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Exogenous interleukin-16 inhibits antigen-induced airway hyper-reactivity, eosinophilia and Th2-type cytokine production in mice

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Summary

Background IL-16 has been described as a natural soluble CD4-ligand with immunosuppressive effects *in vitro*. However, little is known about the effect of IL-16 on immune responses *in vivo*.

Objective In the present study, we examined the effect of IL-16 administration in a murine model of allergic asthma. Next, we determined whether these effects were mediated by modulation of $CD4^+$ T lymphocytes.

Methods and results Intraperitoneal administration of IL-16 completely inhibits antigen-induced airway hyper-responsiveness and largely decreases the number of eosinophils in bronchoalveolar lavage fluid (> 90%) and airway tissue of ovalbumin-sensitized and challenged mice. Firstly, it appears that thoracic lymph node cells isolated from *in vivo* IL-16-treated ovalbumin-challenged animals produce less IL-4 (77%) and IL-5 (85%) upon antigenic re-stimulation, when compared to vehicle-treated mice. Secondly, pre-incubation of lymphocytes with IL-16 *in vitro* reduces antigen-induced proliferation (55%) and Th2-type cytokine production (IL-4; 56%, IL-5; 77%). Thirdly, the presence of IL-16 during priming cultures of TCR transgenic T cells (DO11.10), reduces IL-4 (33%) and IL-5 (35%), but not IL-10 and IFNγ levels upon re-stimulation.

Conclusion It can be concluded that IL-16 has potent immunosuppressive effects on a Th2-dominated allergic airway response.

Keywords airway hyper-responsiveness, allergic asthma, eosinophils, interleukin-16, interleukin-4, interleukin-5, Th2-lymphocytes

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Introduction

$CD4^+$ T cells, following mitogen, antigen or anti-CD3 stimulation, release IL-16. In addition to T lymphocytes, eosinophils, mast cells, dendritic cells, fibroblasts, B cells and epithelial cells can release bioactive IL-16 [1]. Previously, we demonstrated that IL-16 immunoreactivity is present in epithelial cells of ovalbumin-sensitized mice but not in naïve animals [2]. Upon antigen-challenge, increased IL-16 expression was observed in epithelial cells and in both inflammatory cell infiltrates and bronchoalveolar lavage (BAL) fluid [2]. Similarly, IL-16 expression in epithelial cells and presence of bioactive IL-16 in BAL fluid after antigen challenge has been observed in asthmatics [1].

It has clearly been demonstrated that IL-16 uses the CD4 molecule as its receptor and exerts diverse functional activities via CD4 signalling in lymphocytes and eosinophils [1]. IL-16 is a well-known chemoattractant for $CD4^+$ cells *in vitro* [1, 3]. In contrast to this immunostimulatory effect, IL-16 has also been shown to have immunosuppressive effects both *in vitro* and *in vivo*, e.g. inhibition of anti-CD3-induced proliferation, antigen-induced T cell activation in human T cells derived from atopics, and inhibition of rheumatoid synovitis [4–8].

Previously, we demonstrated that administration of antibodies to IL-16 reduces development of airway hyper-responsiveness, without affecting airway eosinophilia in a mouse model of allergic asthma [9]. These data would suggest that endogenous IL-16, in asthmatic inflammation, has a pro-inflammatory effect. To reconcile the differences between the apparent pro-inflammatory effect, observed using neutralizing antibodies, and the direct immunosuppressive effect of IL-16, observed *in vitro*, we investigated the ability of exogenously added IL-16 to alter the inflammatory response in the murine model of allergic asthma. In these studies antigen-sensitized mice were treated with IL-16 [intraperitoneally (i.p)] during the antigen-challenge period and the effect on airway responsiveness to methacholine as well as development of inflammation was assessed. We observed a significant decrease in airway responsiveness after treatment with IL-16. Histological sections and cell differentials from lavage fluid confirmed the lack of eosinophilia and epithelial cell hyperplasia in IL-16-treated animals. The mechanism for the IL-16-induced immunosuppression in this model appears to be related to the ability of IL-16 to preferentially inhibit Th2 T cell cytokine production.

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Antigenic re-stimulation of isolated T cells from lung draining lymph nodes in IL-16-treated mice produced significantly less Th2 cytokines compared with untreated mice. The influence of IL-16 on T cell differentiation was also investigated as a potential mechanism for inhibition of Th2 cytokine production. Stimulation of naïve DO11.10 transgenic T cells with IL-16 resulted in reduced Th2 cytokine production with no inhibition of IFNγ. While the presence of IL-16 in asthmatic lungs has been known for many years, this is the first *in vivo* report of the immunosuppressive role of IL-16 in the development of airway inflammation.

Materials and methods

The animal care committee of the Utrecht University approved all experiments.

