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Suppression of cytokine expression by roflumilast and dexamethasone in a model of chronic asthma

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Summary

Background In a mouse model of mild chronic asthma, both inflammation and remodelling can be suppressed by dexamethasone (a glucocorticoid) and roflumilast (a selective phosphodiesterase-4 inhibitor).

Objective To better understand the underlying molecular mechanisms, we investigated the effects of treatment on airway expression of inflammation-related cytokines, as well as on epithelial expression of growth factors.

Methods BALB/c mice systemically sensitized to ovalbumin were challenged with aerosolized antigen for 6 weeks and treated with roflumilast or dexamethasone during the final 2 weeks. Expression of mRNA, for a variety of cytokines and growth factors, was assessed in selectively dissected proximal airways or in airway epithelium obtained by laser capture microdissection.

Results In the airway wall of vehicle-treated challenged animals, there was significantly elevated expression of mRNA for a variety of pro-inflammatory and T helper type 2 cytokines, as well as for IFN-γ. All these cytokines were suppressed by dexamethasone. Treatment with roflumilast reduced expression of IL-17A, TNF-α, granulocyte-macrophage colony-stimulating factor and IL-6, but did not inhibit other cytokines. Both drugs suppressed the enhanced expression of mRNA for growth factors such as TGF-β1 and FGF-2 in airway epithelium.

Conclusions Whereas dexamethasone non-specifically inhibits numerous mediators involved in inflammation and the immune response, roflumilast selectively inhibits a subset of pro-inflammatory cytokines and growth factors. These mediators and/or the cells that produce them may have critical roles in the pathogenesis of the lesions of chronic asthma.

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Introduction

Asthma is a chronic disease characterized by widespread inflammation of the walls of the airways. Other hallmark lesions include airway hyper-reactivity (AHR) and a variety of structural changes including sub-epithelial fibrosis, goblet cell metaplasia and increased smooth muscle mass, which are collectively referred to as airway remodelling [1]. Both in human studies and in models of allergic bronchopulmonary inflammation, an important pathogenetic role has been ascribed to $CD4^+$ T lymphocytes producing IL-4, -5, -9, -10 and -13 [2, 3], a so-called T helper type 2 (Th2) profile of cytokine expression. These cytokines may not only drive airway inflammation but, by regulating the expression by airway epithelium of growth factors such as TGF-β1, are also likely to play a key role in promoting airway remodelling [4, 5]. In addition, Th1 cells probably contribute to the pathogenesis of asthma [6] and recent studies suggest a role for the newly described Th17 subset of $CD4^+$ T lymphocytes in exacerbations of the disease [7].

Although initial studies in animal models suggested that selective inhibition of Th2 cytokines might be therapeutically effective [8, 9], there have been disappointing results from clinical studies involving blockade of IL-5

with a humanized monoclonal antibody or of IL-4 with a soluble receptor [10]. Approaches targeting cytokines such as IL-9 and IL-13 are under development, but it appears unlikely that inhibition of any single cytokine would significantly benefit patients with chronic asthma [11]. Thus, the mainstay of therapy remains the use of broad-spectrum anti-inflammatory agents, principally inhaled glucocorticoids, or systemic glucocorticoids for management of acute exacerbations. However, these are associated with adverse effects and are ineffective in a subset of patients referred to as steroid resistant [12]. The emergence of a new class of anti-inflammatory agents that target the phosphodiesterase type 4 (PDE4) enzyme, which is present in the majority of leucocytes, is therefore a promising development. Roflumilast is a recently developed investigational drug of this class and is currently being evaluated for treatment of chronic airway diseases including asthma [13, 14].

We have described a mouse model of chronic asthma that involves long-term challenge of sensitized mice with low mass concentrations of aerosolized ovalbumin (OVA) [15]. Key features of this model include, (A) *Changes of chronic asthma resembling the human disease:* airway disease is established by 4 weeks of OVA exposure, with lesions including eosinophil recruitment into the epithelial layer, chronic inflammation in the airway wall with accumulation of T lymphocytes and plasma cells and changes of remodelling such as sub-epithelial fibrosis, epithelial hypertrophy and goblet cell metaplasia. (B) *Minimal parenchymal inflammation:* there is no perivascular or peribronchiolar inflammation in the lungs [15], thus avoiding the disparity between human asthma and models involving uncontrolled exposure to high levels of aerosolized OVA [16], which have an inappopriately exaggerated eosinophilic inflammatory response [17]. Lesions are confined to the conducting airways and AHR to methacholine therefore originates from the airways rather than parenchyma [18]. (C) *A role for both Th2 and Th1 cytokines in pathogenesis:* Studies using gene-targeted animals and neutralizing antibodies demonstrate that airway inflammation and remodelling are dependent on Th2 cytokines [19, 20], and accumulation of the cleaved, potentially biologically active form of TGF-β1 in the airways is regulated by IL-13 [5], but IFN-γ clearly contributes to the pathogenesis of AHR [20].

