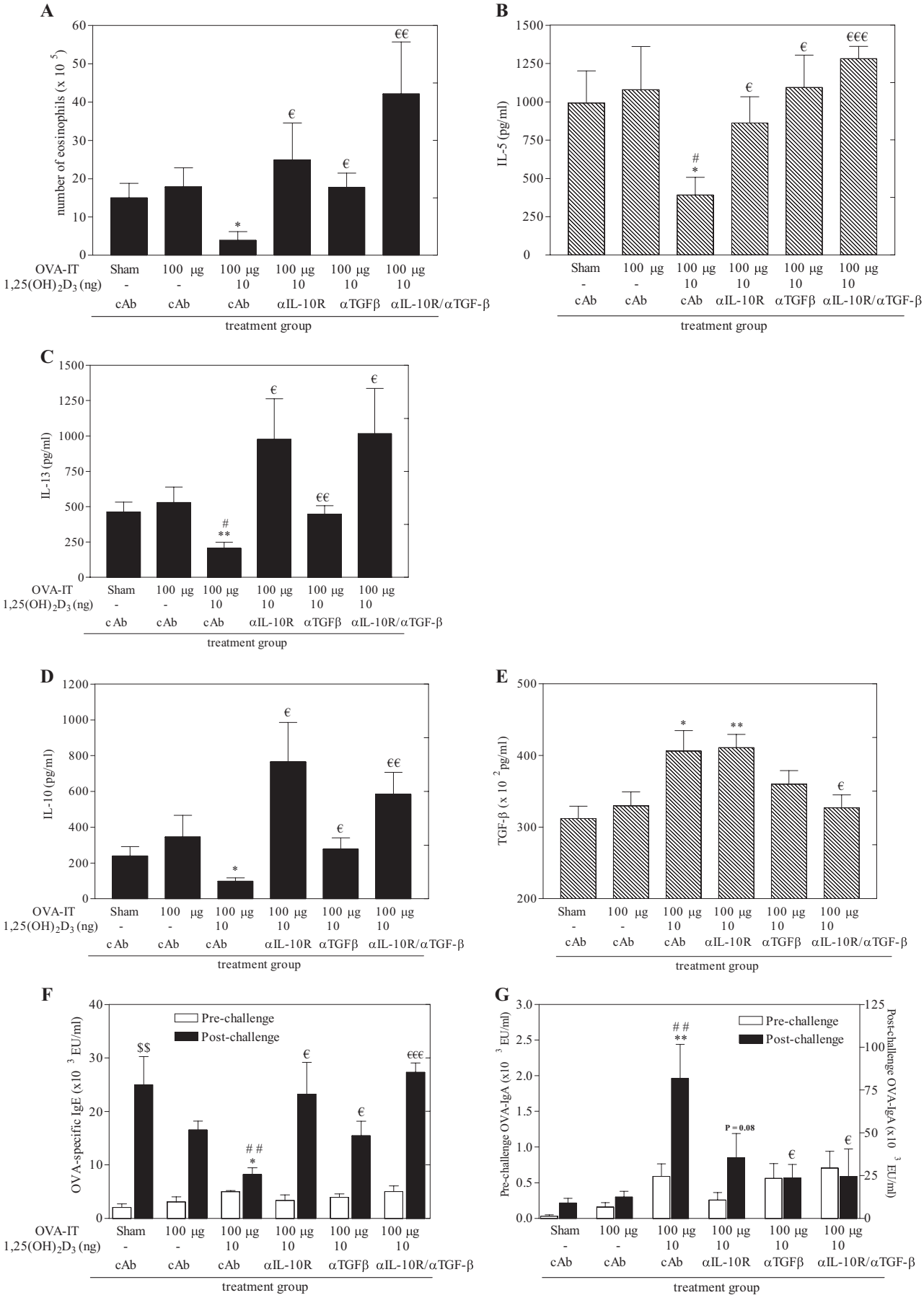

Table III. *Serum OVA-specific Ab levels*[a]

