

Macrophage migration inhibitory factor (MIF) in bronchial asthma

E. YAMAGUCHI*, J. NISHIHARA†, T. SHIMIZU‡, T. TAKAHASHI*,
N. KITASHIRO*, N. HIZAWA*, K. KAMISHIMA§ and Y. KAWAKAMI*

*The First Department of Medicine, School of Medicine, Hokkaido University, †Central Research Institute, School of Medicine, Hokkaido University, ‡Department of Dermatology, School of Medicine, Hokkaido University, §Ebetsu Municipal Hospital, Japan

Summary

Background Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine favouring the secretion of $\text{TNF}\alpha$ and IL-8 and counteracts anti-inflammatory effects of corticosteroids. Airways inflammation is a central feature of bronchial asthma and is characterized by the accumulation of eosinophils.

Objective The aim of this study was to investigate whether MIF is related to asthma symptoms and eosinophil accumulation in the airways.

Methods Serum MIF levels were measured by an enzyme-linked immunosorbent assay in 44 healthy subjects and 44 asthmatics. Levels of MIF in induced sputum were measured in 10 healthy subjects and 15 asthmatics. Levels of eosinophil cationic protein (ECP) in induced sputum were measured by a radioimmunosorbent assay. Fluorescence double immunostaining was conducted to examine cellular source and localization of MIF.

Results Serum MIF levels were significantly increased in asthmatic patients compared with age and sex-matched control subjects. Symptomatic patients had a higher MIF level than asymptomatic patients. Induced sputum obtained from asthmatics contained higher levels of MIF than those from control subjects. MIF levels in induced sputum were correlated with ECP levels in induced sputum. MIF was colocalized with eosinophil peroxidase staining in the cytoplasm of sputum cells.

Conclusion Increased MIF levels are associated with asthma symptoms and one of the cellular sources of MIF in the airways are eosinophils.

Keywords: bronchial asthma, macrophage migration inhibitory factor (MIF)-induced sputum, eosinophil cationic protein (ECP)

Clinical and Experimental Allergy, Vol. 30, pp. 1244–1249. Submitted 16 June 1999; revised 6 December 1999; accepted 25 January 2000.

Introduction

Macrophage migration inhibitory factor (MIF) was originally described to be a T-cell product that inhibited the random migration of macrophages [1]. MIF has been believed to play an important role in the delayed-type hypersensitivity (DTH) reaction, as it is prominently expressed in tuberculin DTH lesions in mice, though the

predominant cellular origin appeared to be the monocyte/macrophage [2,3].

The secretion of MIF is upregulated *in vitro* by $\text{TNF}\alpha$ and $\text{IFN}\gamma$ [3]. MIF in turn augments the secretion of $\text{TNF}\alpha$ and IL-8 [4]. This possible amplification loop of inflammation well characterizes MIF as a pro-inflammatory cytokine. The counteraction of MIF on anti-inflammatory effects of corticosteroids has the potential to further amplify the pro-inflammatory loop [5]. MIF also plays an important regulatory role in the activation of T cells induced by mitogenic or antigenic stimuli [6]. Notably, a prominent induction of MIF mRNA and protein has been observed for

Correspondence: E. Yamaguchi, The First Department of Medicine, School of Medicine, Hokkaido University, Kita-15 Nishi-7 Kitaku, Sapporo 060–8638, Japan.

TH2 clones, but not for TH1 clones [6], suggesting a role in IgE-mediated immune responses.

MIF has been implicated in the pathogenic mechanisms of acute respiratory distress syndrome in humans [4], lethal endotoxemia in rats [7], experimental glomerulonephritis in rats [8], and experimental arthritis in mice [9]. Furthermore, potential importance of MIF in asthma has recently been suggested by Rossi *et al.* They found increased MIF levels in bronchoalveolar lavage fluid from asthmatic patients and demonstrated that circulating eosinophils can produce MIF upon stimulation *in vitro* [10]. However, MIF levels in serum and induced sputum have not been examined. Thus, in view of the increasingly clear importance of MIF as a regulator of inflammatory and immune responses, we investigated the involvement of MIF in the pathophysiology of bronchial asthma and found for the first time that MIF levels in serum was correlated with asthma symptoms. Furthermore, a significant positive correlation was observed between levels of MIF and eosinophil cationic protein (ECP), a marker for eosinophilic airways inflammation, in induced sputum. We also found colocalization of MIF with eosinophil peroxidase in sputum cells.