In a previous study, we compared the anti-inflammatory activity of roflumilast with the glucocorticoid dexamethasone in our model of chronic asthma [21]. While both drugs effectively inhibited inflammation, there were differences in their effects on expression of proteins of the nuclear factor-κB pathway, airway remodelling and sub-epithelial accumulation of TGF-β1. These data indicated that different mechanisms were likely to be involved in the activity of the two drugs. We therefore performed a detailed comparison of the ability of roflumilast and dexamethasone to inhibit local expression of relevant cytokines and correlated this with changes in the inflammatory cell population in the airway wall, in the expectation that this would provide an insight into the cellular and molecular mechanisms critical to the pathogenesis of lesions of asthma.

Materials and methods

Animals

Specific pathogen-free female BALB/c mice aged 8 weeks were obtained from the Animal Resources Centre (Perth, Australia). Animals were housed in a laminar flow holding unit (Gelman Sciences, Sydney, Australia) in autoclaved cages on autoclaved bedding, in an air-conditioned room on a 12 h light/dark cycle. Irradiated food and acidified water were provided *ad libitum*. All experimental procedures were approved by the Animal Care and Ethics Committee of the University of New South Wales (ref. no. 04/06).

Sensitization and inhalational challenge

Animals were sensitized by an intraperitoneal injection of 50 μg of chicken egg OVA (Grade V, ⩾98% pure; Sigma, St Louis, MO, USA; unless otherwise specified, all chemicals were obtained from this source) adsorbed to 1 mg of aluminium hydroxide, 21 and 7 days before the commencement of inhalational challenges. Animals were exposed to aerosolized OVA for 30 min a day, 3 days/week for 6 weeks in a whole-body inhalation chamber as described previously [21]. During exposures, animals were held in wire flow-through cage racks, and filtered air was drawn through the 0.5 m^3 exposure chamber at a rate of 250 L/min. Aerosol was generated from a 2.5% solution of OVA using a sidestream jet nebulizer (Trimed, Sydney Australia). The concentration of aerosol within the chamber was continuously monitored using a real-time particle monitor (DustTrak 8520; TSI, St Paul, MN, USA) and was maintained at ≈ 3 mg/m^3 by controlling the airflow into the nebulizer.

Drug treatment

Each day during weeks 5 and 6 of the inhalational challenge, by which time the animals had established airways' disease [15], mice were administered dexamethasone (1 mg/kg, cyclodextran compound in saline), roflumilast (ALTANA Pharma, Konstanz, Germany) (5 mg/kg, suspended in 2.5% polyethylene glycol 400–4% methylcellulose solution) or vehicle alone by gavage. Age-matched non-sensitized mice that were not exposed to aerosol challenge were used as controls. Experimental groups comprised 16 animals. Based on our previous

studies demonstrating rapid cytokine responses to antigen in this model following a period of chronic challenge [22], mice were killed 4 h after the final airway challenge. Following exsanguination after an overdose of pentobarbital sodium, the lungs were perfused with saline to remove blood from the pulmonary capillary bed.

Assessment of airway inflammation and remodelling

The inflammatory response was quantified in eight animals from each treatment group. Longitudinally oriented 4 μm sections of formalin-fixed, paraffin-embedded tracheas were stained with haematoxylin and eosin for quantification of intraepithelial eosinophils and the total number of cells in the lamina propria of the airway. Immunostaining for T cells was performed on 5 μm acetone-fixed frozen sections in a capillary action system as described previously [23], using rat monoclonal antibodies against CD3 (CD3-12; Novocastra, Newcastle, UK), CD4 (GK1.5, American Type Culture Collection) and CD8 (H35-17.2; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunostaining for expression of eotaxin was performed on paraffin sections using a goat polyclonal antibody to mouse eotaxin (Santa Cruz Biotechnology), as described previously [22], and the intensity of immunoreactivity was semi-quantitatively scored from 0 (absent) to 3 (strong). Sub-epithelial fibrosis was assessed in paraffin sections stained with Gordon & Sweet's reticulin stain. The validity and reliability of the morphometric techniques that we used have been established in previous reports [15, 24].

Isolation of proximal airway tissue

Airway tissue was isolated by blunt dissection [25] from a further eight animals from each group. The trachea and lungs were removed from the thorax and, using two pairs of forceps, lung parenchyma was separated from the larger airways, leaving several generations of airway attached to the trachea. This procedure allowed effective isolation of a reasonable quantity of relevant tissue without significant contamination by irrelevant tissue. The preparation was divided just above the bifurcation of the trachea. Proximal airway tissue was stored in RNAlater (Ambion, Austin, TX, USA) for subsequent homogenization and extraction of RNA. Tracheas were embedded in OCT compound (Miles Inc, Elkhart, IN, USA), frozen in isopentane cooled with liquid nitrogen and stored at − 80 °C for immunohistochemistry.

