

ORIGINAL PAPER

High serum levels of tumour necrosis factor- α and interleukin-8 in severe asthma: markers of systemic inflammation?

M. Silvestri*, M. Bontempelli†, M. Giacomelli†, M. Malerba‡, G. A. Rossi*, A. Di Stefano§, A. Rossi¶ and F. L. M. Ricciardolo*

*Pulmonary Disease Unit, G. Gaslini Institute, Genoa, Italy, †Department of Immunology, Ospedali Riuniti, Bergamo, Italy, ‡Department of Internal Medicine, University of Brescia, Brescia, Italy, §Division of Pulmonary Disease, Salvatore Maugeri Foundation, IRCCS Medical Center of Rehabilitation, Veruno-Novara, Italy and

¶Department of Respiratory Disease, Ospedali Riuniti, Bergamo, Italy

Clinical and Experimental Allergy

Summary

Background Severe asthma is characterized by elevated levels of pro-inflammatory cytokines and neutrophilic inflammation in the airways. Blood cytokines, markers of 'systemic' inflammation, may be a feature of amplified inflammation in severe asthma.

Objective To detect differences in IL-8, TNF- α , IL-16 and IL-13 levels in the serum(s) of stable severe and mild-moderate asthmatics related to blood leucocytes proportion, airway calibre and exhaled nitric oxide (NO) levels.

Methods We assessed cytokine serum levels by ELISA and blood leucocyte counts by an alkaline peroxidase method in 20 healthy controls, 22 mild-moderate [forced expiratory volume in 1 s (FEV₁)_(%pred): 89 $\pm$ 3] and 14 severe asthmatics [FEV₁(%pred): 49 $\pm$ 2].

Results IL-8 and TNF- α levels were higher in severe asthmatics than in mild-moderate asthmatics or in controls ($P < 0.05$). No differences in IL-16 and IL-13 levels were detected. Severe asthmatics showed higher circulating neutrophil and eosinophil number than controls ($P < 0.05$). In severe asthmatics, exhaled NO levels were superior than in controls ($P < 0.05$), but inferior than in mild-moderate asthmatics ($P < 0.05$). We found positive correlation between TNF- α levels and exhaled NO ($r = 0.67$; $P = 0.01$) or circulating neutrophil counts ($r = 0.57$; $P = 0.03$) in severe asthmatics.

Conclusion sTNF- α and sIL-8 are markers of 'systemic' inflammation in severe asthmatics, in conjunction with augmented circulating neutrophils, suggesting the involvement of neutrophil-derived cytokine pattern in severe asthma.

Keywords asthma severity, IL-8, nitric oxide, TNF- α

Submitted 14 July 2005; revised 6 December 2005; accepted 14 March 2006

Correspondence:

Fabio L.M. Ricciardolo, Pulmonary Department, G. Gaslini Institute, Largo G. Gaslini 5, 16147 Genoa, Italy.
E-mail: fabioricciardolo@ospedale-gaslini.ge.it

Introduction

Asthma is a chronic inflammatory disorder characterized by the recruitment of various inflammatory cells and by the release of different cytokines [1]. A recent evidence showed that severe asthma is represented by different phenotypical expressions in the airways: one characterized by a 'classic eosinophilic process' and the other one by a 'pathologically unclassic process' associated with neutrophils [2]. Cytokines/chemokines promote immune cell recruitment and activation playing an integral role in the coordination and persistence of the airway inflammation in asthma [1]. It seems that in severe asthma blood cytokines, markers of 'systemic' inflammation may have a role for the amplification of the disease state. Different cytokines/

chemokines such as IL-8, TNF- α , IL-16 and IL-13 seem to have a role in worsening the severity of asthma.

IL-16 is a cytokine involved in the early accumulation of T cells in the airways after allergen challenge [3] and its release contributes to airway inflammation in allergic asthma. Biopsy studies showed an increased number of IL-16-immunoreactive cells in bronchial mucosa from atopic asthmatic patients 24 h after antigen provocation *in vivo* [4]. No information about the level of IL-16 in the sera of asthmatics has been reported.

The T helper (Th) 2 cytokine IL-13 induces mucus hyper-secretion and airway hyper-responsiveness (AHR), and activates airway stromal cells in releasing chemokines involved in eosinophil recruitment. Overexpression of IL-13 mRNA has been reported in the airway mucosa of atopic

and non-atopic asthmatics [5]. In acute asthma, serum(s) IL-13 levels are elevated compared with control subjects [6].

IL-8, a C-X-C chemokine, is a major chemoattractant for neutrophils [7] and also a potent chemoattractant for eosinophils primed by Th2-derived cytokines [8]. A further evidence for the involvement of IL-8 in asthma comes from a study by Shute *et al.* [9] who demonstrated that increased production of sIL-8 is a marker of asthma as this chemokine was almost undetectable in the serum of controls.

Another cytokine able to promote eosinophil recruitment from blood into inflammatory sites is TNF- α [10]. The latter is considered an amplifier of asthmatic inflammation probably linked to disease severity as it has been shown that the number of TNF- α -positive cells is greater in bronchial biopsies from symptomatic than in those from asymptomatic asthmatics [11]. It has also been observed that sTNF- α level was higher in asthmatic children than in healthy controls [12].

In addition, cytokines are also able to regulate the expression of inducible nitric oxide (NO) synthase (iNOS) isoform [13], which is supposed to be the most important enzymatic source of exhaled NO (FeNO), a non-invasive inflammatory marker, in asthmatics [14].

IL-8, TNF- α , IL-16 and IL-13 are increased in airways of asthmatics, thus we aimed to detect possible differences in the serum levels of these cytokines between the groups of stable severe and mild-moderate asthmatics. We also postulated that serum levels of these cytokines and blood leucocyte counts could correlate with FeNO and airway calibre.

Materials and methods

Study subjects

Three groups of subjects were evaluated (Table 1): subjects with severe asthma ($n = 14$), subjects with mild-moderate

asthma ($n = 22$), and healthy non-atopic control subjects matched for age and gender ($n = 20$). The subjects with mild-moderate or severe asthma were stratified and treated according to the GINA criteria [15]. In the group of severe asthma four of 14 were treated with daily low dose of oral corticosteroid. All the subjects were in stable clinical conditions at least in the 4 weeks preceding the study. Inhaled drugs were withheld at least 12 h before spirometry. None of them had respiratory tract infection in the 2 months preceding evaluation.

