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Interleukin-10 level in sputum is reduced in bronchial asthma, COPD and in smokers

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Interleukin-10 level in sputum is reduced in bronchial asthma, COPD and in smokers. S. Takanashi, Y. Hasegawa, Y. Kanehira, K. Yamamoto, K. Fujimoto, K. Satoh, K. Okumura. ©ERS Journals Ltd 1999.

ABSTRACT: Interleukin (IL)-10 is a potent regulatory cytokine that decreases inflammatory responses. This study investigated whether IL-10 levels in the airway are decreased in chronic airway inflammation associated with asthma or chronic obstructive pulmonary disease (COPD).

Sputum was obtained from 12 healthy nonsmokers, 10 healthy smokers, 16 asthmatic patients and seven patients with COPD by means of the sputum-induction method. The IL-10 level was measured *via* enzyme-linked immunosorbent assay and immunocytochemical analysis.

The IL-10 level in sputum was significantly lower in asthma and COPD patients and healthy smokers compared with that in healthy nonsmokers (nonsmokers, 68.0± 11.3; smokers, 45.3±7.8; asthma, 26.7±4.0; COPD, 18.0±2.3 pg·mL^{-1}; $p<0.05$ for nonsmokers *versus* the other groups). The percentage of IL-10-positive cells in the sputum was also significantly lower in asthma and COPD and in smokers (nonsmokers, 13.2±1.7; smokers, 6.4±1.8; asthma, 5.4±3.5; COPD, 3.5±1.6%; $p<0.05$ for nonsmokers *versus* the other groups). The IL-10-positive cell appeared morphologically to be the macrophage.

These data suggest that the reduced level of interleukin-10 within the airways plays a role in the pathogenesis of chronic airway inflammation in asthma and chronic obstructive pulmonary disease.
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Interleukin (IL)-10 was initially described as a T helper cell (Th)2-derived factor that inhibits cytokine synthesis in murine Th1-type T cells. However, human IL-10 is produced by Th0, Th1 and Th2 and by macrophages [1–3]. IL-10 is a potent regulatory cytokine that reduces inflammatory responses. In animal models, IL-10 has been shown to inhibit immune complex-induced lung injury, reduce tumour necrosis factor-α production and prevent death due to endotoxin injection [4].

Bronchial asthma is a Th2-type disease in which Th2-type cytokines including IL-10 are increased. In a previous study, the number of IL-10 messenger RNA (mRNA)-positive cells in bronchoalveolar lavage (BAL) fluid obtained from asthmatic subjects was shown to be increased compared to that in control subjects [5]. In another study, however, IL-10 was shown to be decreased in the BAL fluid from asthmatic patients [6]. Thus, the level of IL-10 in asthmatic airway remains to be elucidated.

IL-10 inhibits cytokine production in many types of inflammatory cell including eosinophils, and also inhibits antigen-induced cellular recruitment into the airway in the mouse model [7–10]. In another condition involving airway inflammation, cystic fibrosis, less IL-10 was found in the epithelial lining fluid than in that of healthy control subjects [11]. Therefore, it was hypothesized that the diminished production of inhibitory cytokines such as IL-10 could contribute to the chronic airway inflammation found in conditions such as bronchial asthma and chronic obstructive pulmonary disease (COPD). Recently, a sputum induction method involving hypertonic saline was introduced to assess airway inflammation [12, 13]. Using this method, this study examined whether IL-10 levels in the airway are reduced in bronchial asthma and COPD.

Methods

Study subjects

The subjects were 12 healthy nonsmokers, 10 healthy smokers, 16 asthmatic patients and seven patients with COPD (tables 1–3). The healthy nonsmokers lacked a

Table 1. – Subject characteristics

Group	Subjects n	Age yrs	FEV1 % pred
Healthy nonsmoker	12	37.3±1.9	99.1±2.9
Healthy smoker	10	35.5±1.5	94.6±2.3
Asthma	16	44.3±3.3	79.3±5.2
COPD	7	58.7±4.2	52.4±4.1

Data are presented as mean±SEM. FEV1: forced expiratory volume in one second; COPD: chronic obstructive pulmonary disease.