RNA isolation

Blunt dissected airway tissue from individual animals was disrupted in RNA lysis buffer by grinding with a micropestle, and then homogenized by centrifuging through a Qia-Shredder column (Qiagen, Hilden, Germany). Epithelium from two consecutive acetone-fixed 10 μm frozen sections of trachea from individual animals was obtained by laser capture microdissection (LCM) and collected in lysis buffer using laser pressure catapulting according to the procedures recommended by the manufacturer (PALM Microlaser Technologies, Carl Zeiss MicrolmagIng, Bernried, Germany).

RNA was extracted from the airway tissue and LCM samples using an RNeasy micro RNA isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. An on-column DNase digestion was incorporated into the procedure to remove contaminating genomic DNA. One round of linear amplification was performed on RNA samples obtained by LCM, using a SenseAmp RNA amplification kit (Genisphere, Hatfield, PA, USA) according to the manufacturer's instructions.

Polymerase chain reaction

For each treatment group, 1 μg samples of RNA from airway tissue of individual animals were reverse transcribed using Superscript III (Invitrogen, Carlsbad, CA, USA) with poly-dT primers according to the manufacturer's recommendations. Reverse transcription was carried out in a GeneAmp PCR System 2400 thermocycler (Perkin Elmer, Wellesley, MA, USA) using recommended conditions. RNA samples from tissue obtained by LCM were reverse transcribed using 10 μL of amplified RNA and random hexamers.

Preliminary experiments (not shown) used a 96-gene common mouse cytokine cDNA microarray and end-point PCR (SuperArray Bioscience, Frederick, MD, USA) to identify a number of cytokines and growth factors that appeared to be worthy of further investigation. Selected genes were amplified by quantitative real-time PCR using Taqman gene expression assay kits or custom primer/probe sets (Applied Biosystems, Foster City, CA, USA). Real-time reactions were routinely amplified for 45 cycles with 30 s at 95 °C and 1 min at 60 °C using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Threshold cycles were determined using Applied Biosystems Sequence Detector Software (version 1.7), and expression was determined relative to a standard curve generated from serially diluted plasmid DNA. Gene expression for proximal airway tissue samples was normalized to hypoxanthine phosphoribosyltransferase (HPRT) while that for LCM samples was normalized to 18S RNA. The latter was unsuitable as a control for airway tissue samples because of variable non-specific detection of genomic DNA. Control reactions using non-transcribed RNA as a template were also included. Changes in gene expression for individual animals were reported as fold increase or decrease relative to the mean for naïve animals.

Cytokine production by peribronchial lymph node cells

Lymph nodes surrounding the trachea and main bronchi were collected from eight animals in each treatment group and pooled. The isolated cells were cultured in 96-well U-bottom microplates in 100 μL of mixed lymphocyte culture medium at 5×10^6 cells/mL, in the presence or absence of 1 mg/mL of OVA [26]. Culture supernatants were collected 72 h later for measurement of the concentrations of IL-4, -5, -13 and IFN-γ by a sandwich enzyme immunoassay. The sensitivity of detection was 0.5 ng/mL for IL-5, IL-13 and IFN-γ and 0.1 ng/mL for IL-4.

Statistics

Results are presented as mean ± standard error, or as medians (interquartile range) for grading. Differences between groups were assessed using a one-way ANOVA or the Kruskal–Wallis test, followed by Dunnett's or Dunn's post-test as appropriate. The software package GraphPad Prism (version 4.03, GraphPad Software, San Diego, CA, USA) was used for all analyses and preparation of graphs.

Results

Airway inflammation and remodelling

Chronic challenge with OVA on 3 days/week for 6 weeks led to accumulation of eosinophils within the airway epithelial layer, confirming previously published results [15, 21]. Compared with naïve animals, in which eosinophils are rare, challenged mice treated with vehicle alone had significantly increased numbers of intraepithelial eosinophils ($P<0.01$, Fig. 1a). Cell numbers were reduced in challenged mice treated with either dexamethasone or roflumilast ($P<0.01$ for both groups, Fig. 1a). In parallel, there was accumulation of chronic inflammatory cells (i.e. lymphocytes, plasma cells and macrophages) in the lamina propria of the airways in sensitized, chronically challenged mice ($P<0.01$ compared with naïve animals, Fig. 1b). Again, treatment with either dexamethasone or roflumilast inhibited the allergen-induced accumulation of these inflammatory cells ($P<0.01$ for both groups, Fig. 1b).

Immunostaining of tracheal sections with anti-CD3 demonstrated that T lymphocytes were a component of the chronic inflammatory cells in the airway wall (Fig. 2). The increase in the number of these cells compared with naïve mice ($P<0.01$, Fig. 3) was significantly suppressed by treatment with dexamethasone ($P<0.05$) and was completely suppressed by treatment with roflumilast ($P<0.01$). Immunostaining with anti-CD4 and anti-CD8 demonstrated that the majority of the T lymphocytes in the walls of the airways were CD4-positive. Changes in the numbers of CD4-positive cells in the airways of vehicle- and drug-treated animals therefore paralleled those seen for CD3-positive cells (not shown).