Methods

Study subjects

Forty-four patients with bronchial asthma, 20 men and 24 women (median age, 54 years; range, 22–74 years) as defined by the International Consensus Report on the Diagnosis and Management of Asthma [11], were enrolled into the study. All patients had a history of variable degrees of wheezing and coughing associated with chest tightness. Reversibility of airflow obstruction ($FEV_1 > 20\%$) had been documented previously. Patients symptoms were evaluated by an independent clinician using an asthma diary. Symptomatic patients were defined as those presenting asthma symptoms such as wheezing and dyspnea during a 24-hr period prior to blood sampling. Twenty-seven patients were atopic as defined by an increased level of serum total IgE and/or the presence of serum IgE antibodies against one or more of 10 ubiquitous airborne allergens determined by the CAP-RAST (Pharmacia Diagnostics, Uppsala, Sweden). All patients gave informed consent.

Forty-four healthy age and sex-matched subjects, 20 men and 24 women (median age, 52 years; range, 22–73 years), were selected from subjects who received an annual health check-up and served as control subjects. None had a history of lung diseases, symptoms, or abnormal results on chest roentgenography and laboratory data, including complete blood counts, hepatic enzyme activities, a blood urea nitrogen level and urinalysis.

Measurement of MIF and ECP

Sputum was induced from 10 healthy volunteers (nine men and a woman) and 15 patients with bronchial asthma (six men and nine women) as previously described [12]. We collected selected sputum according to a previous report [13]. Sputum was then vigorously dispersed and dissolved in fivefold volume of saline by pipetting and centrifuged for 10 min at 3000 g. Supernatants were collected and stored at -80°C until assayed. Blood was obtained by venipuncture. Serum was separated by centrifugation at 3000 g for 10 min and stored at -80°C until assayed. Levels of MIF in serum and induced sputum were measured by a specific enzyme-linked immunosorbent assay as previously described [14]. Levels of ECP in induced sputum were measured by a specific radioimmunosorbent assay (Amersham Pharmacia Biotech, Uppsala, Sweden).

Immunostaining

Mouse monoclonal antihuman eosinophil peroxidase (EP) antibody was obtained from Nichirei (Tokyo, Japan). FITC-conjugated sheep antimouse IgG and rhodamine-conjugated goat antirabbit IgG were purchased from Pierce (Rockford, IL, USA). Rabbit polyclonal antibody against human MIF was generated by immunizing New Zealand White rabbits with purified recombinant human MIF prepared as previously described [15]. In brief, rabbits were inoculated intradermally with 100 μg of MIF diluted in complete Freund's adjuvant at weeks 1 and 2, and with 500 μg of MIF diluted in incomplete Freund's adjuvant at week 4. Immune serum was collected 1 week after the last inoculation. The IgG fraction was prepared using protein A sepharose (Pharmacia, Uppsala, Sweden) according to the manufacturer's protocol.

Induced sputum was mixed with an equal volume of phosphate-buffered saline (PBS) containing 15 mM dithiothreitol, liquefied by incubation for 15 min at 37°C . Cell suspension was passed through a cell strainer (Falcon 2350, Becton Dickinson Labware, Franklin Lakes, NJ, USA), centrifuged, and washed twice with Hanks' balanced salt solution (HBSS). After being adjusted for cell number, sputum cells were mounted on poly-L-lysine-coated slides by cytocentrifugation, air dried, and fixed in cold acetone at -20°C for 20 min. For double immunostaining, samples were first exposed to both mouse monoclonal anti-EP IgG and rabbit polyclonal anti-MIF IgG by incubation at 37°C for 1 h. After rinsing in PBS, samples were incubated with fluorescein isothiocyanate (FITC)-conjugated sheep antimouse IgG (Pierce, Rockford, IL, USA) and rhodamine red-conjugated goat antirabbit IgG (Pierce) at 37°C for 30 min. After rinsing in PBS, a fluorescence microscope (Eclipse TE-350, Nikon, Tokyo, Japan) was used for immunohistochemical observation.

