Published in: Clinical & Experimental Immunology (2006), vol. 143, iss. 1, pp. 161-166
Status: Postprint (Author's version)

Cytokine production from sputum cells in eosinophilic *versus* non-eosinophilic asthmatics

V. Quaedvlieg, M. Henket, J. Sele and R. Louis

Department of Pneumology, CHU Sart-Tilman Liege, University of Liege, Center for Biomedical Integrative Genoproteomics (CBIG), Belgium

Abstract

The inflammatory pathways involved in asthma are more complex than the sole Th2-mediated eosinophilic airway inflammation. Different phenotypes of asthma have been recently highlighted and are probably underlied by different immunological profiles. The aim of the study was to assess cytokine production from sputum cells in eosinophilic *versus* non-eosinophilic asthmatics. Induced sputum was obtained from 48 consecutive stable mild to moderate asthmatics (20 eosinophilic asthmatics, 28 non-eosinophilic asthmatics) and 31 healthy subjects. Cytokine released from sputum cells were measured by a home-made two-step sandwich immunoassay. Cytokines investigated were interleukin (IL)-4, IL-6, IL-10, tumour necrosis factor (TNF)-α and interferon (IFN)-γ. Sputum cells from eosinophilic asthmatics produced more IL-4 than those from both healthy subjects (P<0.05) and non-eosinophilic asthmatics (P < 0.05). Conversely, sputum cells from eosinophilic asthma were found to release lower amounts of TNF-α than those from healthy subjects (P < 0.05). The group of non-eosinophilic asthmatics did not distinguish from healthy subjects with respect to any cytokines measured. Sputum cells from asthmatics exhibiting eosinophilic airway inflammation release more IL-4 and less TNF-α than those of healthy subjects. By contrast, non-eosinophilic asthmatics did not distinguish from healthy subjects by abnormal cytokine release from their sputum cells.

Keywords: asthma, cytokines, eosinophils, phenotypes, sputum cells

INTRODUCTION

Emphasis has been recently placed on the heterogeneity of airways inflammation in asthma [1]. Although airways eosinophilia governed by Th2 cytokines is regarded as a prominent feature of asthma [2], it has become clear that a substantial part of asthmatics may not exhibit raised number of eosinophils in airways lumen [3] or bronchial walls [4]. The distinction between eosinophilic and non-eosinophilic asthma may not be purely academic as it might be relevant to the response to inhaled corticoids [5] and the propensity to develop asthma exacerbations [6].

Immunological studies comparing eosinophilic *versus* non-eosinophilic asthma are still sparse and potential immune mechanisms involved in non-eosinophilic asthma still largely unknown. Induced sputum was shown to be a useful technique to sample airways cells. Thanks to its relative non-invasiveness and good cell count reproducibility, induced sputum has allowed the definition of normal cell counts derived from large samples of healthy subjects [7,8]. From these studies and our own data it appeared that a sputum percentage of eosinophils greater than 2.2% could be considered as abnormal.

We have demonstrated previously that sputum cells spontaneously release cytokines when cultured *ex vivo* and that this release was modulated by allergen exposure in sensitized subjects [9]. In order to delineate the immunological basis of asthmatic phenotypes, we have compared the cytokine production from sputum cells in eosinophilic *versus* non-eosinophilic stable asthmatics. Interleukin (IL)-4 and interferon (IFN)-γ were chosen as markers of Th2/Th1 balance [10], tumour necrosis factor (TNF)-α and IL-10 as pro- and anti-inflammatory cytokines, respectively [11,12] and IL-6 as a cytokine released in inflammatory state that may stimulate humoral immunity [13].