Sensitization and challenge

Specified pathogen-free male BALB/C mice (6–8 weeks) were obtained from the breeding colony of the Central Animal Laboratory (Utrecht, the Netherlands). Active sensitization – 10 μg ovalbumin (grade V) in 0.5 mL saline [2] – was performed on alternate days (seven times). Four weeks thereafter, mice were exposed (5 min) to eight ovalbumin (2 mg/mL in saline) or saline aerosols (jet nebulizer: Pari IS-2, Pari-Werk GmbH, Starnberg, Germany), on consecutive days (one/day). Before each aerosol the mice were injected i.p. with 1 μg rmIL-16 (in 0.25 mL saline) or vehicle. For the T cell differentiation experiments, spleens were used from DO11.10 TCR transgenic mice (donated by L. Adorini, Department of Inflammation/Autoimmune Diseases, Hoffmann-La Roche Inc., Nutley, NJ, USA) [10].

Airway responsiveness in vivo

Airway responsiveness was measured using whole-body barometric plethysmography (Buxco Corp., Sharon, CT, USA, with a pressure transducer, M45, Validyne Engineering Corp. Northridge, CA, USA; see [11] for more details and validation of this method). Although this technique has many advantages, the contributions of a nasal component in the measurements need to be taken into account, e.g. no discrepancy can be made between nasal congestion and lung responses. As indirect parameter for detection of airway responsiveness to methacholine PENH was used. Baseline PENH values were established by averaging for 3 min (average of 10 valid breaths per PENH value). Mice were subjected to a saline aerosol, followed by increasing concentrations of nebulized methacholine (1–50 mg/mL) for 3 min, followed by a measuring period of 3 min after each aerosol. Aerosol was generated using a jet-nebulizer (Pari IS-2, Par-Werk GmbH).

Bronchoalveolar lavage

Bronchoalveolar lavages were performed as published previously [2] in the same animals that were used for airway hyperresponsiveness measurements.

Ovalbumin-specific IgE enzyme-linked immunosorbent assay

Ovalbumin-specific IgE in serum was measured as published previously [2].

Cytokine production by thoracic lymph node cells in vitro

Thoracic lymph node (TLN) cells were isolated [12] from animals treated *in vivo* with either vehicle or IL-16 and challenged with saline or ovalbumin. A single-cell suspension was prepared and resuspended in RPMI [supplemented with 10% fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, 50 mg/kg gentamycine and 1% glutamax]. After isolation these TLN cells were cultured with or without ovalbumin (40 μg/mL, 1×10^6/mL, 200 μL/well). After 5 days, supernatants were harvested and stored (− 20 °C) for cytokine analysis. IL-4, IL-5, IL-10 and IFNγ levels in supernatants of the TLN cell cultures were determined by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Pharmingen, San Diego, CA, USA).

Pre-incubation of TLN cells with IL-16 in vitro

TLN cells were isolated and cultured from ovalbumin-sensitized and ovalbumin-challenged mice as described above. Cultures were pre-incubated with medium or rmIL-16 (0.01–1 μg/mL, 1 h). Thereafter, cells were cultured with or without ovalbumin (40 μg/mL) and after 5 days supernatant was harvested for cytokine analysis. IL-16 remained present during this culture period. In separate cultures, [^{3}H]thymidine (0.3 μCi/well) was added to each well 48 h after stimulation and left to be incorporated to determine proliferation. [^{3}H] Thymidine uptake was determined after 18 h by liquid scintillation counting.

Effects of IL-16 on generation of DO11.10 CD4$^+$ effector lymphocytes

DO11.10 CD4$^+$ T cells were isolated using immunomagnetic negative selection [see 13 for details]. In short, lymphocytes were isolated (Lympholyte M gradient, Cedarlane, Hornby, Ontario, Canada) from splenocytes (DO11.10 TCR transgenic mice) and incubated (1×10^7cells/mL) with antibodies to MHC-II (MKD6), CD8 (YTS69), Thy1 (YTS154), B220 (RA3–6B2) and CD16/32 (2.4G2) in previously established dilutions (FACS-scan). Negative immunomagnetic selection was performed using magnetic beads coupled to mIgG2a, rIgG2b and mIgM antibodies (Perseptive Biosystems, Boston, MA, USA). The resultant CD4 population was 80–90% pure (FACS-scan, data not shown).

Splenocytes from BALB/c mice were used (1×10^6/mL) as antigen presenting cells (APCs) and cultured (5×10^5/mL) with CD4$^+$ DO11.10 T cells in the presence of ovalbumin$_{323-339}$ (OVA$_{323-339}$) peptide (0.3 μg/mL, 1, 5 mL/well). Supernatant fractions were harvested after 4 days, non-viable cells were removed (Lympholyte M gradient, Cedarlane®) and remaining cells (5×10^5 cells/mL) were re- stimulated (1×10^6/mL APCs) with OVA$_{323-339}$ (0.3 μg/mL). Supernatants were again harvested after 2 days for cytokine analysis. During the first antigen stimulation, cells were cultured with or without rmIL-16 (1 μg/mL). Experiments were performed at least in triplicate.