Statistical analysis

Differences among three groups were assessed by the Kruskal–Wallis test and those between two groups by the Mann–Whitney *U*-test with the Bonferroni correction for multiple testing (SPSS, Chicago, IL, USA). Correlation between levels of MIF and ECP in induced sputum was assessed by the Spearman rank correlation coefficient (SPSS). Differences with a *P*-value of less than 0.05 were considered significant.

Results

The median value of serum MIF in the 44 healthy subjects was 3.8 ng/mL, with a range of 0.8–9.7 ng/mL. Asymptomatic patients with bronchial asthma had MIF levels ranging from 5.6 to 51.3 ng/mL with a median value of 27.7 ng/mL (Fig. 1). When the cut-off value was set at 9.7, all but three asymptomatic patients had an increased serum MIF level. As a group, asymptomatic patients had a significantly higher serum MIF level than did control subjects. Serum MIF levels in symptomatic patients ranged from 13.3 to 107.5 ng/mL, with a median value of 41.6 ng/mL. Every symptomatic patient had a MIF level that was higher than the cut-off value, resulting in a significant difference between symptomatic patients and control subjects (Fig. 1), with symptomatic patients having a significantly higher MIF level than asymptomatic patients. MIF levels in atopic

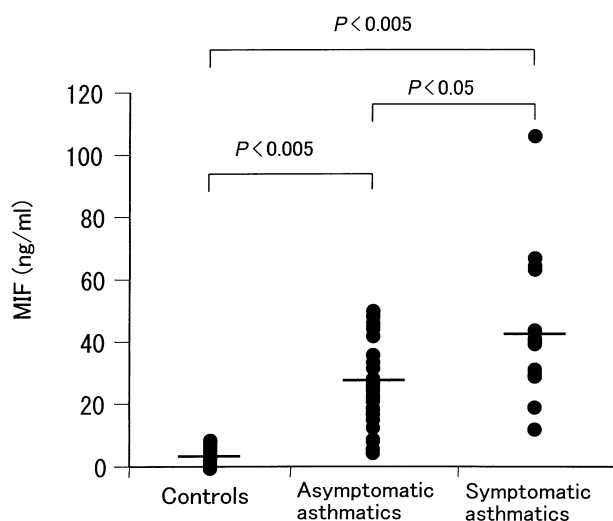


Fig. 1. Serum MIF levels in control subjects ($n = 44$), asymptomatic asthmatic ($n = 31$), and symptomatic asthmatic patients ($n = 13$). Horizontal bars represent medians. The difference among three groups was significant ($P < 0.0005$) by the Kruskal–Wallis test. Significant levels between each pair of groups as calculated by the Mann–Whitney *U*-test and corrected by Bonferroni's method are shown in the figure.

patients did not differ from those in nonatopic patients (data not shown). Seven patients needed oral corticosteroids for the control of their symptoms. They had a significantly higher MIF level (median 42.4 ng/mL; range 18.1–107.5 ng/mL) than those who were not under oral corticosteroid therapy (median 28.1 ng/mL; range 5.6–68.3 ng/mL) at the time of blood sampling (Fig. 2). There was no significant correlation between serum MIF levels and circulating eosinophil count in asthmatic patients (data not shown).

MIF levels in induced sputum from 10 control subjects ranged from 37.5 to 244.0 ng/mL, with a median value of 80.8 ng/mL, whereas those from asthmatics ranged from 66.0 to 1615.5 ng/mL, with a median value of 532.0 ng/mL (Fig. 3). Four of 15 asthmatic patients had a sputum MIF level that was lower than the maximal value (244.0 ng/mL) of the control subjects. Three of the four asthmatic patients with a low sputum MIF level were asymptomatic at the time of sample collection. As a group, asthmatic patients had a significantly higher MIF level than did control subjects (Fig. 3).