Treatment Group	OVA-Specific IgG1 Isotype ($\times 10^6$ EU/ml)		OVA-Specific IgG2a Isotype ($\times 10^4$ EU/ml)	
	Prechallenge	Postchallenge	Prechallenge	Postchallenge
Sham + rIgG + mIgG	0.00 ± 0.00	11.30 ± 2.04*	0.79 ± 0.52	34.87 ± 12.94†
OVA (100 μg)-IT + rIgG + mIgG	9.42 ± 2.60	12.12 ± 2.93	1.88 ± 0.81	29.24 ± 8.05†
OVA (100 μg)-IT/1,25(OH)$_2$D$_3$ (10 ng) + rIgG + mIgG	16.51 ± 2.02	17.14 ± 1.84	11.64 ± 4.25	49.01 ± 16.46
OVA (100 μg)-IT/1,25(OH)$_2$D$_3$ (10 ng) + anti-IL-10R	8.30 ± 2.74	14.82 ± 3.62	0.99 ± 0.55	28.53 ± 10.71†
OVA (100 μg)-IT/1,25(OH)$_2$D$_3$ (10 ng) + anti-TGF-β	10.91 ± 2.13	16.10 ± 1.32	5.32 ± 4.05	37.21 ± 6.90†
OVA (100 μg)-IT/1,25(OH)$_2$D$_3$ (10 ng) + anti-IL-10R + anti-TGF-β	13.65 ± 3.98	17.15 ± 1.33‡	19.54 ± 12.79	31.87 ± 6.41

[a] OVA-sensitized mice were treated with 100 μg of OVA combined with 10 ng of 1,25(OH)$_2$D$_3$ and received i.p. treatment with anti-IL-10R or anti-TGF-β alone or both just before the first OVA aerosol challenge. Levels of OVA-specific IgG1 and IgG2a in serum were measured before (prechallenge) and after (postchallenge) OVA aerosol challenge. Values are expressed as mean ± SEM ($n = 6$).

†, $p < 0.05$ and *, $p < 0.01$ compared to prechallenge levels. ‡, $p < 0.05$ compared to postchallenge levels of sham-treated mice.

as compared with sham-treated mice (Fig. 6). However, coadministration of PS-1145 along with suboptimal OVA-IT significantly suppressed the number of eosinophils by 64% ($p < 0.01$) as well as the levels of IL-5 and IL-13 by, respectively, 62% ($p < 0.01$) and 50% ($p < 0.001$) as compared with sham-treated mice (Fig. 6, *A–C*). Interestingly, compared with sham-treated mice, the level of the immunoregulatory cytokine IL-10 in lung tissue was significantly increased by 85% ($p < 0.01$) after this combination therapy (Fig. 6*D*), whereas the TGF-β level was slightly reduced (24%; $p = 0.09$). The efficacy of combined treatment with PS-1145 was similar to the efficacy of combined treatment with 1,25(OH)$_2$D$_3$ (Fig. 6).

Therefore, similar to 1,25(OH)$_2$D$_3$, coadministration of the selective IKK-2 inhibitor PS-1145 potentiated the immunosuppressive effects of suboptimal OVA-IT on airway eosinophilia and Th2 cytokine levels in lung tissues. Moreover, these immunosuppressive effects were associated with an increase in IL-10 levels in lung tissue.

Discussion

In the present study, it is demonstrated that coadministration of 1,25(OH)$_2$D$_3$ significantly augments the beneficial effects of IT on Ag-induced allergic asthma manifestations in a mouse model. Augmentation of the suppression of allergen-specific IgE levels, eosinophilic airway inflammation, and AHR is associated with reduced IL-5 and IL-13 levels in BAL fluid and lung tissue and reduced production of Th2 cytokines by T cells upon Ag restimulation in vitro. Additionally, we demonstrated that the suppressive effects of IT combined with 1,25(OH)$_2$D$_3$ are associated with increased levels of IL-10 in lung tissue and TGF-β in serum and are completely abrogated after treatment with anti-IL-10R and anti-TGF-β, indicating a crucial role of these immunoregulatory cytokines.

