

Short communication

Low-dose theophylline does not exert its anti-inflammatory effects in mild asthma through upregulation of interleukin-10 in alveolar macrophages

Background: There is accumulating evidence that theophylline has anti-inflammatory or immunomodulatory effects. This may be, in part, mediated via an upregulation in the production of the anti-inflammatory cytokine interleukin (IL)-10. We determined whether low-dose theophylline (LDT) would increase the production of IL-10, and attenuate the production of proinflammatory cytokines by alveolar macrophages.

Methods: In a double-blind, placebo-controlled, crossover study involving 15 steroid-free patients with mild asthma, fiberoptic bronchoscopy and broncho-alveolar lavage (BAL) were performed at the end of the treatment and placebo periods. Alveolar macrophages were cultured *in vitro*, and we measured their release of IL-10, GM-CSF, and TNF- α . We also measured IL-10 production in whole blood together with the number of monocytes and T cells expressing intracellular IL-10 by flow cytometry.

Results: LDT did not increase the production of IL-10, or attenuate the production of GM-CSF or TNF- α by alveolar macrophages. However, after theophylline treatment, there was a significant reduction in mean (SD) (95% CI) BAL eosinophil number from 3.4 (1.7)% (95% CI 2.4–4.4) to 1.7 (1.0)% (95% CI 1.1–2.3) compared with placebo ($P < 0.05$). Similarly, there was no increase in whole-blood IL-10 release or in the number of monocytes and T cells expressing intracellular IL-10 after treatment.

Conclusions: LDT has an anti-inflammatory effect in asthma; however, this effect is not mediated via the production of IL-10 or the attenuation of GM-CSF or TNF- α . The mechanisms of theophylline activity remain to be determined.

**B. Oliver¹, K. Tomita¹, A. Keller²,
G. Caramori³, I. Adcock³, K.
F. Chung³, P. J. Barnes³, S. Lim¹**

¹Respiratory Medicine, ANZAC Research Institute and Concord Hospital, University of Sydney, Sydney, Australia; ²Byk Gulden Pharmaceuticals, Konstanz, Germany; ³Department of Thoracic Medicine, National Heart and Lung Institute, Imperial College School of Medicine, London, UK

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Dr S. Lim
ANZAC Research Institute and
Department of Respiratory Medicine
Concord Repatriation General Hospital
Hospital Road
Concord
NSW 2139
Australia

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Alveolar macrophages from asthmatic patients have been previously shown to release increased amounts of several proinflammatory cytokines including interleukin (IL)-1 β , tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ), IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF). In addition, alveolar macrophages can also elaborate powerful suppressive signals to limit inflammation and maintain local immunologic homeostasis (1).

Theophylline is still widely used worldwide for the treatment of asthma. Recent studies indicate that theophylline has anti-inflammatory effects, and can inhibit the late response to allergen, as well as reduce bronchial mucosal eosinophils in patients with mild asthma (2). In addition, theophylline led to a reduction of mucosal CD4 and CD8 T cells, while withdrawal of theophylline led to an increase in mucosal T cells (3), suggesting a potential immunomodulatory role for theophylline. One of the limitations of theophylline

treatment of asthma in the past has been the side-effects observed in many patients from the traditional bronchodilator doses that achieve plasma levels of theophylline of 10–20 mg/l. However, with the benefits of anti-inflammatory activity at lower plasma theophylline levels (<10 mg/l), the incidence of adverse effects is minimized. Although the clinical utility of theophylline in the management of asthma is not questioned, the mechanisms that underlie its activity continue to elude investigators.