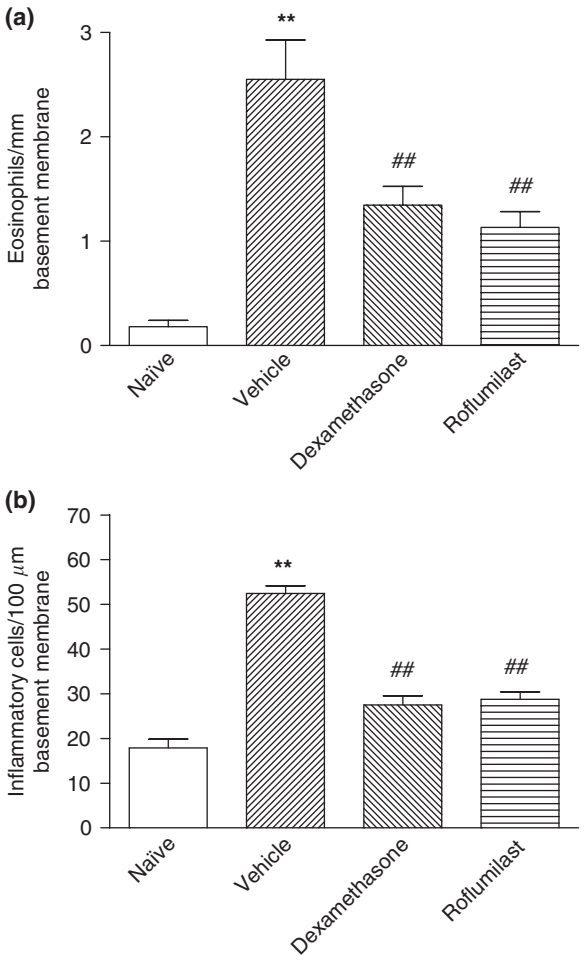

Fig. 1. Profile density of (a) intraepithelial eosinophils and (b) total inflammatory cells in the lamina propria of the trachea. Values are expressed as mean ± SEM; eight animals were assessed per group. Significant differences between chronically challenged mice treated with vehicle alone and naïve controls are shown as $^{**}P<0.01$; between vehicle-treated and drug-treated animals are shown as $^{\#\#}P<0.01$.

Both sub-epithelial fibrosis and airway epithelial hypertrophy induced by chronic challenge with OVA were similarly suppressed by treatment with either dexamethasone or roflumilast ($P<0.01$ for both groups, Fig. 4a and b).

Cytokine expression in the airway wall

Of the candidate mediators selected for further assessment by real-time PCR, increased expression of mRNA was confirmed for IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), CD40 ligand, IL-6, -10, -13, -17A and lymphotoxin-B (LT-B). The relative levels of mRNA for each of these were significantly elevated in vehicle-treated as compared with naïve

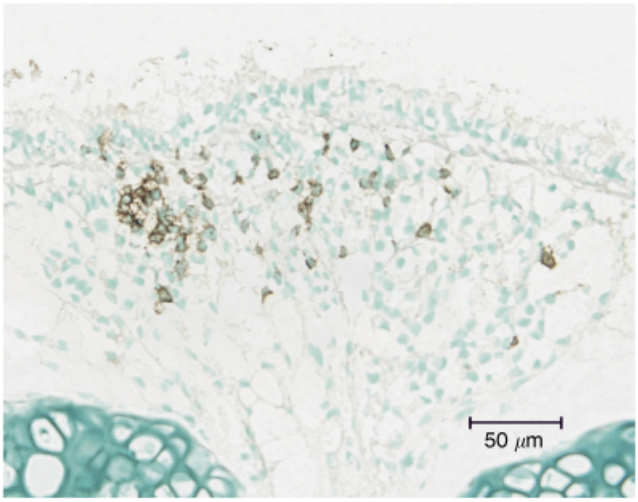

Fig. 2. CD3-positive cells in the lamina propria of the trachea of a BALB/c mouse treated with vehicle alone, demonstrating membrane staining of T cells. Immunoperoxidase-methyl green counterstain, original magnification × 400.