Table 2. – Patient characteristics of the 16 asthmatic patients

No.	Age yrs	Sex	Atopy	Severity step	Total IgE IU·mL^{-1}	PEF_{max} % pred	Therapy
1	52	F	No	1	36	99.5	B2
2	27	F	Yes	2	473	98.0	B2, T
3	23	F	Yes	2	480	97.9	B2, T
4	45	F	Yes	2	450	72.9	B2,
5	42	F	Yes	2	387	70.5	B2, T
6	43	F	Yes	2	491	81.9	B2,
7	44	F	No	2	275	95.6	B2, T
8	34	M	No	2	185	65.5	B2
9	67	F	No	2	10	68.7	B2, T
10	63	M	No	2	38	96.3	B2, IS(800)
11	42	M	Yes	3	283	82.1	B2, T
12	43	F	No	3	81	73.9	B2, IS(800), T
13	27	M	Yes	3	1060	97.0	B2, IS(800), T
14	39	M	Yes	4	1720	92.4	B2, IS(800), T, P(5)
15	55	M	No	4	138	89.2	B2, IS(1200), T, P(5)
16	62	M	No	4	38	52.1	B2, IS(800), T

IgE: immunoglobulin E; PEF_{max}: maximal peak expiratory flow; F: female; M: male; B2: inhaled β_2-agonist; T: oral theophylline; IS: inhaled corticosteroids (beclomethasone daily dose in μg); P: oral prednisone (daily dose in mg).

medical history consistent with atopic disease including asthma, allergic rhinitis and eczema. The healthy smokers were current smokers (>10 pack-yrs) with a normal forced expiratory volume in one second (FEV_1) and no respiratory disease manifestations. The inclusion criteria for asthmatic patients included nonsmoking subjects with relatively stable asthma and documented reversibility following inhaled β-agonist administration of >15% of baseline FEV_1 or >10% of predicted FEV_1. Table 2 shows the clinical profiles of the asthmatic patients. The inclusion criteria for COPD included subjects with stable airflow limitation with an FEV_1 <70% pred, reversibility following inhaled β-agonist administration of <10% of predicted FEV_1 and a smoking history of >20 pack-yrs. In five asthmatic patients whose asthma could not be controlled well (patients 4, 5, 8, 9 and 11 in table 2), sputum was collected before and after a 1-month therapy with inhaled corticosteroids. Each subject gave informed consent before starting the study, and the study protocol was in agreement with the guidelines for clinical study at the authors′ institution.

Sputum induction

Induction of sputum was performed as described by PIN *et al.* [12] with slight modifications. Briefly, 10 min after inhalation of β-agonist, hypertonic saline was nebulized using an ultrasonic nebulizer for 5-min periods. The concentration was increased at 10-min intervals from 3 to 4% and then from 4 to 5%. The FEV_1 was recorded every 5 min during the nebulization. If the FEV_1 fell by >10% of the post-bronchodilator value, the concentration of the hypertonic saline solution was not increased. If the FEV_1 fell by >20% of the post-bronchodilator value, or if any symptoms occurred, nebulization was discontinued. After 10 min, and every 5 min thereafter, the subjects were instructed to rinse their mouths and throats carefully and then try to cough sputum into a container. Nebulization was stopped after 30 min or earlier, if a sputum sample of good quality was obtained.

Table 3. – Patient characteristics of the seven subjects with chronic obstructive pulmonary disease

No.	Age yrs	Sex	IgE IU·mL^{-1}	FEV_1 % pred	Therapy
1	44	M	78	60.6	B2
2	47	M	15	65.8	IO
3	55	M	47	62.0	IO, T
4	56	M	110	51.4	IO, T
5	66	M	7	40.0	B2, IO, T
6	71	F	45	48.0	B2, T
7	72	M	20	39.0	B2, IO, T

IgE: immunoglobulin E; FEV_1: forced expiratory volume in one second; M: male; F: female; B2: inhaled β_2 agonist; IO: inhaled oxitropium; T: oral theophylline.

Sputum handling

The sputum samples were processed as previously described [12]. Briefly, sputum was separated from saliva and mixed with 0.1% dithiothreitol for 5 min. Each sample was diluted with dithiothreitol in Dulbecco′s phosphate-buffered saline (D-PBS) and filtered through a nylon gauze. Then, the sputum cells were separated from the supernatant by centrifugation, and resuspended in D-PBS. For immunocytochemistry, cytospins were prepared and fixed in periodate/lysine/paraformaldehyde for 10 min followed by 15% sucrose for 10 min. Cytospins were kept at -80°C until immunocytochemistry was performed. Diff Quick (Baxter, Miami, IL, USA) was applied for the cell differentials.