Levels of ECP were measured for all induced sputum samples. Asthmatic patients had significantly higher ECP levels than did control subjects (data not shown, $P < 0.001$). When control subjects and asthmatics were combined, there was a significant positive correlation between log-transformed MIF levels and log-transformed ECP levels in induced sputum ($r_s = 0.787$, $P < 0.001$, Fig. 4). Even when analysis was restricted to asthmatics, a similar correlation was observed (data not shown, $r_s = 0.542$, $P < 0.05$).

In order to further clarify the cellular source of MIF, we

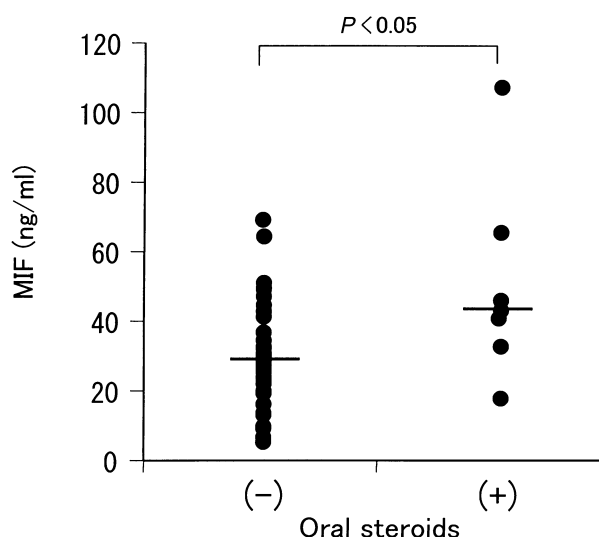


Fig. 2. Serum MIF levels in oral steroid-independent ($n = 37$) and -dependent patients ($n = 7$). The difference was assessed by the Mann–Whitney *U*-test. Horizontal bars are medians.

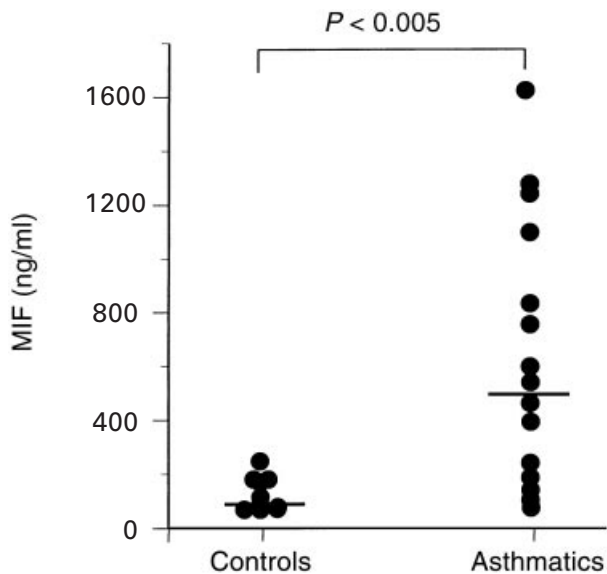


Fig. 3. MIF levels in induced sputum from control subjects ($n=10$) and asthmatic patients ($n=15$). Horizontal bars are medians.

conducted double immunostaining of induced sputum cells. Induced sputum was obtained from an unstable asthma patient in whom 70% of inflammatory cells were eosinophils as determined by Giemsa staining (not shown). MIF was shown to be localized principally in the cytoplasm of cells with a bilobed nucleus (Fig. 5a). Those cells had positive staining for EP, thus demonstrating they were eosinophils (Fig. 5b). Double exposure of rhodamine

and FITC images clearly showed colocalization of MIF with EP (Fig. 5c), demonstrating MIF was expressed by eosinophils.