MATERIALS AND METHODS

Study design and subject characteristics

We recruited 48 consecutive asthmatics from our asthma clinic whose demographic, functional and treatment characteristics are given in Table 1. Asthma was defined by the presence of recurrent symptoms of breathlessness, cough or wheezing associated with bronchial hyperresponsiveness. The demonstration of bronchial hyperresponsiveness was based on either a positive methacholine challenge when baseline forced expiratory volume in 1 s (FEV_1) was greater than 70% predicted or a reversibility of FEV_1 greater than 12% after 400 µg inhaled salbutamol when baseline FEV_1 was lower than 80% predicted. A positive methacholine challenge was defined as a provocative concentration of methacholine causing a fall in FEV_1 of 20% from baseline (PC20M) ≤ than 16 mg/ml. Methacholine challenges were performed according to a slightly modified Cockroft's method, as described previously [14]. Atopy was defined as a positive skin-prick test reaction (wheal ≥ 3 mm compared to control) to common aeroallergens of our area (house dust mites, cat and dog dander, grass, tree and weed pollens, moulds). All asthmatics were in stable condition at the time sputum induction in that none were in a situation to require systemic corticoids or to need transfer to an emergency department. The study was approved by our local ethical committee and each subjects gave its oral informed consent.

Sputum induction and processing

After premedication of the subjects with a 400 µg inhaled salbutamol (pMDI + spacer), sputum was induced by inhalation of an hypertonic saline (NaCl 4.5%) combined with additional salbutamol [15] delivered by an ultrasonic nebulizer (Ultra-Neb 2000, De Vilbiss) with an output set at 0.9 ml/min. Each subject inhaled the aerosol for three consecutive periods of 5 min for a total time of 15 min. For safety reasons, FEV_1 was monitored every 5 min and the induction stopped when FEV_1 fell by more than 20% from post-bronchodilation value.

The whole sputum was collected in a plastic container, weighed and homogenized by adding three volumes of phosphate-buffered saline (PBS), vortexed for 30 s and centrifuged at 800 g for 10 min at 4°C. Supernatant was separated from cell pellet which was resuspended in PBS without Ca^{2+} and Mg^{2+} and used to perform squamous and total cell counts with a manual haemocytometer. Cell viability was checked by trypan blue exclusion. The differential performed on cytospins stained with Diff-Quick. According to our own laboratory reference values obtained on a large sample of healthy non-atopic subjects (n = 62, mean age 34) sputum eosinophil counts greater than 2.2% (90th percentile) and neutrophil counts greater than 57.6% (90th percentile) were considered as abnormally high.

Sputum cell culture and cytokine assays

Cytokines (IL-4, IL-6, IL-10, TNF-α, IFN-γ) were measured by a two-step sandwich type immunoassay. The antibodies and standards were purchased from Biosource (Cytosets, Biosource, Fleurus, Belgium). Fifty µl from standards or sputum cell suspension (2×10^6 non-squamous cells) was incubated at 37°C with 200 µl RPMI-1640 supplemented with 100 U penicillin/ml and 100 µg streptomycin/ml (Cambrex, Verviers, Belgium) and 2% of inactivated fetal calf serum (Cambrex) in apyrogen microwells (Nunc Maxisorp, VWR, Belgium), which were coated previously with specific antibodies directed towards the chosen cytokines. After 24 h cell culture viability was checked by trypan blue and was usually > 80%. The wells were then washed and 150 µl of a solution containing biotinylated detection antibodies (0.23 µg/ml) specific to the cytokines was added for 2 h at room temperature. Then, wells were washed again and filled with a solution containing streptavidin horseradish peroxidase (HRP) for 45 min at room temperature. After washing, 100 µl tetramethylbenzidine (TMB) chromogen solution was added as a chromogen solution for 20 min in the dark. The reaction was stopped by adding 50 µl H_2SO_4 1 M. The amount of substrate converted to products was thereafter detected as optical densities (OD) at 450 nm in an enzyme-linked immunosorbent assay (ELISA) reader (Multiscan Ascent, Thermo Labsystems, Helsinki, Finland). The sensitivities of our assays were 6 pg/ ml for IL-4, 6 pg/ml for IL-6, 4 pg/ml for IL-10, 6 pg/ml for TNF-α and 7 pg/ml for IFN-γ.

Statistical analyses

Results were expressed as median (range). Comparisons between the three groups were performed by Kruskall-Wallis test followed, when significance arose, by Dunn's test for pairwise comparisons. Correlations were tested by using Spearman's coefficient of correlation. *P*-values less than 0.05 were considered as statistically significant.