Enzyme-linked immunosorbent assay for interleukin-10

The IL-10 concentration in the sputum supernatant was assessed using an enzyme-linked immunosorbent assay (R&D, Minneapolis, MA, USA). This assay employed the quantitative sandwich enzyme immunoassay technique.

Immunocytochemical analysis

In order to detect IL-10, a mouse monoclonal antibody to human IL-10 was used at 30 μg·mL^{-1} (R&D).

Table 4. – Total cell counts and differential cell counts in each group

Group	Total cells 10^6 cells·mL^{-1}	Mac %	Neut %	Eos %	Lymph %
Healthy nonsmoker	6.2±1.3	70.0±3.2	25.0±3.0	1.3±0.3	0.8±0.3
Healthy smoker	8.3±2.1	71.2±4.4	21.3±4.4	4.8±1.4	0.8±0.4
Asthma	10.4±2.4	35.5±5.2§	28.1±5.5	31.7±6.3*	0.9±0.2
COPD	33.9±13.8*	18.1±4.4§	76.3±5.4*	3.9±1.7	0.7±0.4

Data are presented as mean±SEM. Mac: macrophages; Neut: neutrophils; Eos: eosinophils; Lymph: lymphocytes; COPD: chronic obstructive pulmonary disease. *: p<0.05 *versus* the other three groups; §: p<0.05 *versus* healthy nonsmokers and smokers.

Immunocytological staining was performed by means of an alkaline phosphatase/anti-alkaline phosphatase method. Appropriate controls and mouse IgG2b (Dako Glostrup, Denmark) directed against IL-10 were used at the same concentration as a negative control. The results were evaluated independently by two observers without any knowledge of the patient profiles.

Data analysis

All values are expressed as mean±SEM. Paired and unpaired t-tests were used when appropriate. Multiple comparisons were made providing that a preliminary two-factor repeated-measures analysis of variance yielded significant F-values. A p-value <0.05 was considered significant.

Results

Characteristics of sputum

All subjects tolerated the sputum induction procedure. Table 4 shows the results of the cytological analysis of sputum. The total cell numbers in sputum were 6.2±1.3, 8.3±2.1, 10.4±2.4 and 33.9±13.8 × 10^6 cells·mL^{-1} in the healthy nonsmokers, healthy smokers, and patients with asthma and COPD, respectively. The total cell number in COPD was significantly greater than that in the other three groups. The percentage of macrophages was significantly lower in asthma and COPD than in the other two groups. However, the absolute number of macrophages did not differ among the groups. The percentage of neutrophils was significantly higher in COPD, and the percentage of eosinophils was significantly higher in asthma than in the other three groups. There was no significant difference in the percentage of lymphocytes among the four groups.

IL-10 levels in sputum

The levels of IL-10 in sputum were 68.0±11.3, 45.3±7.8, 26.7±4.0 and 18.0±2.3 pg·mL^{-1} in the healthy nonsmokers, healthy smokers, and patients with asthma and COPD, respectively (fig. 1). The level of IL-10 was significantly lower in asthma, COPD and healthy smokers than in healthy nonsmokers. In COPD, the level of IL-10 was significantly lower than that in healthy smokers.

Interleukin-10 immunocytochemical evaluation of sputum cells

The percentages of IL-10-positive cells in sputum were 13.2±1.7, 6.4±1.8, 5.4±3.5 and 3.5±1.6% in the healthy nonsmokers, smokers and patients with asthma and COPD, respectively (fig. 2). The percentage of IL-10-positive cells was significantly lower in asthma and COPD and in smokers than in healthy nonsmokers. Morphologically, the majority of IL-10-positive cells were macrophages; some were lymphocytes (fig. 3).