We excluded patients with chronic obstructive pulmonary disease (COPD) from the study. All asthmatics were non-smokers and showed bronchial reversibility (forced expiratory volume in 1 s [FEV₁]: >200 mL or >12% from baseline) after inhaled salbutamol (200 μ g). In addition to the 'standard' clinical evaluation for the identification of the severity of asthma [15] and atopy [16], including spirometry and skin prick test to a panel of the most common aeroallergens [16], these patients underwent FeNO levels measurement and, subsequently, the same day, blood sampling. Asthmatics sensitized to pollens were evaluated out of the pollen season.

The study protocol was approved by the local Ethics Board and all subjects gave their written informed consent to participate to the study, which was conducted in accordance with the Helsinki Declaration.

Blood cell count evaluation

TECHNICON H6000 (Technicon Instrument Corporation, Tarrytown, NY, USA), a system that automatically performs cell counts on peripheral blood samples and differentiates between leucocytes by an alkaline peroxidase method was used. Approximately 12 000 leucocytes were counted. Peripheral blood neutrophil, lymphocyte and eosinophil proportions were evaluated as absolute number.

Table 1. Demographic and clinical data of control subjects and asthmatic patients

	Control subjects ($n = 20$)	Asthmatics		
		All ($n = 36$)	Mild-moderate ($n = 22$)	Severe ($n = 14$)
Age (years)	43.40 $\pm$ 14.41	49.22 $\pm$ 14.79	44.50 $\pm$ 14.42*	56.64 $\pm$ 12.49 [†]
Gender (male/total)	7/20	13/36	7/22	6/14
Atopy (yes/total)	0/20	22/36	14/22	8/14
FEV ₁ (%pred)	97.30 $\pm$ 6.75	73.63 $\pm$ 22.74 ^{†††}	89.33 $\pm$ 12.42***	48.97 $\pm$ 8.48 ^{†††}
FeNO (p.p.b.)	11.51 $\pm$ 1.62	31.38 $\pm$ 15.60 ^{†††}	36.70 $\pm$ 12.77***,§§§	23.01 $\pm$ 16.39 [†]
Inhaled corticosteroid dose (BDP equivalence)	–	1000 (500–1500)	500 (250–1000)	1000 (1000–2000)

Definition of atopy: atopy is a personal or familial tendency to produce IgE antibodies in response to low doses of allergens, usually proteins, and to develop typical symptoms such as asthma, rhinoconjunctivitis, or eczematous dermatitis [16]. The atopic subjects were positive to at least one of the aeroallergen tested in the skin prick test that was confirmed by the presence of specific IgE antibody in the blood by radioallergosorbent test. FEV₁, forced expiratory volume in 1 s; p.p.b., parts per billion.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, comparison between mild-moderate and severe asthmatics. [†] $P < .05$, ^{†††} $P < 0.001$, comparison between severe asthmatics and healthy controls; ^{†††} $P < 0.001$, comparison between asthmatics as a whole and healthy controls; §§§ $P < 0.001$, comparison between mild-moderate asthmatics and healthy controls.

Quantification of interleukin-8, tumour necrosis factor- α , interleukin-16 and interleukin-13 levels in serum

Serum levels of IL-8 (Biosource International, Camarillo, CA, USA), TNF- α (Bender MedSystem, Vienna, Austria), IL-16 (Biosource) and IL-13 (Bender MedSystem) were determined by ELISA, according to the manufacturer's instructions and expressed as pg/mL. The detection limits were 0.02, 0.13, 1.5 and 5 pg/mL for IL-8, TNF- α , IL-13 and IL-16. The reproducibility of the assays for the cytokines detected were as follows: CV for IL-8, 2.6%–5.3%; CV for TNF- α , 4.3%–7.9%; CV for IL-13, 2.1%–6.8%; and CV for IL-16, 4.8%–9.2%.

Pulmonary function test

FEV_{1(%)pred} was measured by spirometry (Med Graphics, Pulmonary Function System 1070 series 2; Med Graphics Corporation, St Paul, MN, USA). On each occasion, three forced expiratory manoeuvres were obtained and the best values were retained.

Detection of exhaled nitric oxide levels

A chemiluminescence analyser (NiOX, Aerocrine AB, Solna, Sweden) sensitive to NO concentrations from 0 to 25 000 parts per billion (p.p.b.) was used and the manoeuvre was performed, according to the American Thoracic Society recommendations [17].

Statistical analysis

Statistical evaluation was performed using the statistical software package GraphPad Prism 3.02 (GraphPad Software Inc., San Diego, CA, USA). Age, FEV_{1(%)pred}, FeNO levels and peripheral blood leucocyte counts are reported as mean with standard deviation of the mean in brackets whereas cytokine levels and inhaled corticosteroid dose (as BDP equivalence) are expressed as median with lower and upper quartiles indicated in brackets.

Unpaired *t*-test or Mann–Whitney *U*-test was used to compare differences in healthy controls and in asthmatic

subjects analysed as a whole. Differences among groups were analysed by ANOVA or Kruskal–Wallis test, followed, when significant, by Bonferroni or Dunn's post-test for comparison among groups. Correlations were determined using Spearman's rank correlation coefficient. The level of statistical significance was set at $P < 0.05$.

Results

Demographic characteristics and clinical data of the studied population

The demographic characteristics and the clinical data of the studied population are reported in Tables 1 and 2. Controls and asthmatics as a whole had comparable age ($P = 0.16$). However, severe asthmatics were significantly older than mild–moderate asthmatics ($P < 0.05$). FEV_{1(%)pred} was significantly different in the three groups ($P < 0.0001$). Particularly, FEV_{1(%)pred} was lower in severe asthmatics than in mild–moderate asthmatics ($P < 0.001$) or in healthy controls ($P < 0.001$). No significant difference in FEV_{1(%)pred} was detected between mild–moderate asthmatics and healthy controls.