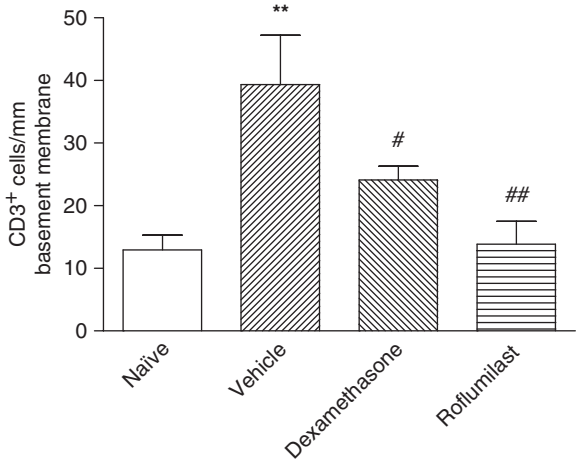

Fig. 3. Profile density of CD3-positive cells in the trachea of chronically challenged mice. Values are expressed as mean ± SEM; six animals were assessed per group. Significant differences between chronically challenged mice treated with vehicle alone and naïve controls are shown as $^{**}P < 0.01$; between vehicle-treated and drug-treated animals are shown as $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$.

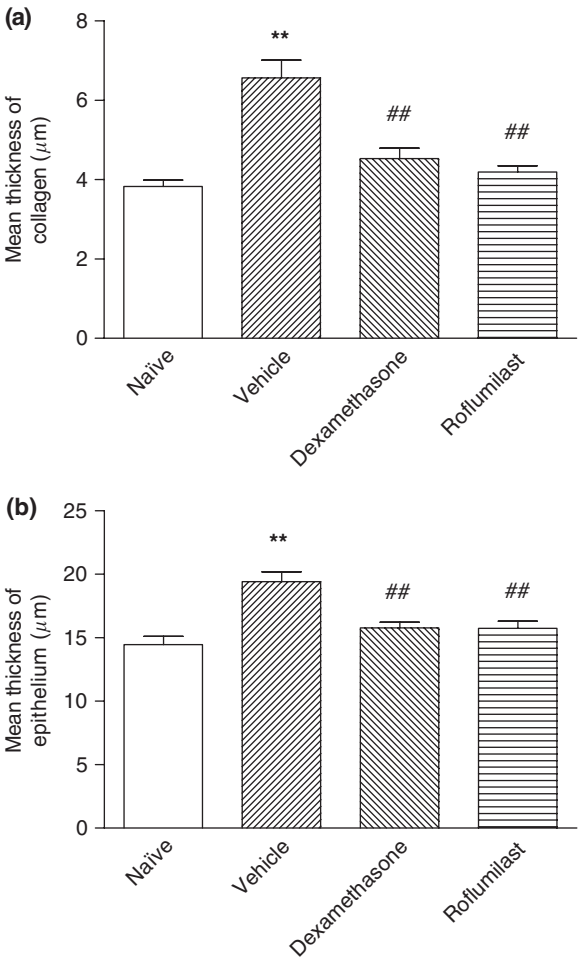

Fig. 4. Sub-epithelial collagenization (a), and epithelial thickness (b) in the trachea. Values are expressed as mean ± SEM; eight animals were assessed per group. Significant differences between chronically challenged mice treated with vehicle alone and naïve controls are shown as $^{**}P < 0.01$; between vehicle-treated and drug-treated animals are shown as $^{\#\#}P < 0.01$.

animals. Expression of mRNA for IL-5 and LT-A was modestly increased, but neither of these was statistically significant (Table 1). Real-time PCR did not demonstrate an increase in mRNA expression for IL-18 in vehicle-treated animals, or for IL-23 (not shown).

Treatment with dexamethasone significantly decreased mRNA expression for all the cytokines that were up-regulated in vehicle-treated animals, in addition to suppressing expression of IL-5 and LT-A markedly. In contrast, treatment with roflumilast significantly decreased expression for IL-17A,TNF-α, GM-CSF and IL-6, but had little effect on expression of mRNA for other inflammatory and Th2 cytokines (Table 1).

Immunostaining for expression of eotaxin revealed that whereas staining was completely absent in naïve control animals (Fig. 5a), there was strong staining in both the airway epithelium and in plasma cells in vehicle-treated mice (Fig. 5b), as reported previously [22]. The median grade of intensity of staining of epithelium in the vehicle-treated animals was 3 (2–3) ($P<0.001$ compared with naïve animals). There was a modest reduction in staining in both dexamethasone- and roflumilast-treated animals, with median grades of 2 (2–3) and 2 (1–3), respectively, but this change was not statistically significant.

Real-time PCR using mRNA extracted from whole tissue homogenates of the walls of the conducting airways did not demonstrate increased expression of any of the

growth factors that were investigated on the basis of preliminary experiments. Therefore, mRNA expression was re-assessed in preparations of airway epithelial cells isolated by LCM. In these samples, there were increased levels of expression of mRNA for TGF-β1 and FGF-2 in vehicle-treated as compared with naïve animals. However, because of the marked variability of replicates, this was statistically significant only for FGF-2 (Table 2). Both dexamethasone and roflumilast suppressed expression of mRNA for these growth factors, as well as for FGF-1 and IGF-1 (Table 2).