IL-10, which is released from alveolar macrophages, monocytes, and T cells (4), is known to inhibit the release of several proinflammatory cytokines such as TNF- α , IL-1 β , IL-6, IL-8, macrophage inflammatory protein-1 α (MIP-1 α), and GM-CSF from monocytes and alveolar macrophages. It also inhibits T-cell proliferation and reduces eosinophil survival. Because IL-10 is produced endogenously by monocytes/alveolar macrophages, it has been proposed that IL-10 has an

autocrine role in downregulating their release of cytokines. There may be a defect in IL-10 transcription and secretion from alveolar macrophages in asthma (5, 6), and treatment with inhaled glucocorticoids resulted in an increase in the production of IL-10 by alveolar macrophages (6). Theophylline led to an increase in the production of IL-10 by peripheral venous blood monocytes, and the increase in IL-10 production might underlie the mechanism of its anti-inflammatory action (7). While many studies have examined the anti-inflammatory effect of theophylline *in vitro*, far fewer clinical studies have directly examined the immunomodulatory activity of theophylline *in vivo*. The principal aim of our study was to investigate in mild, steroid-free asthmatics the effect of low-dose theophylline on the release of IL-10 and the proinflammatory cytokines GM-CSF and TNF- α by alveolar macrophages and in whole blood.

Material and methods

Study subjects

Fifteen mildly atopic, stable asthmatic patients receiving treatment with only the inhaled β_2 -adrenergic agonist salbutamol were recruited. All patients demonstrated >15% improvement in FEV₁ after 200 μ g of salbutamol, and airway hyperresponsiveness to methacholine with a provocative concentration of methacholine producing a 20% fall in FEV₁ (PC₂₀) of <4 mg/ml. They were all nonsmokers. None of the subjects studied had received oral or inhaled glucocorticoids for the preceding 12 months, or any other treatment apart from inhaled β_2 -agonists. We chose this group of steroid-free patients with mild asthma, in order to determine whether theophylline can reduce airway inflammation. The study was approved by the Royal Brompton Hospital Ethics Committee, and all patients gave their informed consent.

Baseline clinical characteristics and steady-state serum theophylline of study subjects

Fifteen steroid-free patients, mean (SD) age: 30.5 (7.85) years, M/F ratio 8/7, FEV₁% predicted: 87.98, PC₂₀ methacholine: 0.85 (1.26) mg/ml, were recruited for the study. The mean (SD) serum theophylline level following the active treatment period was 6.1 mg/l compared with less than 1 mg/l with placebo.

Study design

This was a 14-week, double-blind, randomized, crossover study comparing the effects of low-dose theophylline (Euphylong 250 μ g twice daily) to those of placebo. Each treatment was administered for 5 weeks, separated by a 4-week washout phase. All patients were reviewed at day 28, and spirometry and airway responsiveness to methacholine were measured. At day 35 of each treatment period, venous blood was drawn for the measurement of serum theophylline and whole-blood culture, and fiberoptic bronchoscopy and bronchoalveolar lavage (BAL) were performed.

Fiberoptic bronchoscopy and BAL

Fiberoptic bronchoscopy and BAL were performed from the right middle lobe using warmed 0.9% NaCl with four successive aliquots of 60 ml. BAL cells were spun (500 g; 10 min) and washed twice with

sterile Hanks' balanced salt solution (HBSS, Sigma, Poole, UK). Cytospins were prepared and stained with May-Grünwald for differential cell counts.

Isolation and *in vitro* culture of alveolar macrophages

Isolation and *in vitro* culture of macrophages have been previously described (6). In brief, BAL fluid was filtered through sterile gauze and centrifuged at 500 g for 10 min. Cells were washed twice in HBSS, counted, and plated in sterile media at a concentration of 1×10^6 /ml in a 12-well plate. After 24-h culture, the medium in which BAL cells were cultured was replaced by 1 ml of the same fresh medium. Adherent cells (i.e., alveolar macrophages) from each subject were then cultured for a further 24 h in the presence of medium alone, medium plus human recombinant interleukin 1 β (IL-1 β) (10 ng/ml) (R&D Systems Europe, Abingdon, UK), or medium plus bacterial lipopolysaccharide (LPS) (10 μ g/ml) (Sigma, Poole, UK).

Enzyme-linked immunosorbent assay (ELISA) for IL-10, GM-CSF, and TNF- α

We used three sandwich ELISAs that have been previously described (8). The detection limit of IL-10 and GM-CSF was 15.6 pg/ml, and it was 8 pg/ml for TNF- α .