Discussion

This study represents the first demonstration of increased MIF levels in serum and induced sputum in bronchial asthma. Serum MIF levels roughly correlated with the symptoms of bronchial asthma, symptomatic patients having the highest MIF levels, whereas asymptomatic patients had an intermediate level between controls and symptomatic patients. MIF levels in induced sputum were also increased in asthmatic patients compared with those in control subjects. Our results are consistent with, and extend a previous observation that bronchoalveolar lavage fluid from asthmatic patients contained significantly elevated levels of MIF as compared to nonatopic normal volunteers [10]. Furthermore, we found that there was a significant correlation between levels of MIF and ECP in induced sputum. Ample evidence has indicated ECP is a marker for eosinophilic inflammation of airways in bronchial asthma [16,17]. We have also demonstrated that MIF is colocalized with EP in cytoplasm of sputum cells. Therefore, these results together indicate that one of the cellular sources of MIF is airways eosinophil and hence, MIF levels mirrors the airways inflammation of bronchial asthma. Our observation is consistent with a previous finding that circulating eosinophils can release MIF upon stimulation [10].

Another candidate of cell type responsible for increased MIF levels is the lymphocyte, since MIF can be released from lymphocytes [1] and the activation of lymphocytes is

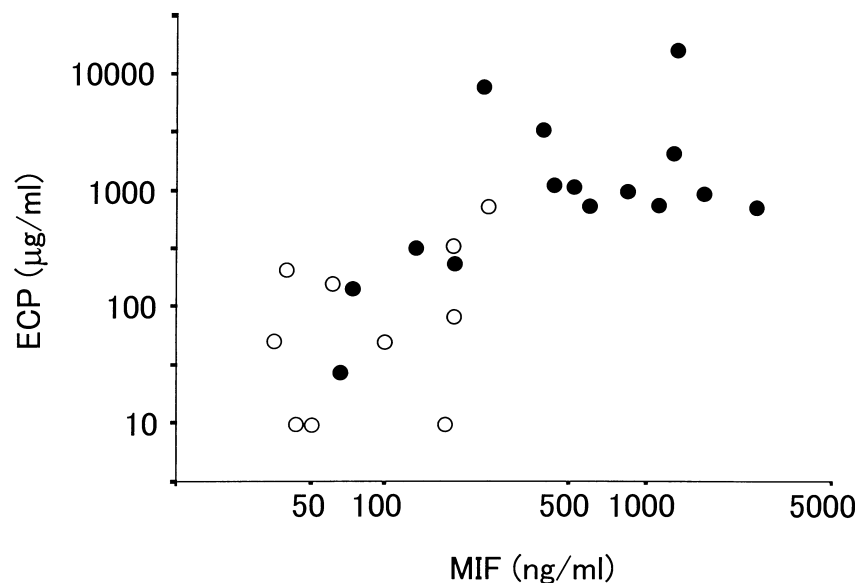


Fig. 4. Correlation between MIF levels and eosinophil cationic protein (ECP) levels in induced sputum. Open circles and closed circles denote control subjects and patients with asthma, respectively.