Statistically significant differences were also reported in FeNO levels among the three groups ($P < 0.0001$). FeNO levels were higher in asthmatics as a whole as compared with healthy controls ($P < 0.0001$) and among asthmatics, FeNO levels were more elevated in mild–moderate asthmatics than either in healthy controls ($P < 0.001$) or in severe asthmatics ($P < 0.01$) (Table 1). In addition, severe asthmatics had higher FeNO levels than healthy controls ($P < 0.05$) (Table 1).

No difference in FeNO levels or in FEV_{1(%)pred} was observed between severe asthmatics treated with oral corticosteroids and severe asthmatics not receiving oral corticosteroids.

Blood leucocyte counts in healthy controls and in mild–moderate and severe asthmatics

Differences in total white blood cell counts and particularly in neutrophil, lymphocyte and eosinophil counts

Table 2. Blood leucocyte counts in control subjects and in asthmatic patients

	Control subjects (<i>n</i> = 20)	Asthmatics		
		All (<i>n</i> = 36)	Mild–moderate (<i>n</i> = 22)	Severe (<i>n</i> = 14)
Blood leukocytes (cells/mm ³)	5.89 ± 0.82	7.23 ± 2.18 ^{†††}	6.67 ± 1.59*	8.21 ± 2.71 ^{†††}
Blood neutrophils (cells/mm ³)	3.32 ± 0.56	4.01 ± 1.71 [†]	3.68 ± 1.27	4.74 ± 2.13 [†]
Blood lymphocytes (cells/mm ³)	1.87 ± 0.58	2.18 ± 0.94	2.11 ± 0.81	2.28 ± 1.11
Blood eosinophils (cells/mm ³)	0.15 ± 0.09	0.47 ± 0.43 ^{†††}	0.40 ± 0.30 [§]	0.49 ± 0.53 [†]

* $P < 0.05$, comparison between mild–moderate and severe asthmatics; [†] $P < 0.05$; ^{†††} $P < 0.001$, comparison between severe asthmatics and healthy controls; [†] $P < 0.05$; ^{†††} $P < 0.001$, comparison between asthmatics as a whole and healthy controls; [§] $P < 0.05$, comparison between mild–moderate asthmatics and healthy controls.

were detected among the three groups ($P < 0.05$). Total white blood cell counts were higher in severe asthmatics than either in healthy control ($P < 0.001$) or in mild-moderate asthmatics ($P < 0.05$) (Table 2). Severe asthmatics showed also higher blood neutrophilia and eosinophilia than healthy controls ($P < 0.05$) (Table 2). Blood eosinophilia were also higher in mild-moderate asthmatics than in healthy controls ($P < 0.05$) (Table 2).

No difference in blood leucocyte counts was observed between severe asthmatics treated with oral corticosteroids and severe asthmatics not receiving oral corticosteroids.

Serum cytokine levels in healthy controls and in mild-moderate and severe asthmatics

All the cytokines detected were above the detection limit of each kit. Differences in sIL-8 levels were detected among the three groups ($P < 0.0001$). sIL-8 levels in healthy controls were lower than in mild-moderate ($P < 0.05$) or in severe asthma ($P < 0.001$). Among asthmatic subjects, sIL-8 levels were also higher in those with severe asthma than in those with mild-moderate asthma ($P < 0.05$) (Fig. 1a).

Detecting sTNF- α levels, we found differences in serum levels of this cytokine among the three groups ($P = 0.0004$). Particularly, sTNF- α levels were higher in severe asthmatics as compared with mild-moderate asthmatics ($P < 0.05$) or to healthy controls ($P < 0.001$) (Fig. 1b). No significant difference was reported in TNF- α between mild-moderate asthmatics and healthy controls.

No statistically significant differences in sIL-16 ($P = 0.06$) and sIL-13 ($P = 0.16$) levels were detected among the three groups and particularly between mild-moderate and severe asthmatics (Figs. 2a and b).

No difference in cytokine levels was observed between severe asthmatics treated with oral corticosteroids and severe asthmatics not receiving oral corticosteroids.

We compared serum levels of different cytokines in atopic and non-atopic asthmatics (as a whole or specifically into the groups of mild and severe asthmatics) without finding any statistically significant difference.

Even though the sample size is rather small particularly in the severe asthma group, the power calculation performed for the primary endpoints (serum cytokines and FeNO) was over 80%.

Correlation between blood leucocyte counts, cytokine levels in the sera and functional parameters

Evaluating possible relationship between systemic and airway parameters, we found that, in the asthmatic group considered as a whole, FEV₁(%pred) as an index of the degree of asthma severity negatively correlated with sIL-8 ($r = -0.37$; $P = 0.028$) (Fig. 3a) and sTNF- α ($r = -0.43$; $P = 0.009$) (Fig. 3b) levels. These correlations were lost considering mild-moderate and severe asthmatics separately.