In humans, specific allergen IT is widely used to treat allergic rhinitis, conjunctivitis, and occasionally allergic asthma. IT reduces symptoms and medication requirements and is currently the only long-term disease-modifying treatment. Although the precise mechanisms of IT are still incompletely understood, recent data suggest an important role for IL-10-producing Tr1 cells and TGF-β-producing Th3-type cells in IT against bee venom, house dust mite, and grass pollen (8–10). In agreement herewith, we recently demonstrated that IL-10 plays a crucial role in the suppression of airway manifestation of asthma after allergen IT in a mouse model (19). Since in the clinic improvement of the efficacy of IT is desirable, we were interested to examine whether the suppressive effects could be potentiated by 1,25(OH)$_2$D$_3$, the active form of vitamin D$_3$.

The biological effects of 1,25(OH)$_2$D$_3$ are mediated by the vitamin D receptor which acts as a transcriptional regulator by binding to vitamin D-responsive elements within the promoters of target genes. The rationale to use 1,25(OH)$_2$D$_3$ in combination with IT is based on the observations that it inhibits the NF-κB protein RelB expression in murine DCs (20). Besides skewing T cells toward Th1 or Th2, DCs are instrumental in the generation of adaptive Treg cells like Th3 and Tr1 cells (11, 12). Immature/tolerogenic DCs induce development of Treg cells by several mechanisms, including production of IL-10 or TGF-β (13, 17). A number of studies has demonstrated that inhibition of NF-κB by 1,25(OH)$_2$D$_3$ induces a persistent immature/tolerogenic phenotype in human and mouse DCs characterized by reduced expression of MHC class II, costimulatory molecules, and IL-12 and strongly enhanced production of IL-10, but not TGF-β (25, 26, 35). DCs modulated by 1,25(OH)$_2$D$_3$ or its analogs induce decreased T cell proliferation and favor development of Treg cells in vitro and in vivo (27, 28, 36). Thus, it is tempting to speculate that combination of 1,25(OH)$_2$D$_3$ with allergen IT promotes the generation of Treg cells that may suppress Th2-driven asthma manifestations at the time of allergen inhalation.

In the present study, we demonstrated that coadministration of either 1,25(OH)$_2$D$_3$ or PS-1145, both potent inhibitors of the NF-κB signaling pathway, potentiated the suppressive effects of IT in a mouse model of allergic asthma. Complete reversal of the suppressive effects of combined IT was observed after blocking of the IL-10R and neutralizing of TGF-β by mAbs at the time of Ag inhalation challenge. These observations may be confounded by effects of the Abs on Ag-induced asthma manifestations per se. Anti-TGF-β treatment was recently shown not to affect Ag-induced airway eosinophilia nor IL-5 levels (37). However, we have previously shown that treatment with anti-IL10R itself potentiates

FIGURE 5. Effects of anti-IL-10R, anti-TGF-β, or both on the immunosuppressive effects induced by suboptimal IT combined with 1,25(OH)$_2$D$_3$. OVA-sensitized mice were treated with 100 μg of OVA combined with 10 ng of 1,25(OH)$_2$D$_3$ and received i.p. treatment with anti-IL-10R or anti-TGF-β or both just before the first OVA aerosol challenge. Number of eosinophils (*A*), IL-5 levels (*B*), IL-13 levels (*C*), IL-10 levels (*D*) in BAL fluid, TGF-β levels in serum (*E*), levels of OVA-specific IgE (*F*), and OVA-specific IgA (*G*) in serum just before (prechallenge) and 1 day after OVA aerosol challenge. Values are expressed as mean ± SEM ($n = 6$). $$, $p < 0.01$ compared with prechallenge. *, $p < 0.05$ and **, $p < 0.01$ compared with sham-treated mice. #, $p < 0.05$; ##, $p < 0.01$ compared with OVA (100 μg)-IT-treated mice. €, $p < 0.05$; €€, $p < 0.01$; and €€€, $p < 0.001$ compared with mice treated with OVA (100 μg)-IT combined with 10 ng of 1,25(OH)$_2$D$_3$ and control Abs (cAb = rIgG plus mIgG).