Table 1. Cytokine mRNA expression in airways

Cytokine	Vehicle	Dexamethasone	Roflumilast
IFN-γ	$1.82 \pm 0.27^{*}$	$0.41 \pm 0.09^{\#\#}$	1.76 ± 0.33
TNF-α	$2.79 \pm 0.20^{**}$	$0.59 \pm 0.04^{\#\#}$	$1.84 \pm 0.33^{\#\#}$
GM-CSF	$1.47 \pm 0.09^{**}$	$0.72 \pm 0.07^{\#\#}$	$0.89 \pm 0.09^{\#\#}$
CD40L	$1.74 \pm 0.16^{*}$	$0.76 \pm 0.16^{\#\#}$	1.46 ± 0.23
IL-5	1.25 ± 0.18	$0.43 \pm 0.08^{\#\#}$	1.39 ± 0.16
IL-6	$3.18 \pm 0.49^{**}$	$0.28 \pm 0.09^{\#\#}$	$1.37 \pm 0.20^{\#\#}$
IL-10	$2.88 \pm 0.35^{**}$	$1.47 \pm 0.15^{\#}$	2.43 ± 0.67
IL-13	$3.35 \pm 0.43^{**}$	$0.77 \pm 0.42^{\#\#}$	2.73 ± 0.45
IL-17A	$2.98 \pm 0.30^{**}$	$1.45 \pm 0.15^{\#\#}$	$1.58 \pm 0.19^{\#\#}$
IL-18	0.88 ± 0.12	1.16 ± 0.13	0.78 ± 0.08
LT-A	1.49 ± 0.37	$0.51 \pm 0.19^{\#}$	1.02 ± 0.22
LT-B	$1.99 \pm 0.36^{*}$	$0.37 \pm 0.06^{\#\#}$	1.53 ± 0.38

Relative gene expression normalized to HPRT, expressed as fold change relative to the mean of naïve animals. Values are expressed as mean ± SEM; eight animals were assessed per group. Significant increases in the vehicle-treated group are shown as $^{*}P < 0.05$ and $^{**}P < 0.01$. Significant differences between vehicle-treated and drug-treated groups are shown as $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$.

HPRT, hypoxanthine phosphoribosyltransferase; GM-CSF, granulocyte-macrophage colony-stimulating factor; LT, lymphotoxin.

Table 2. Growth factor mRNA expression in airway epithelium

	Vehicle	Dexamethasone	Roflumilast
FGF-1	0.99	0.09	$0.00^{\#}$
	(0.39–1.80)	(0.06–0.19)	(0.00–0.01)
FGF-2	351.50^{*}	$0.23^{\#\#}$	28.31
	(67.33–1093.00)	(0.15–16.33)	(0.11–96.80)
TGF-β1	5.80	0.26	0.06
	(0.09–15.69)	(0.00–8.48)	(0.00–0.37)
IGF-1	0.59	0.07	0.14
	(0.05–1.14)	(0.05–0.24)	(0.07–0.35)

Relative gene expression normalised to 18S RNA, expressed as fold change relative to the mean of naïve animals. Values are expressed as median with range; five animals were assessed per group. Significant increases in the vehicle-treated group are shown as $^{*}P < 0.05$. Significant differences between vehicle-treated and drug treated groups are shown as $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$.

Cytokine production by peribronchial lymph node cells

Consistent with the migration of responding lymphocytes from lungs to regional lymph nodes and their proliferation within the nodes in allergic inflammation, peribronchial lymph nodes (PBLNs) from animals sensitized and chronically challenged with OVA were enlarged. From this group, 2.7×10^{7} cells were isolated from nodes pooled from the eight animals. IL-5, IL-13 and IFN-γ were readily detected in supernatants of 72 h cultures of cells re-stimulated with OVA (Table 3) but IL-4 was below the limit of detection. Treatment with dexamethasone was associated with a modest decrease in the total cell yield, to 2.0×10^{7} cells, while the yield from animals treated with roflumilast was somewhat greater at 3.7×10^{7} cells. However, as these were also single pooled samples, no comparison of the cell yield was possible. Nevertheless, there was a moderate decrease in the concentrations of both IL-5 and IL-13 in culture supernatants of cells from both dexamethasone- and roflumilast-treated mice, which was