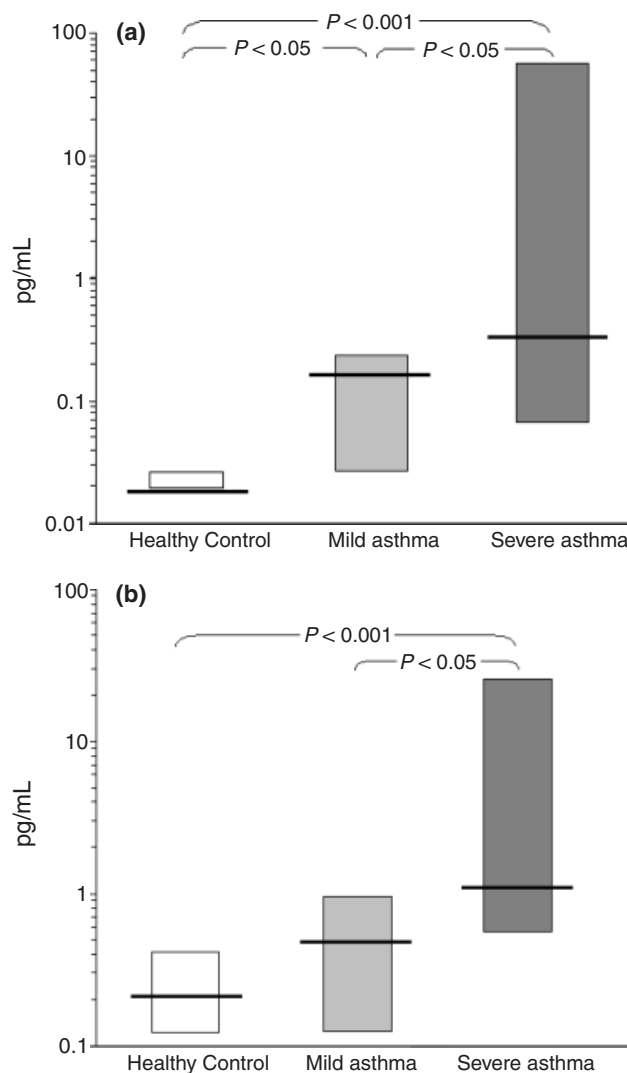


Fig. 1. Serum (s) interleukin IL-8 (a) and sTNF- α (b) of healthy controls ($n = 20$), mild-moderate ($n = 22$) and severe ($n = 14$) asthmatic subjects. Cytokine serum levels are expressed on the ordinate as picogram per millilitre whereas the two groups of asthmatic patients are reported on the abscissa. Horizontal lines indicate the median; boxes indicate the interquartile range.

Data from all asthmatic subjects did not demonstrate any statistically significant correlation between FeNO and cytokine levels. However, evaluating severe and mild-moderate separately, we found a positive correlation between FeNO and sIL-16 levels in mild-moderate asthmatics ($r = 0.48$; $P = 0.026$) (not shown) but not in severe asthmatics ($r = 0.34$; $P = 0.23$) (not shown). In contrast, in severe asthmatics FeNO levels correlated with sTNF- α levels ($r = 0.67$; $P = 0.009$) (Fig. 4) but not in mild-moderate asthmatics ($r = -0.10$; $P = 0.66$) (not shown). No statistically significant correlations were observed between FeNO levels and sIL-8 or sIL-13 in either severe or mild-moderate asthmatics (not shown).

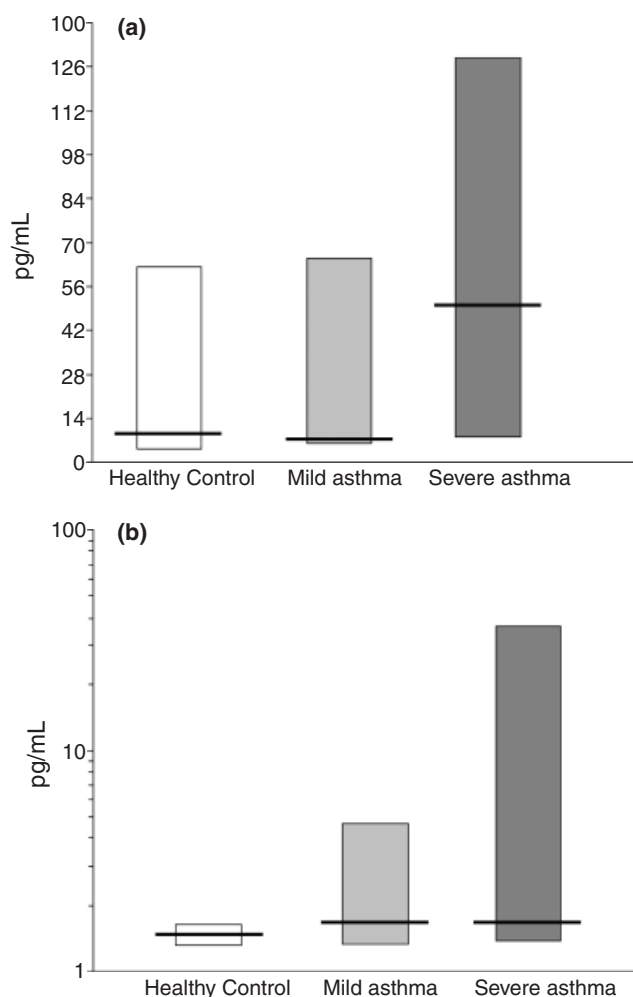


Fig. 2. Serum (s) IL-16 (a) and sIL-13 (b) of healthy controls ($n = 20$), mild-moderate ($n = 22$) and severe ($n = 14$) asthmatic subjects. Cytokine serum levels are expressed on the ordinate as picogram per millilitre whereas the two groups of asthmatic patients are reported on the abscissa. Horizontal lines indicate the median; boxes indicate the inter-quartile range.

In severe asthmatics sTNF- α positively correlated with total white blood cell counts ($r = 0.56$; $P = 0.037$) (Fig. 5a) and to neutrophil counts ($r = 0.57$; $P = 0.033$) (Fig. 5b).

Discussion

Evaluating adult mild-moderate and severe asthmatics, we found that sIL-8 and sTNF- α levels were higher in severe asthmatics as compared with mild-moderate asthmatics and were related to airflow limitation. We also found a positive correlation between sTNF- α levels and FeNO levels or circulating neutrophil number in severe asthmatics.

Although asthma affects nearly 8% of the adult population, most of these patients have mild-to-moderate disease that can be controlled with appropriate treatment. It