Lung histology

Lungs from IL-16-treated or control mice were excised and processed for histology as previously described [2]. Briefly, lung tissue was inflated using 4% paraformaldehyde. Following dehydration the tissue was embedded in paraffin and sectioned at 4-μm thickness. The sections were stained with haematoxylin

and eosin to characterize lung inflammation and identify tissue-associated eosinophils.

Chemicals

Ovalbumin (chicken egg albumin crude grade V) was purchased from Sigma Chemical Company (St Louis, MO, USA). Methacholine (acetyl-β-methylcholine) was purchased from Janssen Chimica (Beerse, Belgium). rmIL-16 used in the cultures was obtained from Pharmingen and rmIL-16 used *in vivo* was produced as previously published [3]. $OVA_{323-339}$ was obtained from the Faculty of Veterinary Sciences, Utrecht University, Utrecht, the Netherlands. Antibodies used in this study were MKD6 (anti-I-A^d, mIgG2a), YTS169 (anti-CD8, rIgG2b), YTS154 (anti-Thy1, rIgG2b), RA3–6B2 (anti-B220, rIgG2b) and 2.4G2 (anti-muFc$_\gamma$RII; CD16/32, rIgG2b). All antibodies were purified by standard protein A or G column affinity using supernatants of hybridoma cultures maintained at Utrecht University, the Netherlands. Hematoxylin and eosin stain were obtained from Fisher Diagnostics (Suwanee, GA, USA).

Data analysis

Unless stated otherwise data are expressed as mean ± standard error of the mean (SEM) and evaluated using an analysis of variance followed by a post-hoc comparison between groups.

Data on proliferation or cytokine levels were analysed using a paired Student's *t*-test. For cell types with low numbers in control animals (i.e. neutrophils and eosinophils) a Poisson distribution was assumed. A difference was considered to be significant when $P < 0.05$. Statistical analyses were carried out using SPSS/PC$^+$, version 4.0.1 (SPSS Inc., Chicago, IL, USA).

Results

Effect of treatment with IL-16 on airway hyper-responsiveness in vivo

In vehicle-treated ovalbumin-challenged mice the increase in airway responsiveness to aerosolized methacholine was enhanced significantly when compared to saline-challenged animals (Fig. 1a) as demonstrated by a significant increase in PENH values, an indirect parameter for airway functioning [see 11 for details and validation]. At the highest concentration of methacholine (50 mg/mL), the PENH in saline-treated animals was 4.5 ± 0.9, whereas in ovalbumin-challenged animals PENH was 9.4 ± 1.7 ($P < 0.05$). Administration of rmIL-16 during the aerosol challenge period completely prevented ($P < 0.05$) ovalbumin-induced airway hyper-responsiveness (Fig. 1b). At the highest concentration of methacholine the PENH in rmIL-16-treated ovalbumin-challenged animals was 5.0 ± 0.5, whereas PENH in saline-challenged mice was 6.2 ± 1.7. These data indicate that the systemic administration of IL-16 results in the complete inhibition of increased airway hyper-responsiveness in this model.

Effect of treatment with IL-16 on number of eosinophils in BAL fluid

To see whether the effect of IL-16 on antigen-induced airway hyper-responsiveness was accompanied by alterations in airway inflammation, the lungs were lavaged and presence of inflammatory cells was determined. Ovalbumin challenge of vehicle-treated animals resulted in a significant ($P < 0.05$) increase in the number of eosinophils in BAL fluid compared to vehicle-treated saline-challenged mice ($162 \pm 85 \times 10^3$ vs. $0.4 \pm 0.3 \times 10^3$ cells, respectively, Fig. 2). Treatment with rmIL-16 during the aerosol challenge period resulted in a significant ($P < 0.05$) decrease of ovalbumin-induced eosinophil infiltration compared to vehicle-treated ovalbumin-challenged animals ($13.9 \pm 7.5 \times 10^3$ cells, Fig. 2). No significant differences in infiltration of mononuclear cells or neutrophils were observed between different groups of animals. Furthermore, no differences were observed in total numbers of cells that were present in BAL fluid (Table 1).