Published in: Clinical & Experimental Immunology (2006), vol. 143, iss. 1, pp. 161-166
Status: Postprint (Author's version)

Table 1. *Subject characteristics.*

	Eosinophilic asthma *n* = 20	Non-eosinophilic asthma *n* = 28	Healthy subjects *n* = 31
Age (years)	43.2 ± 16.8	39.7 ± 17.8	39.8 ± 11.7
Sex (M,F)	11/9	13/12	22/9
Skin-prick +	13	18	0
FEV_1 (L)	3.225 ± 1.117	3.098 ± 0.966	4.066 ± 0.949
FEV_1 (%)	87.4 ± 20.9	90.2 ± 15.5	110.8 ± 17
FVC (L)	4.093 ± 1.517	3.744 ± 1.306	4.983 ± 1.208
FVC (%)	93 ± 24.9	94.6 ± 16.8	110 ± 18
FEV_1/FVC	73.3 ± 9.3	82.4 ± 16.6	85.8 ± 13.7
PC20M (mg/ml)	2.02 (0.37-14)	1.72 (0.35-12)	> 16
Smoking habits	13 NS/3 ES/4 S	15 NS/6 ES/7 S	19 NS/5 ES/7S
IgE (KU/ml)	736 (64-1044)	478 (16.4-1020)	29 (5-90)
Treatment:			
No ICS	11	13	31
ICS	9	15	0

Results are expressed as mean ± s.d. except PC20M which is expressed as geometric mean (range). NS: non smoker, ES: ex smoker, CS: current smoker, ICS: inhaled corticosteroids.

RESULTS

Sputum cell counts in eosinophilic *versus* non-eosinophilic asthma are given in Table 2. The two groups of asthmatics did not differentiate between each other or with healthy subjects with respect to any cell type except eosinophil count (Table 2). In particular, the number of patients exhibiting an abnormally high neutrophil count (> 58%) was not different in eosinophilic (five of 20; 25%) and in non-eosinophilic asthmatics (eight of 28; 28%).

The results regarding the cytokines released from sputum cell culture are given in Table 3. Eosinophilic asthmatics displayed a raised production of IL-4 when compared to both non-eosinophilic asthmatics and healthy subjects (Fig. 1). By contrast, the release of TNF-α was lower in eosinophilic asthmatics when compared to healthy subjects (Fig. 2). There was no difference between the groups regarding IL-6, IL-10 and IFN-γ production (Fig. 3).

With regard to the amount of TNF-α produced [2703 pg/ ml (129-4243) *versus* 2232 pg/ml (0-6771), respectively, $P > 0.05$], those subjects who released IL-4 were not different from the non-IL-4 releasers.

When analysing the data according to the atopic status, no difference in cytokine production could be detected between atopic and non-atopic asthmatics ($P > 0.05$ for each cytokine). In the same line, total serum IgE levels failed to relate significantly to the production of any cytokine considered ($P > 0.05$ for each cytokine).

In each group of asthmatics (eosinophilic and non-eosinophilic), those taking regular inhaled steroids as maintenance treatment did not differ from their steroid naive counterparts with respect to any cytokine considered ($P > 0.05$).