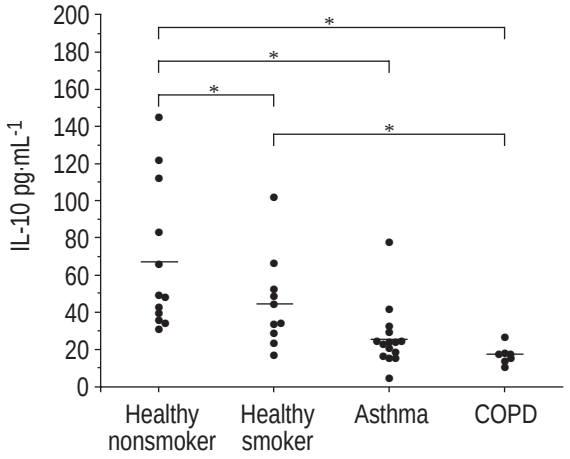

Fig. 1. – Levels of interleukin-10 (IL-10) in sputum obtained from healthy nonsmokers, healthy smokers and patients with asthma and chronic obstructive pulmonary disease (COPD). Horizontal bars represent mean values. *: p<0.05.

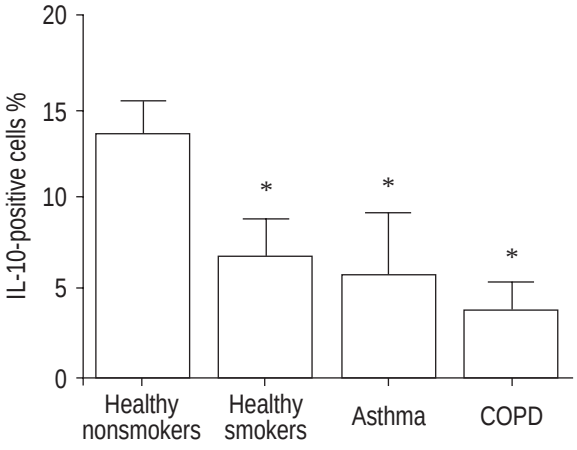

Fig. 2. – Percentages of interleukin-10 (IL-10)-positive cells in sputum, as demonstrated by immunocytochemistry. Data are presented as mean± SEM. COPD: chronic obstructive pulmonary disease. *: p<0.05 *versus* healthy nonsmokers.