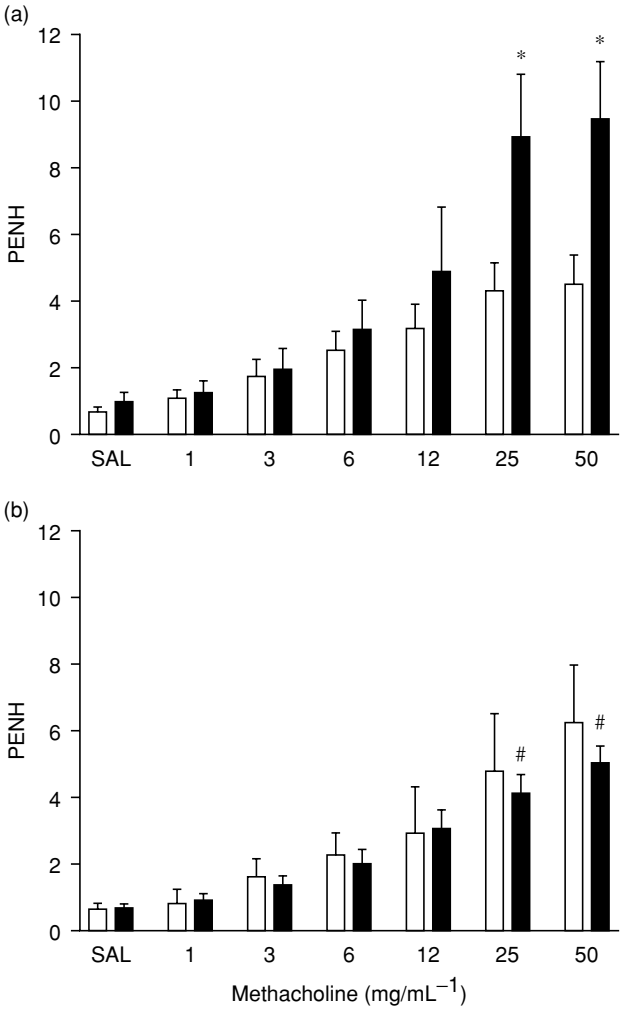

Fig. 1. Increase in PENH values after exposure to nebulized saline (SAL) or increasing concentrations of methacholine (ranging from 1 to 50 mg/mL) in ovalbumin-sensitized mice at 24 h after the last challenge with saline (□) or ovalbumin (■) and treated daily (i.p.) during the challenge period with vehicle (a) or rmIL-16 (1 μg/day; (b) Results are expressed as arithmetic average ± SEM of 5–6 animals per group. *$P < 0.05$, significantly different from saline-challenged animals. #$P < 0.05$, significantly different from vehicle-treated ovalbumin-challenged animals.

Effect of treatment with IL-16 on lung inflammation

A decrease in eosinophils in the BAL fluid suggested that treatment with IL-16 reduced lung inflammation. To confirm this,

following assessment of airway physiology lungs from IL-16-treated or control mice were excised and processed for histology. As depicted in Fig. 3, lungs from ovalbumin-sensitized and challenged mice demonstrated a marked increase in immune cell recruitment that appeared to be both perivascular as well as peribronchial. Epithelial cell hyperplasia was also noted. In contrast, lungs from mice treated with IL-16 demonstrated very little increase in immune cell recruitment as compared with saline-challenged (control) mice. In addition, the thickness of the epithelium appeared to be normal (Fig. 3). Taken together these data indicate that the presence of IL-16 results in significantly less airway hyper-responsiveness and inflammation in this model of allergic asthma.

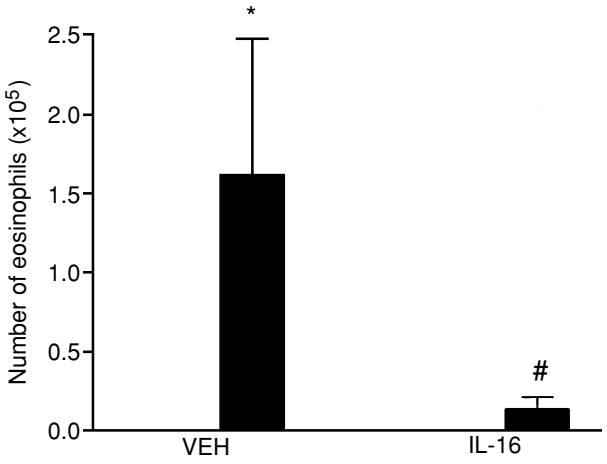

Fig. 2. Number of eosinophils in BAL fluid derived from ovalbumin (■) or saline-challenged (□, hardly any eosinophils were present) animals after i.p. treatment with vehicle (VEH) or rmIL-16. Results are expressed as arithmetic average ± SEM of at least 10 animals per group. *$P < 0.05$, significantly different from saline-challenged animals. #$P < 0.05$, significantly different from vehicle-treated ovalbumin-challenged animals.

Table 1. Absolute numbers of different cells in BAL fluid

Treatment/ challenge	Mononuclear cells ($\times 10^5$)	Neutrophils ($\times 10^3$)	Total number of cells ($\times 10^5$)
VEH/SAL	3.6 ± 0.6	0.6 ± 0.3	3.6 ± 0.6
VEH/OVA	4.9 ± 1.6	3.8 ± 2.9	6.6 ± 2.2
IL-16/SAL	3.5 ± 0.5	0.3 ± 0.2	3.5 ± 0.5
IL-16/OVA	4.0 ± 0.5	0.8 ± 0.3	4.1 ± 0.5

Animals were treated i.p. with either vehicle (VEH) or rmIL-16 (1 μg/day), before each ovalbumin (OVA) or saline (SAL) aerosol. Data are expressed as arithmetic average ± SEM of at least six animals per group. The numbers of eosinophils present in BAL fluid are presented in Fig. 2.