Whole-blood cultures

A volume of 2 ml of whole peripheral venous blood for each patient was collected in heparinized tubes and was separated in two tubes (1 ml each). One volume was cultured without stimulation, and the second one was stimulated with 10 ng/ml of LPS (Sigma, Poole, UK) for 20 h and further incubated for 4 h with 10 μ g/ml of brefeldin A (Sigma, Poole, UK), an inhibitor of intracellular protein secretion.

Intracellular expression of IL-10 in monocytes and T cells

The methods have been previously described (9). In brief, after 24-h culture, red blood cells were lysed and the remaining leukocytes incubated with fluorescent conjugated antibodies (CD14-FITC; UCHM1 or CD3-FITC; UCHT1, Serotec, Oxford, UK), and fixed. The cells were further incubated with rat antihuman IL-10-PE (JES3-9DF; Serotec Ltd) in permeabilizing buffer. The cells were washed and fixed with 1% paraformaldehyde. Immunofluorescence was analyzed by FACScan flow cytometry (Becton Dickinson, San Jose, CA, USA). Isotype antibodies (Serotec, UK) were incubated as negative control. Lymphocytes (10 000 events) or monocytes (5000 events) were acquired by gating on forward (FSC) and side-angle scatter (SSC) properties. We gated mononuclear cells by specific forward- and side-scatter characteristics. Monocytes were then gated for CD14 or IL-10 expression. CD3-positive lymphocytes were also gated by size and granularity characteristics. All results are expressed as a percentage of IL-10-positive cells in the total CD14-positive population or in the total CD3-positive cells.

Statistical analysis

Data are reported as mean (SD). Data obtained within the asthmatic group during the placebo and active periods were compared by the paired *t*-test. A *P* value of <0.05 was considered to be significant.

Results

Effect of theophylline on FEV₁, PC₂₀ methacholine, and BAL cells

There was no significant improvement in lung function after treatment with theophylline. The mean (SD) FEV₁

Table 1. Cytokine release from alveolar macrophages and whole blood after placebo and theophylline treatment

Cytokine release	Stimulation	Placebo			Theophylline		
		Cont	LPS	IL-1 β	Cont	LPS	IL-1 β
AM	IL-10 production (pg/ml)	83.52 (213.8)	789.0 (642)*	561.3 (615.8)*	186.2 (376.8)	919.7 (660.7)**	458.1 (634.5)**
	TNF- α production (ng/ml)	15.41 (38.16)	54.9 (48.1)*	10.3 (15.9)*	11.6 (29.8)	54.9 (45.9)**	16.4 (32.5)**
	GM-CSF production (pg/ml)	471.3 (842.3)	1095.0 (887.1)*	527.3 (850.5)*	419.5 (767.6)	1109.0 (1006)**	491.1 (673)**
	IL-10 production (pg/ml)	163.8 (213.4)	792.9 (1179)*		112.8 (113.6)	655.7 (954.4)**	
WB	% IL-10-positive monocytes	30.3 (16.2)	43.1 (16.1)*		20.0 (7.4)	34.2 (12.2)**	
	% IL-10 +ve T cells	8.9 (7.2)	10.8 (9.3)*		6.4 (3.7)	8.4 (4.9)**	

Data shown as mean (SD).

AM: alveolar macrophages; WB: whole blood; cont: control; LPS: lipopolysaccharide; IL: interleukin.

* and ** indicate significant differences in cytokine release within placebo and theophylline treatment arms when compared to control values.

was 3.27 (0.61) l during placebo and 3.42 (0.32) l after theophylline treatment. After low-dose theophylline, there was no significant change in airway hyperresponsiveness to methacholine, PC₂₀ placebo mean (95% CI) 0.94 (-0.01, 1.89), and theophylline PC₂₀ 1.24 (0.46, 1.99). However, after theophylline treatment, there was a significant reduction in mean (SD) (95% CI) BAL eosinophil number from 3.4 (1.7)% (95% CI 2.4–4.4) to 1.7 (1.0)% (95% CI 1.1–2.3) compared with placebo ($P < 0.05$) (Fig. 1).