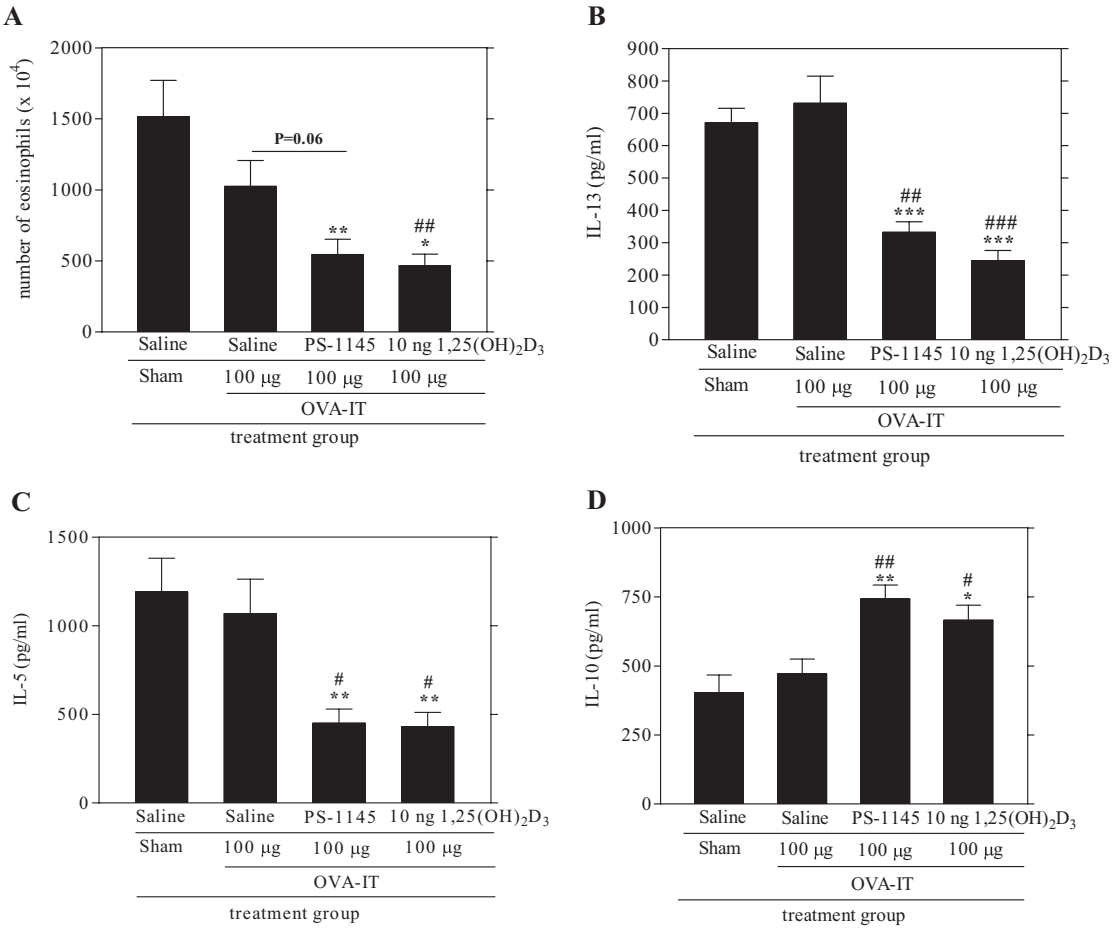

FIGURE 6. Effects of coadministration of PS-1145 or $1,25(OH)_2D_3$ on the efficacy of suboptimal IT. OVA-sensitized mice received IT with 100 μg of OVA combined with 50 mg/kg PS-1145 or with 10 ng of $1,25(OH)_2D_3$. Number of eosinophils (*A*) and levels of IL-13 (*B*), IL-5 (*C*), and IL-10 (*D*) in lung tissue of mice 1 day after OVA aerosol challenge. Values are expressed as mean $\pm$ SEM ($n = 6$). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with sham-treated mice. #, $p < 0.05$; ##, $p < 0.01$; and ###, $p < 0.001$ compared with OVA (100 μg)-IT-treated mice.