Effect of treatment with IL-16 on ovalbumin-specific IgE levels in serum

A decreased inflammatory response in this model could arise as a result of insufficient production of ovalbumin-specific IgE antibodies. To determine if this was a contributing factor, ovalbumin-specific IgE antibody was assessed. Ovalbumin challenge of vehicle-treated animals resulted in an almost fivefold increase ($P < 0.05$) in serum levels of ovalbumin-specific IgE when compared to saline-challenged mice (745 ± 209 vs. 174 ± 55 units/mL, respectively, $n = 5$). Ovalbumin challenge of IL-16-treated animals also resulted in a significant ($P < 0.05$) increase of serum IgE levels when compared to saline-challenged animals (768 ± 50 vs. 271 ± 84 units/mL, respectively, $n = 5$). No significant differences were observed between serum IgE levels of vehicle or IL-16-treated animals challenged with either saline or ovalbumin.

Effect of treatment with IL-16 in vivo *on antigen-specific cytokine production* in vitro

To investigate whether the inhibitory effects of IL-16 on airway hyper-responsiveness and eosinophilia were related to altered cytokine production by T lymphocytes, lymph node-derived T cells from IL-16-treated or control animals were isolated and cultured in presence or absence of antigen. No significant differences were observed in the total number of cells derived from thoracic lymph nodes from either vehicle or IL-16-treated saline or ovalbumin-challenged animals (data not shown). No IFNγ could be detected in supernatant of TLN cells derived from vehicle- or rmIL-16-treated, saline- or ovalbumin-challenged animals, cultured *in vitro* in the presence or absence of ovalbumin (data not shown, detection limit 0.3 ng/mL). TLN cells derived from vehicle-treated ovalbumin-challenged animals produced more IL-4 ($P < 0.05$) and IL-5 after culturing in the presence of antigen when compared to cells derived from saline-challenged animals (Figs 4a and b). *In vivo* treatment with rmIL-16 during the aerosol challenge period with ovalbumin significantly inhibited the production of IL-4 and IL-5 after stimulation with ovalbumin *in vitro* (Figs 4a and b). No

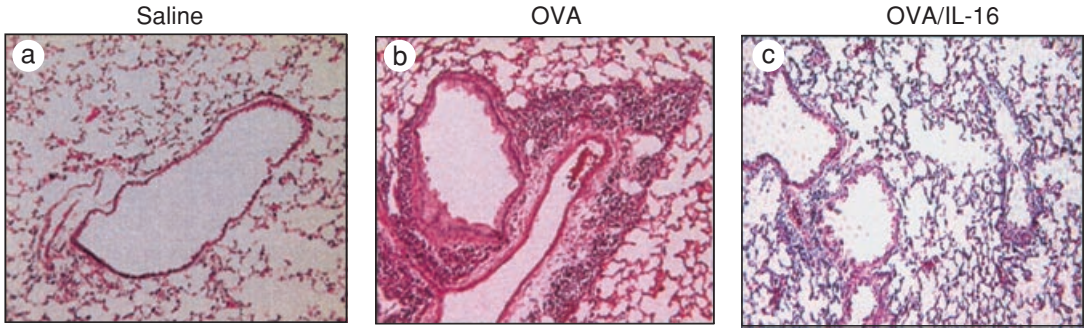

Fig. 3. Histological staining of lungs from ovalbumin (OVA)-sensitized animals. Haematoxylin and eosin staining of lung sections from animals aerosol challenged with saline (a); animals treated with vehicle (VEH) prior to challenged with ovalbumin (b); and animals treated with IL-16 prior to OVA challenge (c).