Effects of theophylline on IL-10, GM-CSF, and TNF- α release from alveolar macrophages

There was no significant increase in IL-10 release from alveolar macrophages after theophylline treatment. Similarly, there was no significant reduction in GM-CSF or TNF- α (Table 1) production by alveolar macrophages after low-dose theophylline treatment.

Effects of theophylline on IL-10 release from whole blood and percentage of mononuclear cells expressing intracellular IL-10

Whole-blood stimulation with LPS led to a significant increase in the whole-blood production of IL-10 and in the percentage of monocytes and lymphocytes expressing intracellular IL-10. However, after theophylline treatment, there was no significant increase in consti-

tutive or LPS-stimulated release of IL-10 from whole blood, nor was there any significant change in the percentage of monocytes or T cells expressing intracellular IL-10. The results are summarized in Table 1.

Discussion

We have confirmed that low-dose theophylline has the ability to reduce airway inflammation *in vivo* in patients with mild asthma; however, this was not mediated by an increase in the production of IL-10 by monocytes, alveolar macrophages, and T cells or a decrease in the production of GM-CSF or TNF- α by alveolar macrophages. The reduction in airway eosinophils may explain the beneficial effects of theophylline that were not totally explained by bronchodilation in several clinical studies using theophylline at doses to produce serum levels below the conventional bronchodilator levels (10). Although there was no significant improvement in lung function, we studied patients with mild asthma and normal baseline lung function. Indeed, the lack of response in lung function suggests that the beneficial effects previously observed may be a result of immunomodulation rather than bronchodilation.

Theophylline inhibits *in vitro* the degranulation and release of mediators, including platelet-activating factor, leukotriene C₄, cationic proteins, and superoxide anion (11, 12), from eosinophils, granulocytes, and alveolar macrophages. It inhibits the proliferation and activation of T cells and has also been shown to cause eosinophil apoptosis (13). However, of the few studies that have examined the effect of theophylline *in vivo*, one study suggests that theophylline may reduce sputum eosinophil chemotactic activity (14). Clearly, there must be a disparity between the effects of theophylline *in vitro* and its effect *in vivo*. We have chosen to investigate the ability of low-dose theophylline to increase the expression of IL-10 and to decrease the production of GM-CSF and TNF- α , on the basis of previous work suggesting that this may be the underlying basis of its anti-inflammatory activity (7). Our data suggest that this is unlikely to be the mechanism in alveolar macrophages. Moreover, our data are in

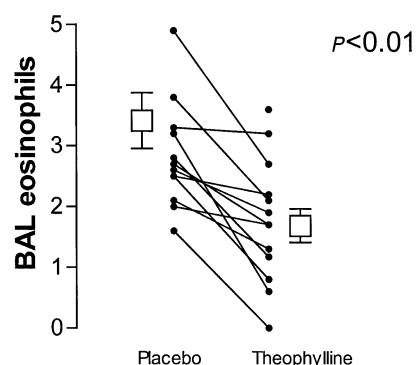


Figure 1. Bronchoalveolar lavage eosinophils and granulocytes after placebo and theophylline treatment periods. Results are expressed as mean (SD) in each group ($P < 0.05$).

keeping with the data of Seldon et al. (15), which suggest that IL-10 does not mediate the inhibitory effects of phosphodiesterase inhibitors on cytokine production by monocytes. Other studies have also demonstrated that low concentrations of theophylline decreased the eosinophil survival induced by IL-5 and GM-CSF, and that this is independent of PDE inhibition and changes in cyclic AMP (16). These observations may account for the decrease in BAL eosinophil number seen in our patients.

The clinical utility of theophylline in the management of asthma is not controversial, and several recent studies have confirmed the role of theophylline in

asthma management (10, 17). However, the lack of clarification of theophylline's anti-inflammatory activity has limited its acceptance as a disease-modifying agent. In summary, our study clearly indicates that low-dose theophylline has the ability to reduce airway inflammation in patients with mild persistent asthma; however, the mechanisms of theophylline's anti-inflammatory activity *in vivo* need to be elucidated in further clinical studies.