Table 2. *Sputum cell count.*

	Eosinophilic asthmatics	Non-eosinophilic asthmatics	Healthy subjects
Total cells			
$\times 10^6$/ml	0.75 (0.12-0.5)	0.7 (0.01-22.6)	0.53 (0.05-2.4)
Cell viability			
%	51 (29-98)	70.5 (37-98)	63 (36-100)
Squamous			
%	17 (0-52)	20.5 (0-55)	18 (0-61)
Macrohages			
%	30.5 (3.2-77)	44.8 (0.8-91.2)	42 (1.6-96)
$\times 10^6$/g	0.18 (0.03-2.48)	0.24 (0.01-2.62)	0.29 (0.01-1.8)
Lymphocytes			
%	1.3 (0-11.4)	0.8 (0-14.4)	2.2 (0-10)
x 10^6/g	0.015 (0-0.57)	0.01 (0-0.34)	0.01 (0-0.24)
Neutrophils			
%	23.2 (0-82.6)	40 (0.8-95.9)	30 (0-87)
$\times 10^6$/g	0.18 (0-2.3)	0.2 (0-21.7)	0.25 (0.01-1.04)
Eosinophils			
%	11 (3.4-94)	0.4 (0-2)	0.2 (0-3.6)
$\times 10^6$/g	0.095 (0.01-3.9)	0.01 (0-0.41)	0.01 (0-0.04)
Epithelial cells			
%	7.8 (0.2-28.2)	4.2 (0.2-61.6)	9.8 (0.4-66.4)
$\times 10^6$/g	0.05 (0-0.53)	0.03 (0-0.26)	0.06 (0-0.35)

Results are expressed as median (range).

Table 3*. Cytokine production from sputum cells.*

Cytokine (pg/ml)	Eosinophilic asthmatics	Non-eosinophilic asthmatics	Healthy subjects
IL-4	1.6 (0-1183)*	0 (0-51)	0 (0-0.25)
IL-6	35 (0-1370)	21 (0-1281)	29.2 (0-807)
IL-10	37 (12-870)	26.4 (0-2000)	43 (1-253)
IFN-γ	0 (0-1000)	0 (0-94)	0 (0-3420)
TNF-α	1139 (130-4000)†	2107 (0-6771)	2290 (140-389)

Results are expressed as median (range). *$P < 0.01$ *versus* non-eosinophilic asthmatics and *versus* healthy subjects. $^\dagger P < 0.05$ *versus* healthy subjects. IL: interleukin; IFN: interferon; TNF: tumour necrosis factor.

Published in: Clinical & Experimental Immunology (2006), vol. 143, iss. 1, pp. 161-166
Status: Postprint (Author's version)

***Fig. 1**. Sputum cell production of interleukin (IL)-4 in non-eosinophilic asthmatics (filled square), eosinophilic asthmatics (open triangle) and healthy subjects (filled circle).*