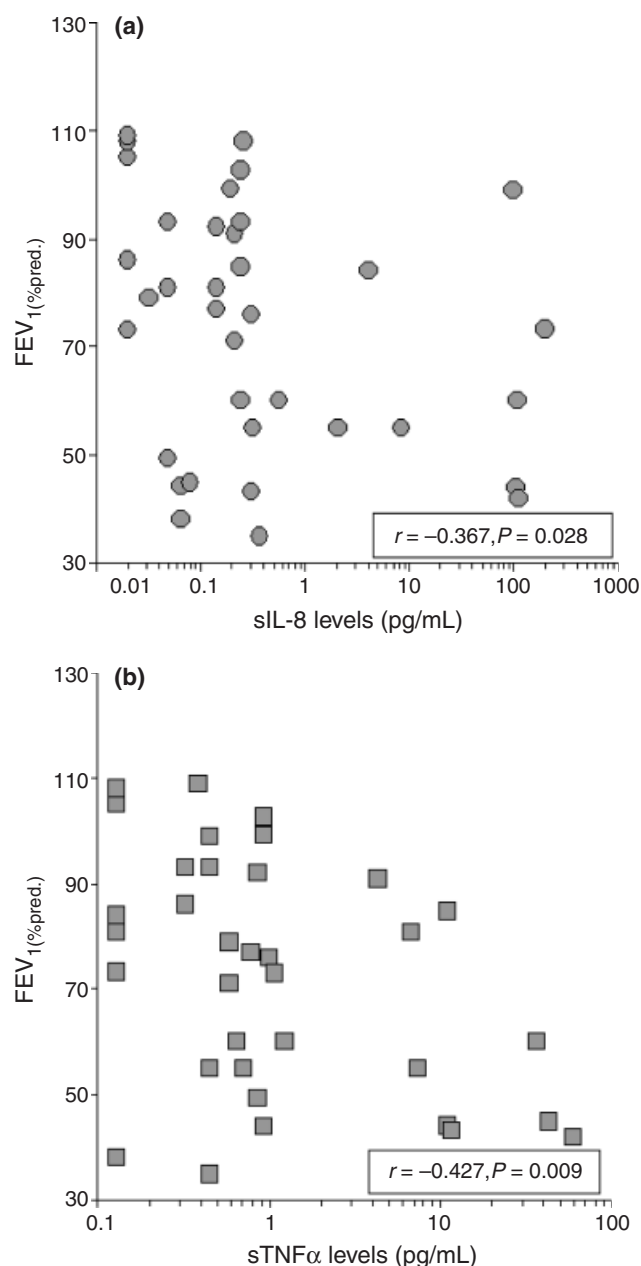


Fig. 3. Correlation between the level of airway obstruction and serum (s) IL-8 (a) or sTNF- α (b) in the whole asthmatic population ($n = 36$). Airway obstruction evaluated as forced expiratory volume in 1 s (FEV₁(%pred.)) is reported on the ordinate whereas serum cytokine levels, expressed as picogram per millilitre, are reported on the abscissa. FEV₁, forced expiratory volume in 1 s.

is estimated, however, that 5%–10% of patients with asthma have severe disease that is relatively responsive to corticosteroids. One of the goals of this study was to verify whether serum cytokines could be markers of asthma severity.

We showed that severe asthmatics had higher concentrations of IL-8 in serum than mild-moderate asthmatics or healthy controls. This finding confirms, at least in part,

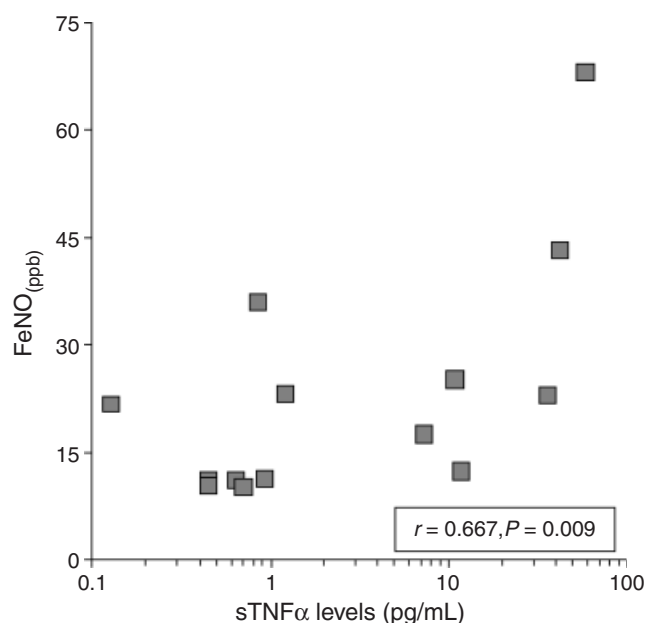


Fig. 4. Correlation between exhaled nitric oxide (FeNO) and serum(s) TNF- α in severe asthmatics ($n = 14$). FeNO levels expressed as parts per billion (p.p.b.) are reported on the ordinate whereas serum cytokine levels, expressed as picogram per millilitre, are reported on the abscissa.

previous observations that in severe asthma IL-8 levels were increased both in sputum [18] and in peripheral blood [9]. However, the latter [9] was made on serum from asthmatics defined by the authors as 'severe' but including also subjects with FEV₁(%pred) higher than 97%. In contrast, our data show that sIL-8 was higher in severe asthmatics (mean FEV₁(%pred): 49%) than in mild-moderate asthmatics or healthy controls, despite the high-dose of inhaled steroid treatment. This finding might reflect the inability of these drugs to significantly suppress production of this cytokine by airway cellular sources including epithelial cells [19] and inflammatory cells (granulocytes and mononuclear cells) [20]. In addition, the bioavailability of free IL-8 can be limited by IL-8 autoantibodies of the IgG and IgA class [21] and it is supposed that in patients with severe asthma there may be an imbalance between IL-8 production and the blocking capacity of IL-8 autoantibodies [22]. Evidence from patients with COPD suggests that neutrophilic inflammation may be insensitive to corticosteroid therapy [23] and this observation may account for the sustained elevation of IL-8 (and neutrophilic airway inflammation) in severe persistent asthma.

We showed high levels of sTNF- α in severe asthma suggesting an increased production of this cytokine from the airways of severe asthmatics. Probably, the role of TNF- α in severe asthma is to amplify airway inflammation recruiting neutrophils and to potentiate AHR [24]. On the other hand, neutrophils can also produce a wide range of cytokines including TNF- α [25] that may be linked to