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References

- LOHMANN-MATTHES ML, STEINMULLER C, FRANKE-ULLMANN G. Pulmonary macrophages. *Eur Respir J* 1994;**7**:1678–1689.
- SULLIVAN P, BEKIR S, JAFFAR Z, et al. Anti-inflammatory effects of low-dose oral theophylline in atopic asthma. *Lancet* 1994;**343**:1006–1008.
- KIDNEY J, DOMINGUEZ M, TAYLOR PM, et al. Immunomodulation by theophylline in asthma. Demonstration by withdrawal of therapy. *Am J Respir Crit Care Med* 1995;**151**:1907–1914.
- YSSEL H, DE WAAL MR, RONCAROLO MG, et al. IL-10 is produced by subsets of human CD4⁺ T cell clones and peripheral blood T cells. *J Immunol* 1992;**149**:2378–2384.
- BORISH L, AARONS A, RUMBYRT J, et al. Interleukin-10 regulation in normal subjects and patients with asthma. *J Allergy Clin Immunol* 1996;**97**:1288–1296.
- JOHN M, LIM S, SEYBOLD J, et al. Inhaled corticosteroids increase IL-10 but reduce macrophage inflammatory protein-1 α , granulocyte-macrophage colony-stimulating factor, and interferon-gamma release from alveolar macrophages in asthma. *Am J Respir Cell Mol Biol* 1998;**157**:256–262.
- MASCALI JJ, CVIETUSA P, NEGRI J, et al. Anti-inflammatory effects of theophylline: modulation of cytokine production. *Ann Allergy Asthma Immunol* 1996;**77**:34–38.
- LIM S, ROCHE N, OLIVER B, et al. Balance of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 from alveolar macrophages in cigarette smokers. Regulation by interleukin-10. *Am J Respir Crit Care Med* 2000;**162**:1355–1360.
- ROUSSEAU Y, HAEFFNER-CAVAILLON N, POIGNET JL, MEYRIER A, CARRENO MP. *In vivo* intracellular cytokine production by leukocytes during haemodialysis. *Cytokine* 2000;**12**:506–517.
- EVANS DJ, TAYLOR DA, ZETTERSTRÖM O, et al. A comparison of low-dose inhaled budesonide plus theophylline and high-dose inhaled budesonide for moderate asthma. *N Engl J Med* 1997;**337**:1412–1418.
- CALHOUN WJ, STEVENS CA, LAMBERT SB. Modulation of superoxide production of alveolar macrophages and peripheral blood mononuclear cells by beta-agonists and theophylline. *J Lab Clin Med* 1991;**117**:514–522.
- SHUTE JK, TENOR H, CHURCH MK, et al. Theophylline inhibits the release of eosinophil survival cytokines: is Raf-1 the protein kinase A target? *Clin Exp Allergy* 1998;**28**:47–52.
- TOMITA K, CHIKUMI H, TOKUYASU H, et al. Functional assay of NF-kappa B translocation into nuclei by laser scanning cytometry: inhibitory effect by dexamethasone or theophylline. *Naunyn Schmiedeberg Arch Pharmacol* 1999;**359**:249–255.
- LOUIS R, BETTIOL D, CATALDO J, et al. Effect of a four week treatment with theophylline on sputum eosinophilia and sputum eosinophil chemotactic activity in steroid-naïve asthmatics. *Clin Exp Allergy* 2000;**30**:1151–1160.
- SELDON PM, BARNES PJ, GIEMBYCZ MA. Interleukin-10 does not mediate the inhibitory effect of PDE-4 inhibitors and other cAMP-elevating drugs on lipopolysaccharide-induced tumor necrosis factor- α generation from human peripheral blood monocytes. *Cell Biochem Biophys* 1998;**29**:179–201.
- OHATA K, SAWAMOTO S, NAKAJIMA M, et al. The prolonged survival of human eosinophils with interleukin-5 and its inhibition by theophylline via apoptosis. *Clin Exp Allergy* 1996;**26**:10–15.
- UKENA D, HARNEST U, SAKALAUSKAS R, et al. Comparison of addition of theophylline to inhaled steroid with doubling of the dose of inhaled steroid in asthma. *Eur Respir J* 1997;**10**:2754–2760.