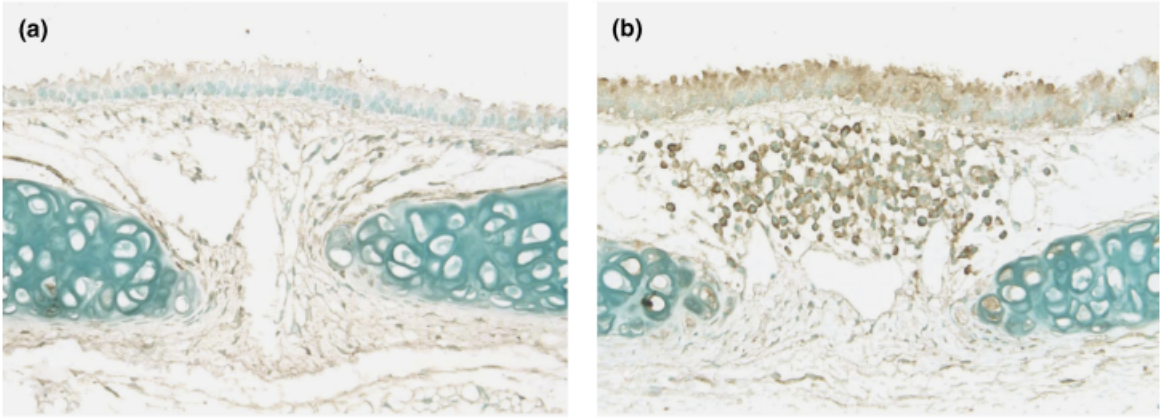

Fig. 5. Immunostaining for expression of eotaxin in the trachea of a naïve animal (a) and a vehicle-treated mouse (b), demonstrating reactivity of airway epithelium and plasma cells. Immunoperoxidase-methyl green counterstain, original magnification × 400.

Table 3. Cytokine production by peribronchial lymph node cells

	Vehicle	Dexamethasone	Roflumilast
IL-5	0.95 ± 0.035	$0.77 \pm 0.007^{**}$	$0.79 \pm 0.002^{**}$
IL-13	0.71 ± 0.030	$0.50 \pm 0.016^{**}$	$0.52 \pm 0.009^{**}$
IFN-γ	0.84 ± 0.013	0.80 ± 0.070	0.82 ± 0.033

Expressed as ng/mL in supernatants of ovalbumin-restimulated cells cultured at 5×10^6 cells/mL for 72 h; significant differences compared with vehicle-treated animals are shown as $^{**}P < 0.01$.

statistically significant ($P<0.01$ for both compared with vehicle-treated animals). The concentrations of IFN-γ in the supernatants were not decreased.

Discussion

In this study, we demonstrated that in a model of mild chronic asthma, expression of mRNA for a number of inflammation-related cytokines was significantly increased in whole tissue homogenates of the walls of the conducting airways. mRNA was up-regulated for Th2 (IL-13 and a borderline increase in IL-5) and Th1 (IFN-γ and a small increase in LT-A) cytokines, as well as for a variety of pro-inflammatory mediators. Treatment with either the glucocorticoid dexamethasone or the PDE4 inhibitor roflumilast effectively suppressed inflammation, including accumulation of T cells (primarily $CD4^+$) in the airways, and changes of remodelling. However, the effects of the two drugs in regulating gene expression were markedly dissimilar. Whereas dexamethasone suppressed all the cytokines up-regulated in airway wall tissue, roflumilast inhibited a subset of mediators, in particular, the cytokines IL-17A, TNF-α, GM-CSF and IL-6. Notably, roflumilast had relatively little effect on the expression of Th2 cytokines or IFN-γ. Both drugs suppressed the expression of mRNA for various growth factors by airway epithelial cells. Because the inflammatory response in this model is mild, levels of cytokines in lavage fluid or tissue lysates are generally below the limits of detection by enzyme immunoassay, making it impracticable to assess changes in protein levels between treatment groups. Attempts to assess expression of relevant cytokines by immunostaining were unsuccessful (not shown).

In this model, the immunological environment in the airways is biased towards expression of Th2 cytokines [27] and we have demonstrated previously that suppression of key Th2 cytokines such as IL-5 and IL-13 ameliorates inflammation and remodelling in experimental chronic asthma [20]. Nevertheless, the findings of the present study indicate that suppression of Th2 cytokines is not essential for inhibition of inflammation and/or remodelling, because despite the relatively selective action of roflumilast, its effects were essentially equivalent to those of dexamethasone. The lack of effect of roflumilast on expression of Th2 cytokines is consistent with previous reports of the activity of PDE4 inhibitors [28, 29], whereas the failure to suppress IFN-γ was unexpected, but might reflect the altered inflammatory and immunological response in the chronic challenge model of asthma.

Importantly, these data imply that the cytokines targeted by roflumilast, namely IL-17A, TNF-α, GM-CSF and IL-6, may play critical roles in the inflammation associated with chronic asthma. Of interest in this context is that a newly defined subset of $CD4^+$ T cells, designated Th17 cells [30, 31], exhibits up-regulated expression of mRNA for and secretion of IL-17A, TNF-α and GM-CSF [30, 32, 33]. These cells were also initially reported to secrete IL-6, although that has now been challenged [34]. The observed pattern of suppression of mediators by roflumilast raises the intriguing possibility that this drug, which inhibits various kinase pathways and the transcription factor NF-κB [35], may target the accumulation and/or activation of Th17 cells. In turn, this raises interesting questions about the possible contribution of Th17 cells towards the pathogenesis of chronic asthma. Whether the T cells accumulating in the airway wall in this model express a Th17 profile of cytokine expression remains to be established.