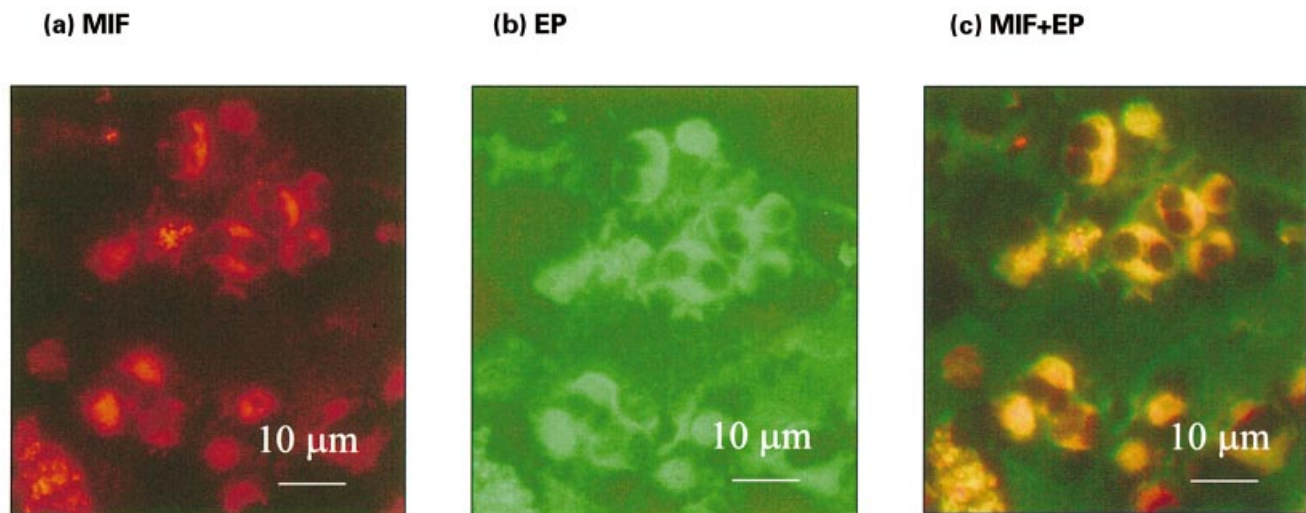


Fig. 5. (a) The rhodamine red image for MIF showed that the cytoplasm of cells with a bilobed nucleus were positive for staining. (b) The FITC image for eosinophil peroxidase (EP) indicated that most bilobed nuclear cells were eosinophils. (c) The yellow colour represents colocalization of MIF with EP.

an integral part of airways inflammation in bronchial asthma. It is therefore possible that parallel and cooperative activation of airways lymphocytes and eosinophils contribute to heightened expression of MIF.

The airways inflammation in bronchial asthma is characterized by the predominance of TH2-type cytokines, which include IL-4, IL-5, IL-6, and IL-13 [18–21]. It is further enhanced by other cytokines that are not directly relevant to TH2-type immune responses. For instance, bioactive TNF is present in bronchoalveolar lavage fluid from patients with symptomatic asthma [20]. Elevated release of TNF α and even IFN γ by resting and stimulated bronchoalveolar lavage cells has been reported for patients with asthma [22]. Constitutional variation in the capacity to secrete TNF α has been suggested to be associated with an increased risk of asthma [23]. Inasmuch as MIF expression can be induced by TNF α and IFN γ [3], and MIF in turn augments the secretion of TNF α and IL-8 [4], the involvement of MIF in a pro-inflammatory loop further strengthens the inflammatory nature of the pathogenesis of bronchial asthma.

Another implication of our findings on MIF is its possible role in steroid-resistant cases of bronchial asthma. The present study revealed that steroid-dependent asthmatic patients had a higher serum MIF level than those who did not need oral corticosteroids. It is possible that the use of corticosteroids *per se* induced the heightened expression of MIF as has been demonstrated in an *in vitro* study [5]. However, this is unlikely since our preliminary study revealed serum levels of MIF within the normal range in nonasthmatic patients who were under treatment with

systemic corticosteroids at doses comparable to those for asthmatic patients in the present study (not shown). The large majority of patients with steroid-resistant asthma appear to have an acquired defect, likely as the result of cytokine stimulation [24,25]. The abundance of cytokines at the site of disease can profoundly affect the pharmacological effects of corticosteroids on inflammatory cells, leading to steroid resistance. MIF has also been shown to antagonize the inhibitory effects of dexamethasone on the production of cytokines such as TNF α , IL-1 β , IL-6, and IL-8 by LPS-stimulated human monocytes [5]. Similar incapacitating effects of MIF on corticosteroids has been observed for the production of IL-2 and IFN γ by mitogen-stimulated T cells [6]. As most of these cytokines are involved in the pathogenic mechanism of asthma, MIF released in the airways may be, to some extent, responsible for intractability of asthma in steroid-resistant cases.

In conclusion, our study provides further evidence for the expression of MIF as an inflammatory component of bronchial asthma. Our observations indicate a new aspect of airways inflammation distinct from that characterized by TH2-type cells. Anti-MIF therapeutic strategies may prove to be a means to modulate the pathological cytokine milieu, leading to restoration of airways inflammation in bronchial asthma.

Acknowledgements

We are grateful to Y. Mizue of Sapporo Immunodiagnostic Laboratory for her excellent technical assistance.