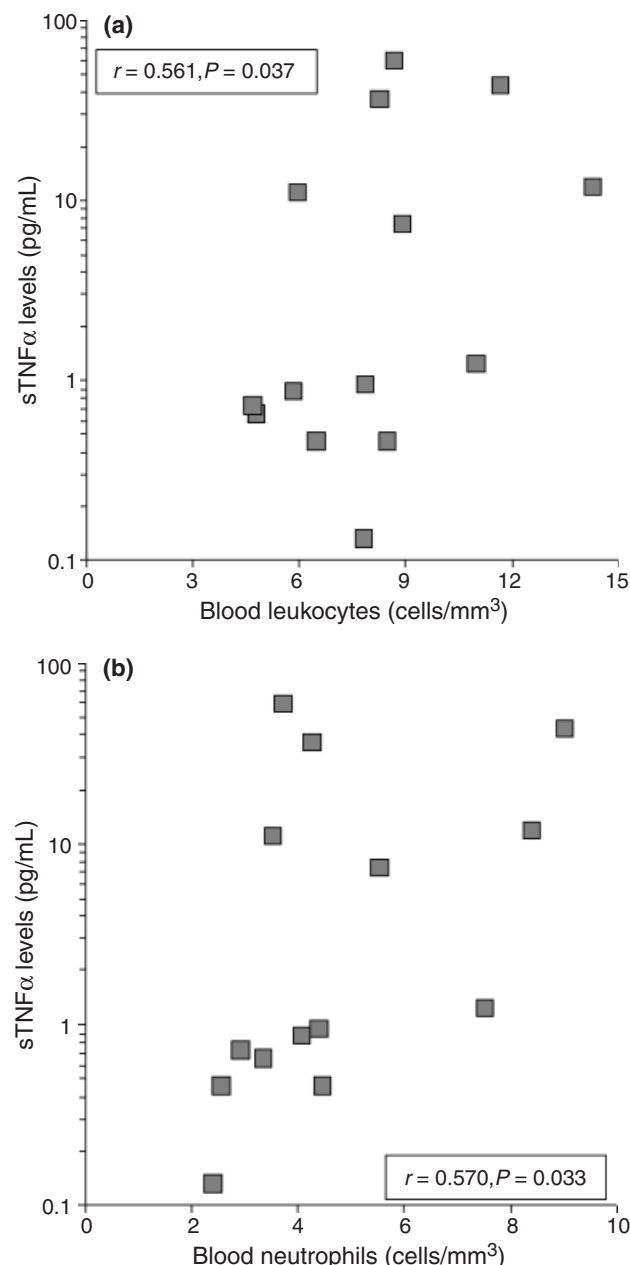


Fig. 5. Correlation between serum (s) TNF- α and white blood cell counts (a) and neutrophil counts (b) in severe asthmatics ($n = 14$). sTNF- α expressed as picogram per millilitre are reported on the ordinate whereas white blood cell counts and neutrophil counts, expressed as cells per mm³ are reported on the abscissa.

disease severity in asthma. Our data demonstrated for the first time that sTNF- α levels, in conjunction with elevated circulating leucocyte and neutrophil number as reported previously [26], were higher in severe than in mild-moderate asthmatics and healthy controls. These findings can be explained, at least in part, by the data by Wenzel et al. [27] who reported that severe asthmatics, treated with high dose of inhaled corticosteroids, demonstrated a

twofold increase of neutrophils in bronchoalveolar lavage and in bronchial and transbronchial biopsy specimens than both mild-moderate asthmatics and normal subjects. Similarly, Meagher et al. [28] found that glucocorticosteroids activate neutrophil progenitors, increase circulating neutrophil counts and inhibit neutrophil apoptosis. Probably, this class of drugs may enhance neutrophil function through increased leukotrienes and superoxide production, as well as inhibition of apoptosis [29]. In addition, high levels of sTNF- α and systemic corticosteroid therapy could be involved in determining the skeletal muscle weakness, a feature of chronic severe asthma [30–32].

We did not find any difference in the sIL-16, marker of T cell recruitment, levels among the healthy controls and mild-moderate or severe asthmatics. These results are in line with previous data on sputum showing no difference between mild and severe asthmatics in the proportion of lymphocytes [18, 33]. Indeed, T cell chemotaxis in response to allergen within the airways is an important component of mucosal inflammation in both mild and severe asthma. Even though the mechanisms that underlie its generation and the profile of chemotactic factors might vary depending on disease severity [27]. A recent *in vivo* study [34] demonstrated an up-regulation of IL-16 expression in allergic nasal mucosa after antigen challenge that may have implications in the accumulation of CD4⁺ cells in response to antigen exposure [34].

In this study, it has also been shown a normal range of sIL-13 levels in severe asthma suggesting that this cytokine has not a role in systemic inflammation induced by severe asthma. Previous study showed elevation of sIL-13 levels in acute asthma compared with control subjects [6] indicating a systemic involvement during exacerbation. This Th2 cytokine is involved in various airway responses (responsiveness, mucus production and remodelling) and in the progression of the disease, but our data at the systemic level showed not to be a marker of chronic severe asthma. This result is in line with others suggesting that corticosteroid treatment may prevent the increase in IL-13 observed in patients with asthma [35].

We found that severe asthmatics were significantly older than mild-moderate asthmatics. This is not surprising as multiple cross-sectional and longitudinal studies [36, 37] demonstrated a small annual decline (1% of predicted per year) in lung function among patients with asthma. In addition, it has been demonstrated that older age is associated with progressive remodelling of the airway wall [38].

We found that both sIL-8 and sTNF- α levels were negatively related to FEV₁(%pred) suggesting an association between 'systemic' inflammation and a marker of pulmonary function in asthma severity. This finding is partially confirmed by the increasing evidence that neutrophilic inflammation may reflect asthma severity [39].

A similar relationship between sputum IL-8 concentration (or sputum neutrophil counts) and maximal FEV₁ after a course with oral corticosteroids was found in chronic asthma suggesting a role for the IL-8-dependent recruitment of neutrophils in the development of irreversible airway obstruction in chronic severe asthma [40].

We showed that FeNO, a marker of airway inflammation, significantly correlates with the 'systemic' inflammatory marker sIL-16 in mild-moderate asthma. Several reports demonstrated increased FeNO in asthma reflecting airway inflammatory events [41]. Besides being produced by neutrophils [42] and eosinophils [43] NO is also produced by epithelial cells and macrophages in asthma and has an inhibitory effect on Th1 cells allowing the activation of Th2 cells which are normally suppressed by IFN- γ [44]. Th2 cells can be recruited at sites of inflammation by IL-16, whose relevance in the pathogenesis of asthma has been well demonstrated [45]. We did not find a positive correlation between FeNO and sIL-16 in severe asthmatics possibly because FeNO levels in this group were lower than in mild-moderate asthmatics because of the treatment with higher doses of corticosteroids [46].

A further strong correlation between FeNO levels and sTNF- α was found in severe asthmatics, and it could be explained taking into account that TNF- α induces iNOS expression not only in human lung epithelial cells [47] but also in blood mononuclear cells [48]. Therefore, sTNF- α levels might reflect FeNO levels. We did not show a correlation between these two parameters in mild-moderate asthmatics as this group has low sTNF- α -levels as similar as those detected in healthy controls.