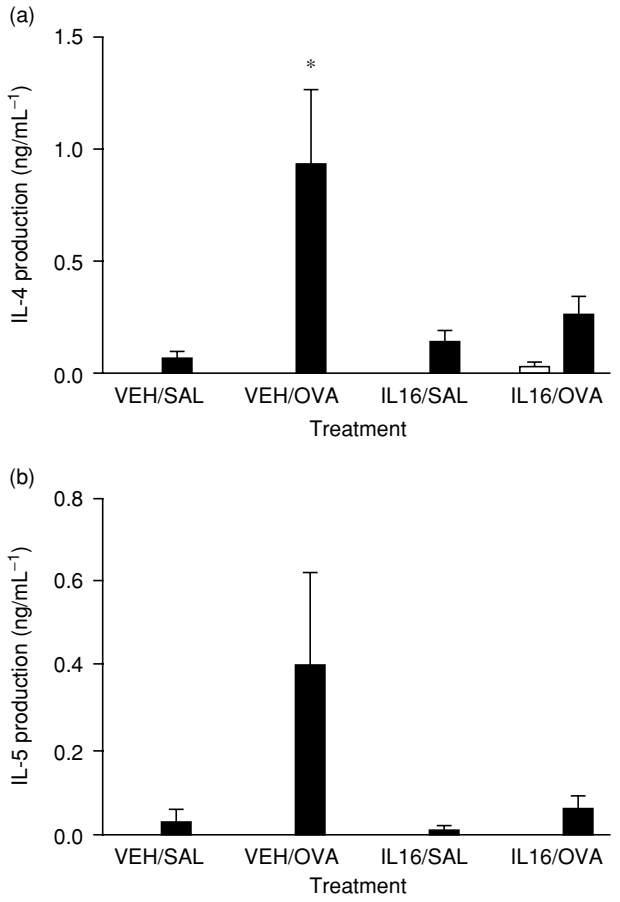

Fig. 4. Cytokine production by thoracic lymph node cells isolated from ovalbumin-sensitized animals challenged with saline (SAL) or ovalbumin (OVA). Animals were treated i.p. during the challenge period with either vehicle (VEH) or rmIL-16 *invivo*. TLN cells were re-stimulated *invitro* in the absence (□) or presence of ovalbumin (■). Hardly any cytokine production was detectable in supernatant of TLN cells cultured in the absence of ovalbumin. Depicted are IL-4 (a) and IL-5 production (b). Results are expressed as arithmetic average ± SEM of 5–6 animals per group. *$P < 0.05$, significantly different from vehicle-treated saline-challenged animals.

differences were observed in IL-4 and IL-5 production by TLN cells between saline-challenged animals treated with rmIL-16 or with vehicle (Figs 4a and b). These data indicate that IL-16 administration *in vivo* resulted in antigen-unresponsive Th2 cells when stimulated *in vitro*.

Effect of pre-incubation with IL-16 on TLN cells in vitro

The immunosuppressive effect of IL-16 seen with *in vivo* treatment could occur by a number of different mechanisms. To determine whether this was a direct effect on Th2 cells, TLN cells were isolated following ovalbumin sensitization and airway challenge, and then treated with or without IL-16 prior to antigenic stimulation. Antigen-specific re-stimulation *in vitro* of TLN cells derived from ovalbumin-sensitized and challenged animals resulted in an increase in IL-4 production when compared to cells cultured with medium alone (2.25 ± 1.55 vs. 0.01 ± 0.01 ng/mL). In a concentration-dependent fashion, IL-16 decreased IL-4 production up to 56% at 1 μg/mL. IL-5 production was also enhanced ($P < 0.05$) by ovalbumin stimulation *in vitro* when compared to cells cultured with medium alone (1.45 ± 0.46 vs. 0 ng/mL). Pre-incubation of TLN cells *in vitro* with rmIL-16 also significantly decreased ($P < 0.05$) ovalbumin-induced IL-5 production up to 77% at 1 μg/mL. TLN cells stimulated with ovalbumin *in vitro* displayed a significant ($P < 0.05$) increase in proliferation when compared to cells cultured without ovalbumin ($11\,447 \pm 2509$ vs. 1618 ± 472[^{3}H]-incorporation in cpm). Pre-incubation of TLN cells with rmIL-16 (1 μg/mL) significantly ($P < 0.05$) decreased *in vitro* proliferation up to 55% after stimulation with ovalbumin. IFNγ levels in supernatants derived from ovalbumin-stimulated TLN cells were below detection levels (0.3 ng/mL, data not shown). Culturing TLN cells with IL-16 in the absence of ovalbumin did not induce cytokine production or proliferation (data not shown). Stimulation of T cells by IL-16 therefore results in a decrease in antigen-induced Th2 cytokine production. This effect appears to be direct as similar results were obtained using only TLN cells *in vitro*.

Effect of IL-16 on generation of DO11.10 effector lymphocytes

A decrease in Th2 cytokine production can occur as a result of inhibiting Th2 T cell responsiveness to antigenic stimulation and/or by affecting T cell differentiation. Because *in vivo* treatment with IL-16 decreases Th2-type cytokine production by TLN cells stimulated with antigen *in vitro* we investigated whether the observed *in vivo* anti-inflammatory effect of IL-16 could be caused by alteration of T cell differentiation. To determine whether IL-16 has an influence on the differentiation of $CD4^+$ T cells into Th1- or Th2-type T cells, we used $CD4^+$ T cells isolated from DO11.10 mice. $CD4^+$ T cells were isolated and cultured for 4 days in the presence of APCs and $OVA_{323\text{-}339}$ with or without rmIL-16. Thereafter, non-viable cells were removed and remaining cells were re-stimulated with antigen and freshly isolated APCs and cultured for an additional 2-day period in the absence of IL-16. Cytokine levels in supernatants were determined both at the end of the first stimulation period and after the second stimulation period with $OVA_{323\text{-}339}$.