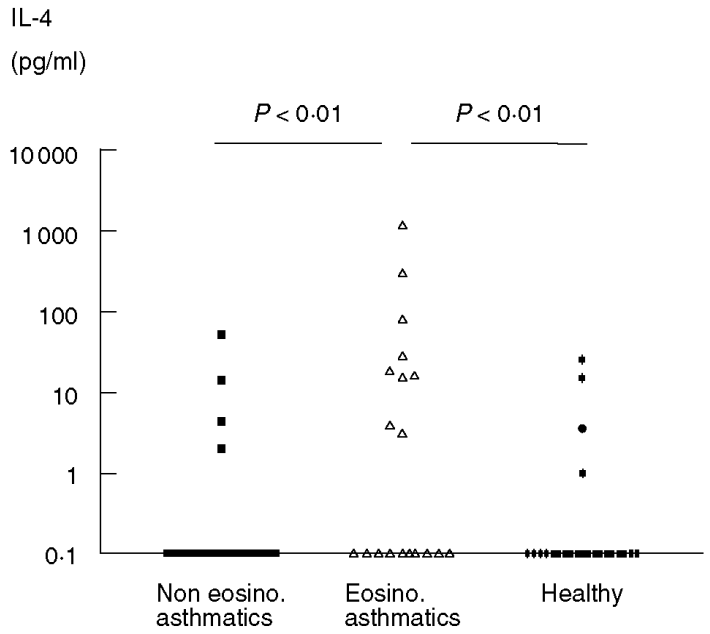
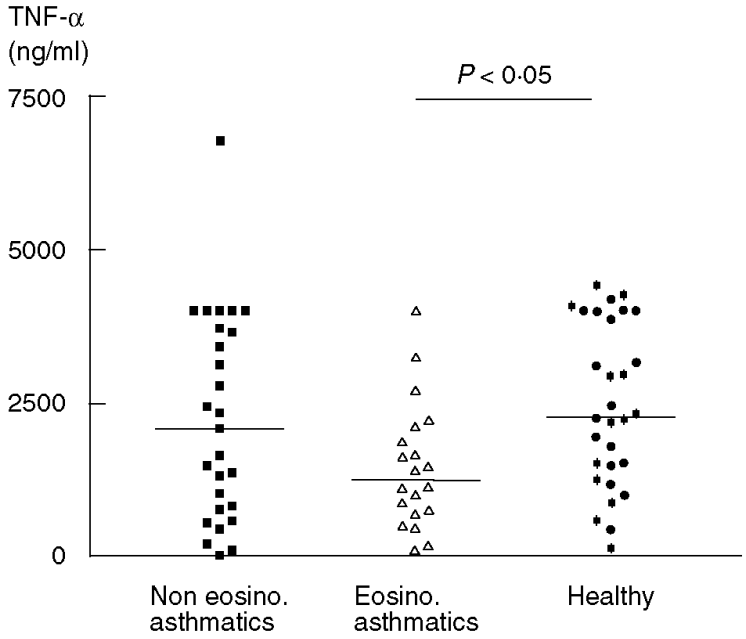
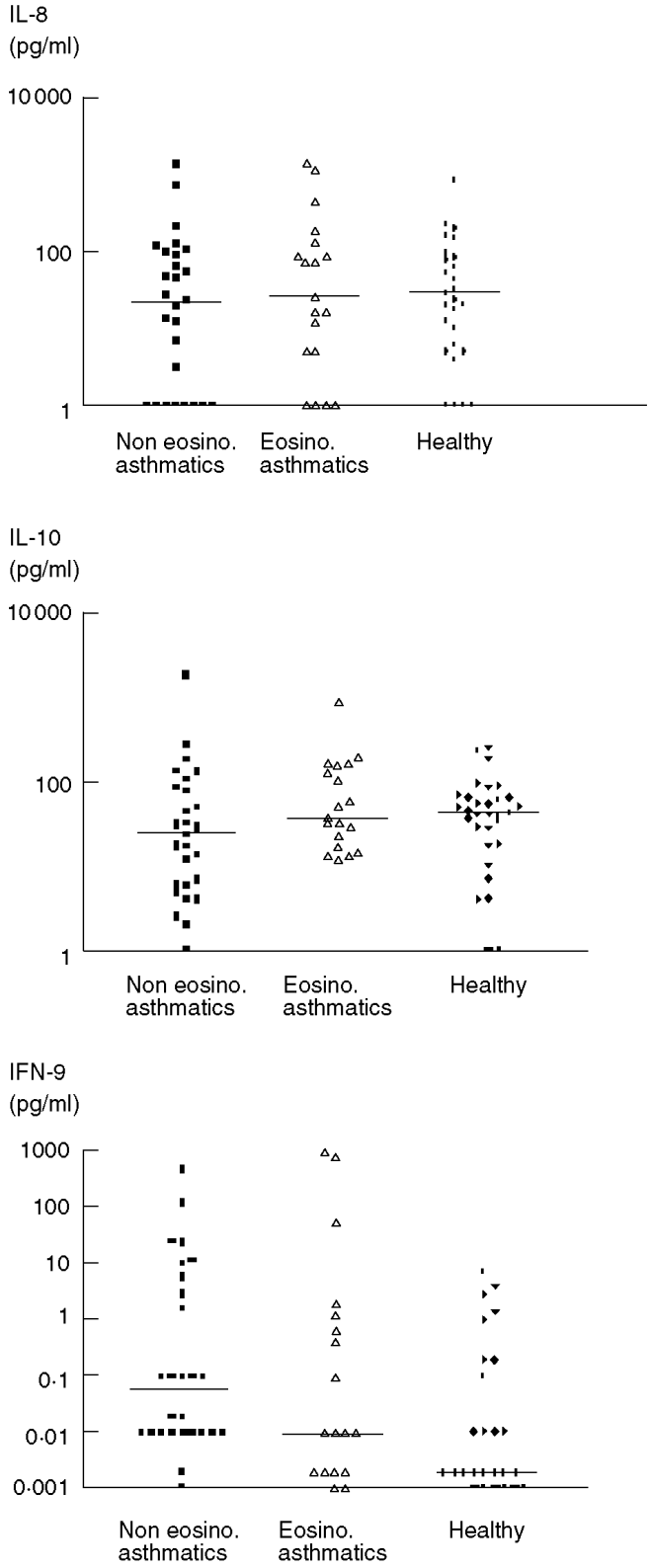