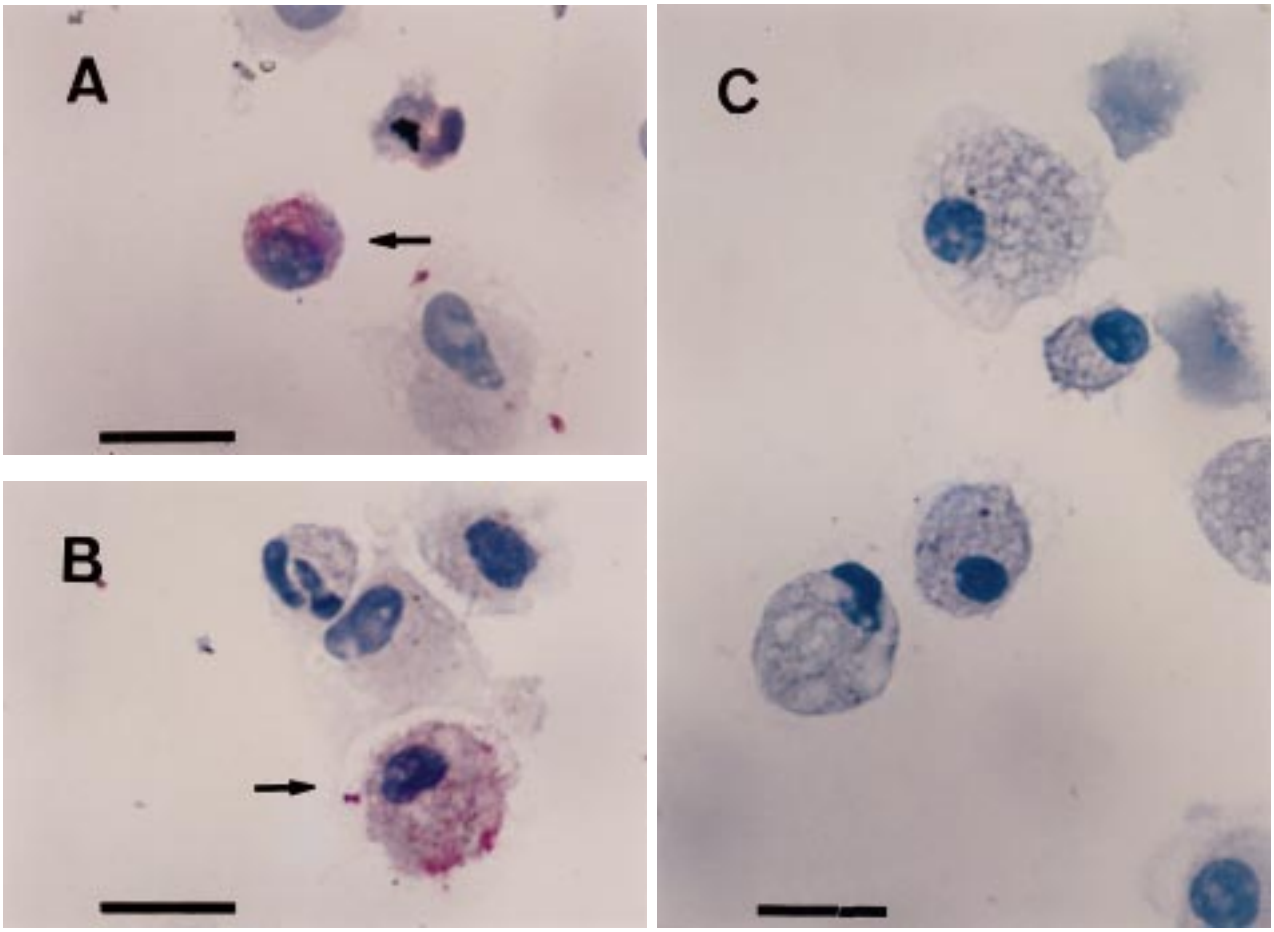

Fig. 3. – Immunocytochemical staining of sputum obtained from a healthy nonsmoker. Cytospins were stained with an antihuman interleukin-10 (IL-10) antibody by means of the alkaline phosphatase/anti-alkaline phosphatase method. a, b) Macrophages contain IL-10 (indicated by arrows). c) Control. (Internal scale bars=25 μm.)

Changes in interleukin-10 levels after inhaled steroid therapy

In five asthmatic patients, 800 μg beclomethasone·day^{-1} (400 μg, twice daily) was inhaled for 1 month, and the sputum induction repeated. Mean peak flow increased from 71.9±2.8 to 83.8±4.9% pred (p=0.03). In all cases, the percentage of eosinophils in sputum decreased from 23.3±6.4 to 4.8±1.9% (p=0.03) after inhaled beclomethasone. However, neither the levels of IL-10 nor the percentages of IL-10-positive cells in the sputum changed (fig. 4).

Discussion

In this study, a significant reduction in IL-10 levels and a small number of IL-10-expressing cells in the sputum obtained from patients with asthma and COPD and smokers was demonstrated compared with those found in the sputum of healthy nonsmokers. To the best knowledge of the authors′, this is the first report describing the concentration and localization of IL-10 in sputum. It has been demonstrated that induced sputum samples are more concentrated and richer in airway secretions than BAL samples [14]. The analysis of induced sputum revealed similar findings to those of the bronchial wash. Therefore, induced sputum analysis represents conditions involving airway inflammation well.

IL-10 was initially described as a Th2-derived factor inhibiting cytokine synthesis in murine Th1 T-cell clones [1]. It is now appreciated that human IL-10 is produced by both Th1 and Th2 lymphocytes, although monocytes and tissue macrophages are important major sources of IL-10 [15]. It is an anti-inflammatory cytokine with major downregulatory effects on inflammation. This molecule has been shown to inhibit the release of cytokines from monocytes, lymphocytes, neutrophils and eosinophils [7–9, 16].

It has been reported that numbers of IL-10 mRNA-expressing cells in BAL fluid from subjects with asthma are significantly increased compared with those of healthy control subjects [5]. However, protein levels were not measured. In contrast, another human BAL study showed decreased IL-10 mRNA and IL-10 in BAL fluid from asthmatic patients compared with that from healthy subjects [6], consistent with the present results. Further, a relatively low level of IL-10 and low number of IL-10-expressing cells was demonstrated in sputum from COPD patients. A recent study showed that IL-10 is constitutively produced from normal bronchial epithelial cells and is downregulated in cystic fibrosis [11]. Taken