Taking together, these data suggest that sIL-8 and sTNF- α are important markers of 'systemic' inflammation in severe asthmatics and correlate with the degree of the airflow limitation. The role of TNF- α as marker of 'systemic' inflammation is supported by the demonstration of a significant correlation between the levels of this cytokine in the serum of severe asthmatics and the degree of airway inflammation, as judged by FeNO. Several cytokine and chemokine inhibitors are in development as future therapy for different diseases. In rheumatoid arthritis and inflammatory bowel disease a blocking humanized monoclonal antibody to TNF- α (infliximab) and soluble TNF receptors (etanercept) produce remarkable clinical responses [49, 50]. Inhibitors of TNF- α -converting enzyme, a matrix metalloproteinase-related enzyme critical for the release of TNF from the cell surface, are also in development [51]. Recently, etanercept treatment in severe corticosteroid-dependent asthmatics improved respiratory symptoms and lung function identifying anti-TNF- α treatment as a new therapeutic option in severe asthma [52]. The present data may have clinical relevance in order to support future therapeutic strategies with drugs aimed at blocking the release or the activity of TNF- α for treating severe asthma.

Acknowledgements

The authors thank Dr. Angela Pistorio (Clinical Epidemiology and Biostatistic Unit, G. Gaslini Institute, Genoa, Italy) who provided statistical support in power calculation. This study was supported by the Italian Institutes of Health, Research (Ricerca Corrente).

References

- Chung KF, Barnes PJ. Cytokines in asthma. *Thorax* 1999; 54:825–57.
- 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.
- Mashikian MV, Tarry RE, Saukkonen JJ et al. Identification of IL-16 as the lymphocyte chemotactic activity in the bronchoalveolar lavage fluid of histamine-challenged asthmatic patients. *J Allergy Clin Immunol* 1998; 101:786–92.
- Laberge S, Pinsonneault S, Varga EM et al. Increased expression of IL-16 immunoreactivity in bronchial mucosa after segmental allergen challenge in patients with asthma. *J Allergy Clin Immunol* 2000; 106:293–301.
- Naseer T, Minshall EM, Leung DY et al. Expression of IL-12 and IL-13 mRNA in asthma and their modulation in response to steroid therapy. *Am J Respir Crit Care Med* 1997; 155:845–51.
- Lee YC, Lee KH, Lee HB, Rhee YK. Serum levels of interleukins (IL)-4, IL-5, IL-13, and interferon-gamma in acute asthma. *J Asthma* 2001; 38:665–71.
- Kunkel SL, Standiford T, Kasahara K, Strieter RM. Interleukin-8 (IL-8): the major neutrophil chemotactic factor in the lung. *Exp Lung Res* 1991; 17:17–23.
- Walker C, Bauer W, Braun RK et al. Activated T cells and cytokines in bronchoalveolar lavages from patients with various lung diseases associated with eosinophilia. *Am J Respir Crit Care Med* 1994; 150:1038–48.
- Shute JK, Vrugt B, Lindley IJ et al. Free and complexed interleukin-8 in blood and bronchial mucosa in asthma. *Am J Respir Crit Care Med* 1997; 155:1877–83.
- Rothenberg ME. Eosinophilia. *N Engl J Med* 1998; 338:1592–600.
- Ackerman V, Marini M, Vittori E, Bellini A, Vassali G, Mattoli S. Detection of cytokines and their cell sources in bronchial biopsy specimens from asthmatic patients. Relationship to atopic status, symptoms, and level of airway hyperresponsiveness. *Chest* 1994; 105:687–96.
- Najam FI, Giasuddin AS, Shembesh AH. Tumour necrosis factors in childhood asthma. *Ind. J Pediatr* 2001; 68:217–22.
- Ricciardolo FL, Sterk PJ, Gaston B, Folkerts G. Nitric oxide in health and disease of the respiratory system. *Physiol Rev* 2004; 84:731–65.
- Hansel TT, Kharitonov SA, Donnelly LE et al. Selective inhibitor of inducible nitric oxide synthase inhibits exhaled breath nitric oxide in healthy volunteers and asthmatics. *FASEB J* 2003; 17:1298–300.
- National Institutes of Health NHLBI. Burden of asthma. In: *Global Strategy for Asthma Management and Prevention*. NIH Publication. National Institutes of Health NHLBI, 2002; 11–27 Available from: <http://www.ginasthma.com/workshop.pdf>.
- Johansson SGO, Hourihane JO'B, Bousquet J et al. A revised nomenclature for allergy. An EAACI position statement from EAACI nomenclature task. *Allergy* 2001; 56:813–24.
- American Thoracic Society–Medical Section of the American Lung Association. Recommendations for standardized procedures for the on-line and off-line measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide in adults and children – 1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors. *Am J Respir Crit Care Med* 1999; 160:2104–17.
- Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med* 1999; 160:1532–9.
- Levine SJ, Larivee P, Logun C, Angus CW, Shelhamer JH. Corticosteroids differentially regulate secretion of IL-6, IL-8, and G-CSF by a human bronchial epithelial cell line. *Am J Physiol* 1993; 265:L360–8.
- Mukaida N, Shiroo M, Matsushima K. Genomic structure of the human monocyte-derived neutrophil chemotactic factor IL-8. *J Immunol* 1989; 143:1366–71.
- Crabtree JE, Peichl P, Wyatt JI, Stachl U, Lindley IJ. Gastric interleukin-8 and IgA IL-8 autoantibodies in *Helicobacter pylori* infection. *Scand J Immunol* 1993; 37:65–70.
- Vrugt B, Djukanovic R, Bron A, Aalbers R. New insights into the pathogenesis of severe corticosteroid-dependent asthma. *J Allergy Clin Immunol* 1996; 98:S22–6.
- Keatings VM, Jatakanon A, Worsdell YM, Barnes PJ. Effects of inhaled and oral glucocorticoids on inflammatory indices in asthma and COPD. *Am J Respir Crit Care Med* 1997; 155:542–8.
- Thomas PS, Heywood G. Effects of inhaled tumour necrosis factor alpha in subjects with mild asthma. *Thorax* 2002; 57:774–8.
- Sampson AP. The role of eosinophils and neutrophils in inflammation. *Clin Exp Allergy* 2000; 30 (Suppl. 1):22–7.
- European Network for Understanding the Mechanism of Severe Asthma (ENFUMOSA). Cellular composition of induced sputum in severe persistent asthma. *Am J Respir Crit Care Med* 1999; 159:A700.
- Wenzel SE, Szeffler SJ, Leung DYM, Sloan SI, Rex MD, Martin RJ. Bronchoscopic evaluation of severe asthma: persistent inflammation associated with high dose glucocorticoids. *Am J Respir Crit Care Med* 1997; 156:737–43.
- Meagher LC, Cousin JM, Seckl JR, Haslett C. Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. *J Immunol* 1996; 156:4422–8.
- Cox G. Glucocorticoid treatment inhibits apoptosis in human neutrophils. Separation of survival and activation outcomes. *J Immunol* 1995; 154:4719–25.
- Picado C, Fiz JA, Montserrat JM et al. Respiratory and skeletal muscle function in steroid-dependent bronchial asthma. *Am Rev Respir Dis* 1990; 141:14–20.
- Akkoca O, Mungan D, Karabiyikoglu G, Misirligil Z. Inhaled and systemic corticosteroid therapies: do they contribute to inspiratory muscle weakness in asthma? *Respiration* 1999; 66:332–7.
- Tracey KJ, Morgello S, Koplin B et al. Metabolic effects of cachectin/tumor necrosis factor are modified by site of production. Cachectin/tumor necrosis factor-secreting tumor in skeletal muscle induces chronic cachexia, while implantation in brain induces predominantly acute anorexia. *J Clin Invest* 1990; 86:2014–24.