***Fig. 2.** Sputum cell production of tumour necrosis factor (TNF)-α in non-eosinophilic asthmatics (filled square), eosinophilic asthmatics (open triangle) and healthy subjects (filled circle).*



Published in: Clinical & Experimental Immunology (2006), vol. 143, iss. 1, pp. 161-166
Status: Postprint (Author's version)

Fig. 3. *Sputum cell production of interleukin (IL)-6 (upper panel), IL-10 (middle panel) and interferon (IFN)-γ (lower panel) in non-eosinophilic asthmatics (filled square), eosinophilic asthmatics (open triangle) and healthy subjects (filled circle).*



Published in: Clinical & Experimental Immunology (2006), vol. 143, iss. 1, pp. 161-166
Status: Postprint (Author's version)

DISCUSSION

Our study shows that sputum cells from eosinophilic asthmatics produced more IL-4 than those from both healthy subjects and non-eosinophilic asthmatics. Conversely, sputum cells from eosinophilic asthma were found to release lower amounts of TNF-α than those from healthy subjects. The group of non-eosinophilic asthmatics did not distinguish from healthy subjects with respect to any cytokines measured. Our finding of a raised production of IL-4 in eosinophilic asthmatics is in keeping with the observation that a prominent Th2 cytokine profile is associated with eosinophilic inflammation in asthmatic airways [16]. One way through which local release of IL-4 into the airways may favour the recruitment of eosinophils is the induction of eotaxin release from epithelial cells [17]. As we have used the global sputum cell population, we can only speculate about the cell type involved in cytokine production. The main source of IL-4 in asthma has been thought to be CD4 T lymphocytes [18]. Although found to be increased in sputum from asthmatics, CD4 lymphocytes represent only a minority of cells recovered from sputum [19]. One alternative explanation for our observation might be the fact that eosinophils themselves produce most of the IL-4 measured from the cell culture. If that were true, high local IL-4 production would be then considered as a key event in the perpetuation of local eosinophilic inflammation. Supporting this view are the facts that eosinophils from peripheral human blood were shown to synthetize and release IL-4 [20] and that IL-4 was found to be localized within eosinophils contained in the bronchial mucosa of asthmatics [21].

So far, IL-4 has been considered mainly as a pivotal cytokine to drive IgE production from B cells [22]. Interestingly, in our study the raised IL-4 production did not relate to atopic status nor to total serum IgE levels in asthmatics. The rise in IL-4 production in non-atopic asthmatics fits with data reported by Humbert *et al.* [23]. Of course, it could be argued that the production of IL-4 in the airways is mainly relevant to the local production of IgE, which is not necessarily reflected by serum levels [24].

One surprising observation of our study is the lower TNF-α production in the group of eosinophilic asthma. Macrophages certainly represent an important source of TNF-α production in the airways [11]. However, although slightly reduced in eosinophilic asthma, the percentage of macrophages did not differ significantly between the groups. Therefore it is unlikely that the low TNF-α release can be accounted for only by the sole reduced proportion of macrophages in the cell culture. An additional explanation might be that high local release of IL-4 reduces the production of TNF-α from macrophages [25]. However, the fact that those who produced IL-4, as opposed to the non-producers, failed to show reduced production of TNF-α indicates that the mechanisms regulating TNF-α production are more complex and depend on other factors than IL-4. Monoclonal antibodies towards TNF-α have been developed recently and proved to be efficient in rheumatoid arthritis [26] and Crohn's disease [27] and some trials are currently ongoing in asthma. Our results suggest that a group of mild to moderate eosinophilic asthma might not be the best target for using anti-TNF-α in asthma.