OVA challenge-induced BAL eosinophilia but not IL-5 levels nor AHR (19). In line herewith, treatment with both Abs increased BAL eosinophilia but not IL-5 levels in combined IT-treated mice as compared with control Ab sham-treated mice, reflecting the effect of anti-IL10R on eosinophilia. Altogether, we conclude that both IL-10 and TGF-β mediate the suppressive effects on asthma manifestations after combined IT. Furthermore, we speculate that IL-10- and TGF-β- producing Treg, e.g., respectively, Tr1 and Th3 cells, are induced during combined IT and become reactivated to exert their antiasthmogenic effects during Ag inhalation challenge. Interestingly, after combined IT levels of the immunoregulatory cytokines IL-10 and TGF-β were significantly increased in lung tissue and serum, respectively, after Ag challenge, but we did not observe increased levels of these cytokines in BAL fluid. It is striking that these immunoregulatory cytokines are increased at different locations, which may indicate that they are secreted by different (sub)sets of cell types. T cells appear to be the most likely source of these cytokines considering the long-term immunosuppressive effects of IT (19). However, IL-10 secretion upon restimulation of T cells isolated from lung draining lymph nodes was decreased after IT, which indicates that regulation does not appear to occur at the level of the draining lymph nodes. Although these latter findings do not necessarily support the concept that induction of cytokine- producing Treg cells mediate the immunosuppressive effects, it remains possible that Treg cells exert their immunosuppression at another location.

In addition to keeping DCs in an immature/tolerogenic state, $1,25(OH)_2D_3$ may also locally increase TGF-β production. Whereas $1,25(OH)_2D_3$ has not been shown to directly induce TGF-β secretion by DCs (36), it has been demonstrated that it induces TGF-β among other fibroblasts (38). Therefore, TGF-β produced in the local microenvironment may act in concert with tolerogenic DCs in the generation of TGF-β-producing Th3-type cells (39). $1,25(OH)_2D_3$ has also been shown to directly affect Th cell polarization by augmenting Th2 development, independent of an effect on APCs (40). However, it appears unlikely that this plays a role in the induction of a memory-suppressive response as observed after IT in the mouse model (19).

It is well-known that TGF-β plays a critical role in the isotype switch of B cells toward IgA (41, 42). Likewise, increased levels of OVA-specific IgA in serum were detected after combination of IT with $1,25(OH)_2D_3$ while administration of anti-TGF-β significantly suppressed the increased serum IgA levels. Similarly, in human studies, it has been clearly demonstrated that TGF-β secretion by allergen-specific T cells is increased after IT with house dust mites, which is associated with increased serum Der p1-specific IgA levels (9). Interestingly, by binding to FcαRI on leukocytes, serum IgA may have potent anti-inflammatory effects mediated by intracellular recruitment of the tyrosine phosphatase SHP-1 (43). In agreement herewith, Schwarze et al. (44) demonstrated that allergen-specific IgA completely prevented the development of AHR to methacholine,

reduced eosinophilic airway inflammation, and increased allergen-specific IgG2a in a mouse model of allergic asthma. Thus, the TGF-β/IgA/FcαRI pathway may by one of the effector mechanisms to suppress asthma manifestations after IT.

In summary, we demonstrate in a mouse model of allergic asthma that coadministration of $1,25(OH)_2D_3$ or PS-1145, both potent inhibitors of the NF-κB signaling pathway, augments the efficacy of IT to prevent Th2-driven development of asthma manifestations. The immunoregulatory cytokines IL-10 and TGF-β play a crucial role in these beneficial effects of this combined therapy. Inhibition of NF-κB may be a novel strategy to improve allergen immunotherapy for the treatment of allergic diseases, asthma included.

Disclosures

A.J.M.v.O. holds a pending patent application regarding vitamin D_3 and allergen immunotherapy.

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