Cells maintained in medium and stimulated with $OVA_{323\text{-}339}$ produce IL-5, low levels of IL-4 and IL-10, and a high amount of IFN-γ (Table 2). Cytokine levels in supernatants derived from cells cultured in medium with rmIL-16 after the first

Table 2. Effect of IL-16 on priming of antigen-specific DO11.10 lymphocytes

Stimulation period	I	II	I	II
IL-16 presence	–	–	+	–
IL-4	0.05 ± 0.03	0.9 ± 0.2	0.05 ± 0.03	0.6 ± 0.2*
IL-5	1.4 ± 0.5	8.4 ± 3.7	1.5 ± 0.5	5.5 ± 3.2*
IL-10	0.0 ± 0.0	7.0 ± 0.4	0.6 ± 0.3	8.3 ± 1.2
IFN-γ	10.0 ± 0.1	9.2 ± 0.7	9.8 ± 0.3	8.5 ± 1.1

Cytokine levels (ng/mL) in supernatant fractions from DO11.10 $CD4^+$ T cells, stimulated with $OVA_{323\text{-}339}$ presented by APCs (BALB/c splenocytes) after the first culturing period (I) with (+) or without IL-16 (–, 1.0 μg/mL) and after the second stimulation period (II). During the second stimulation period with $OVA_{323\text{-}339}$, presented by APCs, cultures were maintained in medium only. Results are expressed as arithmetic average ± SEM of four separate experiments. *$P < 0.05$, significantly different from the cytokine level measured in supernatant fractions from cells cultured without IL-16 present during the first antigen stimulation.

stimulation period did not differ from levels measured in cultures without IL-16 (Table 2). During the second culture period, $OVA_{323-339}$ induced production of IL-4, IL-5, IL-10 and IFN-γ (Table 2), which is indicative of a Th0-type cytokine profile. Upon re-stimulation with antigen, cells generated in presence of IL-16 produce less IL-4 ($P < 0.05$) and IL-5 ($P < 0.05$), whereas IL-10 and IFN-γ levels were not altered in these supernatant fractions (Table 2). As IL-16 was not present during the second stimulation period, the decrease in Th2 cytokines would suggest the presence of less Th2 cells in cultures previously stimulated with IL-16. These data suggest that the presence of IL-16 not only inhibits Th2 cell activation and cytokine production, but also may influence the differentiation of Th1 and Th2 cells.

Discussion

In this study we demonstrate for the first time, that administration of IL-16 during the antigen-challenge period has potent immunosuppressive effects in a murine model of allergic asthma. IL-16 administration inhibited antigen-induced eosinophil infiltration and airway hyper-responsiveness but did not affect up-regulation of antigen-specific IgE levels in serum. The beneficial effects of IL-16 appear to be caused by inhibition of proliferation and cytokine production of antigen-specific type-2 lymphocytes.

The Th2-type $CD4^+$ T lymphocyte is of main importance for development of airway hyper-responsiveness and eosinophilia [14–18]. In murine models it has been shown that IL-4 and IL-13 play essential roles in antigen-induced airway hyper-responsiveness and eosinophilia. However, a crucial role for IL-5 in the development of airway hyper-responsiveness is still under debate [9, 19]. Since IL-16 uses CD4 as its receptor [1], the $CD4^+$ T lymphocyte is a likely candidate for the direct effects of IL-16 treatment. However, we can not exclude that IL-16 exerts its *in vivo* immunosuppressive actions through an alternative receptor, as the possibility exists that CD4 is not required for IL-16-induced signalling in cells of the monocyte lineage [20].

At present, little is known about the effect of IL-16 on type-2 cytokine production by T lymphocytes *in vivo*. Here we demonstrate that treatment with IL-16 *in vivo* inhibits antigen-stimulated production of IL-4 and IL-5 *in vitro* by lymphocytes isolated from lung draining lymph nodes. Moreover, pre-incubation of TLN cells derived from sensitized and antigen-challenged animals with IL-16 *in vitro* also inhibits antigen-specific Th2-type cytokine production and proliferation. This is consistent with the reports that addition of rhIL-16 to antigen-stimulated human peripheral blood mononuclear cell (PBMC) cultures significantly reduces the amount of IL-5 [8]. However, in the PBMC cultures of human atopics IFN-γ levels were significantly increased, suggesting a further shift towards a Th1-type cytokine profile [8]. This observation could not be confirmed in our studies as IFN-γ could not be detected in either TLN cultures from IL-16-treated animals, or in supernatants from *in vitro* pre-incubated TLN cultures upon antigen re-stimulation.

In agreement with our data, IL-16 has been shown to suppress the mixed lymphocyte reaction as well as antigen or anti-CD3-induced proliferation of human $CD4^+$ T cells *in vitro* when added prior to stimulation [4, 5]. Moreover, the anti-inflammatory activity of IL-16 is supported by the fact that *in vivo* administration of IL-16 results in inhibition of $CD4^+$ T cell mediated responses in a murine model of human rheumatoid synovitis [7], and prevents skin graft rejection in an *in vitro* model [6]. Similar to the down-regulatory actions of IL-16, other CD4 ligands such as antibodies to CD4 and HIV-1 gp120 have also been shown to be immunosuppressive in various models [21–24]. While the mechanism for the immunosuppressive effect of any of these CD4 ligands has not been elucidated, it is likely attributable to the ability to disrupt CD4 autoaggregation and CD4–T cell receptor complex formation, both requirements for optimal T cell activation.