Somewhat unexpectedly, we did not find increased IL-6 production in asthma. IL-6 is considered to be a non-specific inflammatory cytokine as it is released in several types of inflammation. Circulating levels of this cytokine were shown to rise during an asthma exacerbation [28] and secretion of IL-6 from blood leucocytes in response to lipopolysaccharide were found to be increased in both atopic and no-atopic asthma when compared to healthy subjects [29]. From our data, IL-6, in contrast to IL-4, did not appear to be related closely to eosinophilic inflammation.

Recent interest has emerged for IL-10, a cytokine produced by T regulator lymphocyte and thought to curb inflammation and to protect against diseases such as asthma [12]. Sputum levels of IL-10 were reported to be lower in asthma when compared to healthy subjects [30]. No significant difference regarding IL-10 could be observed between the different groups in our study, even when the levels of IL-10 were slightly lower in asthmatics compared to healthy subjects. As far IFN-γ is concerned, the cytokine was poorly detectable in our model and not different between the groups.

Although sharing similar clinical characteristics with its eosinophilic counterpart, the group of non-eosinophilic asthmatic patients did not distinguish from that of healthy subjects regarding cytokine production, nor with regard to sputum cell counts. In particular, in our series only eight of 28 patients had abnormally high sputum neutrophil counts (> 58% according to our laboratory reference values in healthy subjects). This indicates that, in the majority of non-eosinophilic patients, mild to moderate asthma cannot be considered as neutrophilic. Therefore our data emphasize that the clinical asthma phenotype defined by symptoms and bronchial hyperresponsiveness may exist without marked airway leucocyte infiltration and disturbed cytokine release from cells contained in airway secretions. Recent data have highlighted the importance of remodelling of extracellular matrix and airway smooth muscle in determining the asthmatic phenotype [31]. In the absence of a good soluble

biochemical marker, these aspects of asthma may not be identified by the analysis of sputum itself.

We conclude that eosinophilic asthmatics exhibit an overproduction of IL-4 together with a reduced production of TNF-α from their sputum cells when compared to healthy subjects. In contrast, their non-eosinophilic counterparts did not differentiate from healthy subjects with respect to sputum cell counts and *ex vivo* cytokine release.

References

1 O'Donnell RA, Frew AJ. Is there more than one inflammatory phenotype in asthma? Thorax 2002; 57:566-8.

2 Robinson DS, Hamid Q, Ying S *et al.* Predominant TH2-like bron-choalveolar T-lymphocyte population in atopic asthma. N Engl J Med 1992; 326:298-304.

3 Douwes I, Gibson P, Pekkanen I, Pearce N. Non-eosinophilic asthma: importance and possible mechanisms. Thorax 2002; 57:643-8.

4 Wenzel SE, Schwartz LB, Langmack EL *et al.* Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. Am J Respir Crit Care Med 1999; 160:1001-8.

5 Pavord ID, Brightling CE, Woltmann G, Wardlaw AI. Non-eosinophilic corticosteroid unresponsive asthma. Lancet 1999; 353:2213-4.

6 Green RH, Brightling CE, McKenna S *et al.* Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. Lancet 2002; 360:1715-21.

7 Belda J, Leigh R, Parameswaran K, O'Byrne PM, Sears MR, Har-greave FE. Induced sputum cell counts in healthy adults. Am J Respir Crit Care Med 2000; 161:475-8.

8 Spanevello A, Confalonieri M, Sulotto F *et al.* Induced sputum cel-lularity. Reference values and distribution in normal volunteers. Am J Respir Crit Care Med 2000; 162:1172-4.

9 Bettiol I, Sele I, Henket M *et al.* Cytokine production from sputum cells after allergenic challenge in IgE-mediated asthma. Allergy 2002; 57:1145-50.

10 Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today 1996; 17:138-46.

11 Thomas PS. Tumour necrosis factor-alpha: the role of this multifunctional cytokine in asthma. Immunol Cell Biol 2001; 79:132-40.