Each of the four cytokines suppressed by roflumilast is produced by various types of cells other than T lymphocytes, notably macrophages, epithelial cells and eosinophils. PDE4 inhibitors are well known to suppress the production of TNF-α by monocytes/macrophages [28, 29] and have also been shown to inhibit the production of GM-CSF by airway epithelial cells [36]. Therefore, further studies will be required, both to define the cellular source of the cytokines down-regulated by roflumilast and to assess whether it has a selective effect on Th17 cells as compared with glucocorticoids.

Also of interest are the specific roles of each of these cytokines, given that inhibition of this relatively small subset prevents the progression of inflammation and remodelling in this model of asthma and leads to the reversal of established lesions. TNF-α has long been recognized as a potentially important mediator of inflammation and AHR in asthma [37]. Its importance is emphasized by recent studies of the effectiveness of treatment of severe asthma using a TNF-α receptor antagonist [38]. GM-CSF may play important roles in both the induction and the prolongation of the Th2 inflammatory response [39]. The role of IL-6 in asthma is less well defined, but this cytokine may contribute to the induction of a Th2 [40] or a Th17 [30] response in asthma. Furthermore, all these cytokines have well-recognized pro-fibrotic effects [41–43]. More recent studies have shown that IL-17A contributes to asthmatic inflammation and may also be involved in changes of remodelling [7, 44, 45].

Additional relevant questions are whether the demonstrated reduction in the numbers of T lymphocytes in the

lamina propria of the airways following treatment with dexamethasone or roflumilast is related to diminished recruitment of these cells and whether reduced cytokine production is primarily the result of reduction in the numbers of cells or their functional activity. Glucocorticoids may suppress lymphocyte accumulation via inhibition of the production of chemoattractant cytokines and down-regulation of the expression of endothelial adhesion molecules [46]. PDE4 inhibitors have been shown to reduce chemotaxis of lymphocytes *in vitro* [47]. Further investigation will be required to assess the relative contributions of direct inhibition of lymphocyte chemotaxis, inhibition of chemokine release and/or altered expression of adhesion molecules to the decrease in the numbers of T cells in the airway wall. However, data from the experiments using re-stimulated PBLN cells support the notion that in addition to suppressing T cell accumulation, treatment with both dexamethasone and roflumilast directly inhibited cytokine production by these cells.

Assessing the relative expression of cytokine mRNA in airway wall tissue has limitations, because the denominator in all such calculations is affected not only by the choice of housekeeping gene (a potentially significant source of error [48]) but also by the increase in the total number of cells resulting from recruitment as part of the inflammatory response. As a consequence, it is possible for substantially increased expression by a minority of cells to be completely masked [49]. This could account for our observation that in homogenates of airway wall tissue, levels of mRNA expression for growth factors were apparently moderately decreased in vehicle-treated chronically challenged mice relative to naïve animals, as well as in drug-treated animals (not shown). The presence of mRNA from the large number of recruited inflammatory cells in these whole tissue homogenates would have diluted growth factor mRNA produced by airway epithelial or other structural cells.

This problem was overcome by assessing expression of mRNA for growth factors in airway epithelium obtained by laser capture microdissection, which revealed enhanced relative expression of TGF-β1 and FGF-2 in vehicle-treated animals. The data for TGF-β1 are of particular interest in the context of our previous report of progressive accumulation of TGF-β1 in parallel with subepithelial fibrosis [5]. However, the scatter of the replicate data was much greater than for whole tissue homogenates, so that the increase was statistically significant only for FGF-2. Various technical factors are likely to have contributed to the scatter, including degradation of mRNA during sample preparation by LCM and potential non-linear effects of amplification. Whether these factors affected demonstration of a relative increase in expression of cytokine mRNA for other growth factors in vehicle-treated animals is unclear. Nevertheless, expression of mRNA for TGF-β1, IGF-1, FGF-1 and FGF-2 was inhibited by treatment with both dexamethasone and roflumilast. It seems reasonable to infer that the effectiveness of these drugs in preventing the development of subepithelial fibrosis and epithelial hypertrophy is at least in part related to their capacity to suppress growth factor expression.

In summary, our results indicate that in this model of mild chronic asthma, expression of mRNA for pro-inflammatory mediators is up-regulated within the airway wall, and expression of mRNA for growth factors is up-regulated in airway epithelium. The anti-inflammatory drugs roflumilast and dexamethasone, which inhibit the expression of several of these cytokines, suppress the accumulation of eosinophils and of chronic inflammatory cells including T lymphocytes, as well as the development of subepithelial fibrosis and epithelial hypertrophy. The effectiveness of roflumilast, which is relatively selective in its action when compared with dexamethasone, implies that pro-inflammatory cytokines such as IL-17A, TNF-α, GM-CSF and IL-6 play important roles in asthmatic inflammation. These mediators, or cells that preferentially express them such as the Th17 subset of $CD4^+$ T cells, may be particularly appropriate therapeutic targets in chronic asthma.

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