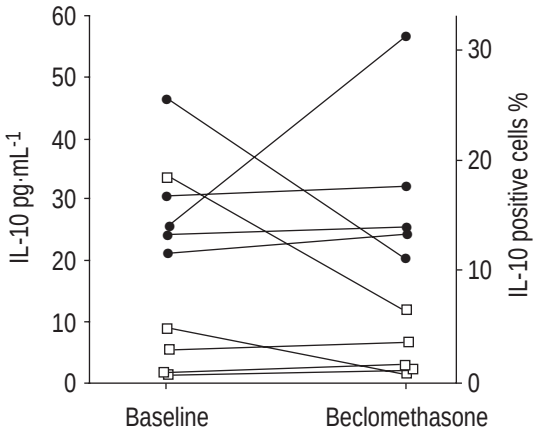

Fig. 4. – Changes in interleukin-10 (IL-10) levels (●) and percentages of IL-10-positive cells (□) after therapy with inhaled beclomethasone in asthmatic patients.

together, decreased IL-10 levels in the airway may be one of the important pathogenetic factors in chronic airway inflammation. Interestingly, a decreased level of IL-10 was found in healthy smokers. Smoking is widely recognized as a primary risk factor for COPD. In this study, the smokers were moderate smokers and no difference could be found in total cell numbers and cell differentials in sputum between smokers and healthy nonsmokers. Smoking affects the immune-system in the lung. For example, smokers have significantly increased cell counts with proportionately more macrophages and neutrophils and an alteration in T cell subsets in BAL fluid compared with nonsmokers [17]. These data indicate that a decreased level of IL-10 might be one of the factors which cause COPD. Thus, decreased IL-10 levels in the airway may be a nonspecific feature of chronic airway inflammation.

Another explanation for the decreased IL-10 levels in the sputum might be increased clearance from airways. The present preliminary study showed that co-incubation of recombinant human IL-10 with sputum obtained from asthmatic patients for 1 h did not affect the level of IL-10. This suggests that clearance mechanisms such as proteases and IL-10 receptors were not responsible for the decreased level of IL-10.

Before measuring IL-10 levels in sputum, the effect of dithiothreitol was examined. Recombinant human IL-10 was submitted to the same procedure as used in sputum handling, and it was found that the level of IL-10 was not changed after exposure to dithiothreitol. Another source of IL-10 such as salivary contamination may be responsible for the increased level of IL-10 in the sputum of healthy nonsmokers, but IL-10 was not detected in saliva from healthy nonsmokers and smokers. Therefore, the sputum handling procedure *per se* did not affect the levels of IL-10 in the sputum.

IL-10 localized to the macrophages in the sputum, especially in healthy nonsmokers. In sputum from asthma and COPD patients, the percentage of macrophages was lower; however, the total cell number in sputum was greater. Therefore, the number of macrophages was not significantly different between the four groups. Therefore the lower percentage of macrophages was not responsible for the low levels of IL-10 in the sputum in asthma and COPD.

Macrophages are widely recognized as cells that play an important role in the regulation of immune and inflammatory activities. Alveolar macrophages are major sources of IL-10, and alveolar macrophages from asthmatic subjects release less IL-10 *in vitro* than those from healthy subjects. Similarly, the peripheral blood mononuclear cells in the patients with asthma were demonstrated to have reduced spontaneous IL-10 production compared with those in normal subjects [6]. Macrophages usually elaborate powerful suppressive signals to limit the proliferative potential of T cells, thus maintaining local immunological homeostasis [18]. A reduction in the capacity of macrophages to produce IL-10 may influence the progression of airway inflammation.

The present data suggest that inhaled corticosteroid modified neither the level of IL-10 nor the number of IL-10-positive cells in sputum asthmatic patients, although the number of patients was small. A recent report showed that, in alveolar macrophages from asthmatic patients but not blood monocytes, the ability to produce IL-10 is increased after treatment with inhaled corticosteroid [6]. In contrast, methylprednisolone inhibits the lipopolysaccharide-stimulated production of IL-10 *in vitro* by peripheral blood mononuclear cells obtained from normal subjects [19]. Interestingly, theophylline also induces IL-10 production from mononuclear cells *in vitro* [20]. It is suggested that these drugs mitigate chronic inflammation through induction of IL-10, thus contributing to their clinical efficacy. Further *in vivo* studies are required to clarify the effect of steroids on IL-10 production in the airway.

In summary, the present data suggest that reduced levels of anti-inflammatory cytokines such as interleukin-10 in the airways may contribute to the development of chronic airway inflammation including asthma and chronic obstructive pulmonary disease, and that macrophages may play an important role in this mechanism.

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