- 33 Dent G, Hosking LA, Lordan JL *et al.* Differential roles of IL-16 and CD28/B7 costimulation in the generation of T-lymphocyte chemotactic activity in the bronchial mucosa of mild and moderate asthmatic individuals. *J Allergy Clin Immunol* 2002; **110**:906–14.
- 34 Laberge S, Durham SR, Ghaffar O *et al.* Expression of IL-16 in allergen-induced late-phase nasal responses and relation to topical glucocorticosteroid treatment. *J Allergy Clin Immunol* 1997; **100**:569–74.
- 35 Fushimi T, Okayama H, Shimura S, Saitoh H, Shirato K. Dexamethasone suppresses gene expression and production of IL-13 by human mast cell line and lung mast cells. *J Allergy Clin Immunol* 1998; **102**:134–42.
- 36 Peat JK, Woolcock AJ, Cullen K. Rate of decline of lung function in subjects with asthma. *Eur J Respir Dis* 1987; **70**:171–9.
- 37 Lange P, Parner J, Vestbo J, Schnohr P, Jensen G. A 15-year follow-up study of ventilatory function in adults with asthma. *N Engl J Med* 1998; **339**:1194–200.
- 38 Bai TR, Cooper J, Koelmeyer T, Pare PD, Weir TD. The effect of age and duration of disease on airway structure in fatal asthma. *Am J Respir Crit Care Med* 2000; **162**:663–9.
- 39 Lamblin CL, Gosset P, Tillie-Leblond I *et al.* Bronchial neutrophilia in patients with noninfectious status asthmaticus. *Am J Respir Crit Care Med* 1998; **157**:394–402.
- 40 Little SA, MacLeod KJ, Chalmers GW, Love JG, McSharry C, Thomson NC. Association of forced expiratory volume with disease duration and sputum neutrophils in chronic asthma. *Am J Med* 2002; **112**:446–52.
- 41 Kharitonov SA, Barnes PJ. Exhaled markers of pulmonary disease. *Am J Respir Crit Care Med* 2001; **163**:1693–722.
- 42 Zanardo RCO, Costa E, Ferreira HHA *et al.* Pharmacological and immunohistochemical evidence for a functional nitric oxide synthase system in rat peritoneal eosinophils. *Proc Natl Acad Sci USA* 1997; **94**:14111–4.
- 43 Iijima H, Duguet A, Eum SY, Hamid Q, Eidelman DH. Nitric oxide and protein nitration are eosinophil dependent in allergen challenged mice. *Am J Respir Crit Care Med* 2001; **163**:1233–40.
- 44 Barnes PJ, Liew FY. Nitric oxide and asthmatic inflammation. *Immunol Today* 1995; **16**:128–30.
- 45 Taha RA, Laberge S, Hamid Q, Olivenstein R. Increased expression of the chemoattractant cytokines eotaxin, monocyte chemotactic protein-4, and interleukin-16 in induced sputum in asthmatic patients. *Chest* 2001; **120**:595–601.
- 46 Silkoff PE, McClean P, Spino M, Erlich L, Slutsky AS, Zamel N. Dose response relationship and reproducibility of the fall in exhaled nitric oxide after inhaled beclomethasone dipropionate therapy in asthma patients. *Chest* 2001; **119**:1322–8.
- 47 Kwon S, George S. Synergistic cytokine-induced nitric oxide production in human alveolar epithelial cells. *Nitric Oxide* 1999; **3**:348–57.
- 48 Allanore Y, Borderie D, Hilliquin P *et al.* Low levels of nitric oxide (NO) in systemic sclerosis: inducible NO synthase production is decreased in cultured peripheral blood monocyte/macrophage cells. *Rheumatology* 2001; **40**:1089–96.
- 49 Markham A, Lamb HM. Infliximab: a review of its use in the management of rheumatoid arthritis. *Drugs* 2000; **59**:1341–59.
- 50 Jarvis B, Faulds D. Etanercept: a review of its use in rheumatoid arthritis. *Drugs* 1999; **57**:945–66.
- 51 Barlaam B, Bird TG, Lambert-Van DB, Campbell D, Foster SJ, Maciewicz R. New alpha-substituted succinate-based hydroxamic acids as TNF-alpha convertase inhibitors. *J Med Chem* 1999; **42**:4890–908.
- 52 Howarth PH, Babu KS, Arshad HS *et al.* Tumour necrosis factor (TNFalpha) as a novel therapeutic target in symptomatic corticosteroid dependent asthma. *Thorax* 2005; **60**:1012–8.