In addition to inhibition of Th2 T cell activation, IL-16 may also influence the development of T cell differentiation. It is well known that the CD4 molecule plays a role in differentiation of T cells into Th2-type effector cells [13], besides having an accessory function in TCR-induced signalling. We found that the presence of IL-16 during priming cultures of naive DO11.10 $CD4^+$ T cells induces an effector phenotype that displays a decreased production of IL-4 and IL-5 but normal levels IL-10 and IFN-γ upon re-stimulation. As already mentioned, both IL-4 and IL-5 are necessary for development of allergy-associated parameters, whereas both IL-10 and IFN-γ have been demonstrated to be potent inhibitors of Th2-type T cell-mediated responses *in vivo* [25, 26]. Such a shift in the balance between pro- and anti-allergic cytokines may contribute to the observed *in vivo* effects of exogenous IL-16. However, it should be stressed that we used an artificial *in vitro* T cell differentiation system using DO11.10 T cells, which are highly specific for $OVA_{323-339}$. Furthermore, as already discussed, *in vivo* treatment with IL-16 does not induce IFN-γ production by T cells isolated from lung draining lymph nodes and therefore further research is required to elucidate the importance of this observation for our *in vivo* findings.

It is interesting to note that IL-16-treated DO11.10 cells did not display decreased cytokine production after the first antigen stimulation. This is consistent with the concept that only memory cells are susceptible to CD4-induced unresponsiveness, whereas naive T cells are not [27]. Moreover, freshly isolated DO11.10 $CD4^+$ T cells demonstrated a naive phenotype, as was indicated by high expression of $CD45^{RB}$ and CD62L/L-selectin (data not shown), whereas TLN cells, isolated from antigen-sensitized and repeatedly challenged animals are likely to have a memory/effector phenotype and are therefore more susceptible to CD4-mediated unresponsiveness. Indeed, it has been shown that $CD4^{high}CD62L^{low}$ lymphocytes are responsible for the passive transfer of allergic inflammation [17]. Finally, it is well known that CD4 expression increases when T cells are exposed to antigen, which could render these T cells more susceptible for CD4 ligands.

Previously, we have observed that administration of anti-IL-16 antibody during the challenge period partially decreased airway hyper-responsiveness, whereas eosinophilic infiltration was not affected [2]. Those data would initially appear to be contradictory with the data presented in this study. However, the two protocols are addressing two different concepts of IL-16 biology. The earlier study investigated the potential role of endogenous IL-16 by using neutralizing antibodies. In that model the major cell source of IL-16 is the airway epithelium. Production of low levels of IL-16 selectively in the lung, by virtue of establishing a tissue-specific concentration gradient,

could attract $CD4^+$ cells to the lungs, contributing to the inflammatory response. Neutralization of the IL-16 would result in fewer recruited cells. The magnitude of the inhibition would reflect the contribution of IL-16 bioactivity to airway hyperreactivity and inflammation. In the current model, systemic administration of IL-16 results in high circulating levels of IL-16. Under these conditions, it is likely that the effects of IL-16 are similar to what is seen *in vitro*, inhibition of T cell activation and cytokine production. As potentially all $CD4^+$ T cells would be exposed to IL-16, the magnitude of the inhibitory effect would be greater. This concept would support our data where IL-16 treatment resulted in almost complete inhibition of increased airway hyper-reactivity and inflammation. It is unclear at present whether endogenous levels of IL-16 reach levels required for induction of immunosuppression. Understanding the *in vivo* role of IL-16 in allergic inflammation will be greatly enhanced with data generated from an IL-16 knockout mouse.

Finally, in our study, IgE production was not inhibited by administration of IL-16, whereas IL-4 production by TLN cells was decreased. This seems to contradict the generally accepted essential role of IL-4 in IgE-synthesis. However, upon repeated encounter with antigen, IgE production by B cells is less dependent on cognate B–T cell contact and on the presence of IL-4 [28]. It could thus be hypothesized that lower levels of IL-4 in our model would not significantly alter IgE production by memory B cells.

In conclusion, we are the first to demonstrate that IL-16, a natural CD4 ligand, has immunosuppressive effects *in vivo* on both antigen-induced inflammatory responses as well as airway hyper-responsiveness. Although the precise mechanism remains to be elucidated, we demonstrate that *in vivo* immunosuppressive effects of IL-16 are associated with inhibition of Th2-type cytokine production. As IL-16 has been detected in association with a number of inflammatory conditions, it is tempting to speculate that this process for regulating Th2 T cell activation is not restricted to the lung but may represent a more widely utilized mechanism in the development of allergic inflammation.

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