12 Koulis A, Robinson DS. The anti-inflammatory effects of interleukin-10 in allergic disease. Clin Exp Allergy 2000; 30:747-50.

13 Kishimoto T, Akira S, Narazaki M, Taga T. Interleukin-6 family of cytokines and gp130. Blood 1995; 86:1243-54.

14 Louis R, Leyder E, Malaise M, Bartsch P, Louis E. Lack of association between adult asthma and the tumour necrosis factor alpha-308 polymorphism gene. Eur Respir J 2000; 16:604-8.

15 Delvaux M, Henket M, Lau L *et al.* Nebulised salbutamol administered during sputum induction improves bronchoprotection in patients with asthma. Thorax 2004; 59:111-5.

16 Kay AB. TH2-type cytokines in asthma. Ann NY Acad Sci 1996; 796:1-8.

17 Fujisawa T, Kato Y, Atsuta I *et al.* Chemokine production by the BEAS-2B human bronchial epithelial cells: differential regulation of eotaxin, IL-8, and RANTES by TH2- and TH1-derived cytokines. J Allergy Clin Immunol 2000; 105:126-33.

18 Ricci M, Rossi O, Bertoni M, Matucci A. The importance of Th2-like cells in the pathogenesis of airway allergic inflammation. Clin Exp Allergy 1993; 23:360-9.

19 Louis R, Shute I, Biagi S *et al.* Cell infiltration, ICAM-1 expression, and eosinophil chemotactic activity in asthmatic sputum. Am J Respir Crit Care Med 1997; 155:466-72.

20 Moqbel R, Ying S, Barkans I *et al.* Identification of messenger RNA for IL-4 in human eosinophils with granule localization and release of the translated product. J Immunol 1995; 155:4939-47.

21 Moller GM, de Jong TA, van der Kwast TH *et al.* Immun-olocalization of interleukin-4 in eosinophils in the bronchial mucosa of atopic asthmatics. Am J Respir Cell Mol Biol 1996; 14:439-43.

22 Ryan JJ. Interleukin-4 and its receptor: essential mediators of the allergic response. J Allergy Clin Immunol 1997; 99:1-5.

23 Humbert M, Durham SR, Ying S *et al.* IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and non-atopic asthma: evidence against 'intrinsic' asthma being a distinct immunopathologic entity. Am J Respir Crit Care Med 1996; 154:1497-504.

24 Ying S, Humbert M, Meng Q *et al.* Local expression of epsilon germline gene transcripts and RNA for the epsilon heavy chain of IgE in the bronchial mucosa in atopic and nonatopic asthma. J Allergy Clin Immunol 2001; 107:686-92.

25 Sone S, Yanagawa H, Nishioka Y *et al.* Interleukin-4 as a potent down-regulator for human alveolar macrophages capable of producing tumour necrosis factor-alpha and interleukin-1. Eur Respir J 1992; 5:174-81.

26 Maini RN, Elliott MJ, Brennan FM, Feldmann M. Beneficial effects of tumour necrosis factor-alpha (TNF-alpha) blockade in rheumatoid arthritis (RA). Clin Exp Immunol 1995; 101:207-12.

27 Targan SR, Hanauer SB, van Deventer SI *et al.* A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. N Engl J Med 1997; 337: 1029-35.

28 Yokoyama A, Kohno N, Fujino S *et al.* Circulating interleukin-6 levels in patients with bronchial asthma. Am J Respir Crit Care Med 1995; 151:1354-8.

29 Bettiol I, Bartsch P, Louis R *et al.* Cytokine production from peripheral whole blood in atopic and nonatopic asthmatics, relationship with blood and sputum eosinophilia and serum IgE levels. Allergy 2000; 55:1134-41.

30 Takanashi S, Hasegawa Y, Kanehira Y *et al.* Interleukin-10 level in sputum is reduced in bronchial asthma, COPD and in smokers. Eur Respir J 1999; 14:309-14.

31 Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodeling. Am J Respir Crit Care Med 2000; 161:1720-45.