References

- 1 Bloom BR, Bennett B. Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. *Science* 1966; 153:80–2.
- 2 Bernhagen J, Bacher M, Calandra T, Metz CN, Doty SB, Donnelly T et al. An essential role for macrophage migration inhibitory factor in the tuberculin delayed-type hypersensitivity reaction. *J Exp Med* 1996; 183:277–82.
- 3 Calandra T, Bernhagen J, Mitchell RA, Bucala R. The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. *J Exp Med* 1994; 179:1895–902.
- 4 Donnelly SC, Haslett C, Reid PT, et al. Regulatory role for macrophage migration inhibitory factor in acute respiratory distress syndrome. *Nature Med* 1997; 3:320–3.
- 5 Calandra T, Bernhagen J, Metz CN, et al. MIF as a glucocorticoid-induced modulator of cytokine production. *Nature* 1995; 377:68–71.
- 6 Bacher M, Metz CN, Calandra T, et al. An essential regulatory role for macrophage migration inhibitory factor in T-cell activation. *Proc Natl Acad Sci USA* 1996; 93:7849–54.
- 7 Bernhagen J, Calandra T, Mitchell RA, et al. MIF is a pituitary-derived cytokine that potentiates lethal endotoxemia. *Nature* 1993; 365:756–9.
- 8 Lan HY, Bacher M, Yang N, et al. The pathogenic role of macrophage migration inhibitory factor in immunologically induced kidney disease in the rat. *J Exp Med* 197 (185):1455–65.
- 9 Mikulowska A, Metz CN, Bucala R, Holmdahl R. Macrophage migration inhibitory factor is involved in the pathogenesis of collagen type II-induced arthritis in mice. *J Immunol* 1997; 158:5514–7.
- 10 Rossi AG, Haslett N, Greening AP, et al. Human circulating eosinophils secrete macrophage migration inhibitory factor (MIF). Potential role in asthma. *J Clin Invest* 1998; 101:2869–74.
- 11 International Consensus Report on Diagnosis and Treatment of Asthma. National Heart, Lung, and Blood Institute, Publication no. 92–3091. *Eur Respir J* 1992; 5:601–41.
- 12 Pin I, Gibson PG, Kolendowicz R, et al. Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax* 1992; 47:25–9.
- 13 Spanevello A, Beghe B, Bianchi A, et al. Comparison of two methods of processing induced sputum: selected versus entire sputum. *Am J Respir Crit Care Med* 1998; 157:665–8.
- 14 Wada S, Fujimoto S, Mizue Y, Nishihira J. Macrophage migration inhibitory factor in the human ovary: presence in the follicular fluids and production by granulosa cells. *Biochem Mol Biol Int* 1997; 41:805–14.
- 15 Nishihira J, Kuriyama T, Nishino H, Ishibashi T, Sakai M, Nishi S. Purification and characterization of human macrophage migration inhibitory factor: evidence for specific binding to glutathione and formation of subunit structure. *Biochem Mol Biol Int* 1993; 31:841–50.
- 16 Shoji S, Kanazawa H, Hirata K, Kurihara N, Yoshikawa J. Clinical implication of protein levels of IL-5 in induced sputum in asthmatic patients. *Am J Respir Crit Care Med* 1998; 157:665–8.
- 17 Fujimoto K, Kubo K, Matsuzawa Y, Sekiguchi M. Eosinophil cationic protein levels in induced sputum correlate with the severity of bronchial asthma. *Chest* 1997; 112:1241–7.
- 18 Robinson DS, Hamid Q, Ying S, et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992; 326:298–304.
- 19 Humbert M, Durham SR, Ying S, et al. IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and nonatopic asthma: evidence against (intrinsic (asthma being a distinct immunopathologic entity. *Am J Respir Crit Care Med* 1996; 154:1497–504.
- 20 Broide DH, Lotz M, Cuomo AJ, Coburn DA, Federman EC, Wasserman SI. Cytokines in symptomatic asthma airways. *J Allergy Clin Immunol* 1992; 89:958–67.
- 21 Naseer T, Minshall EM, Leung DYM, 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.
- 22 Cembrzynska-Nowak M, Szklarz E, Inglot AD, Teodorczyk-Injeyan JA. Elevated release of tumor necrosis factor- α and interferon- γ by bronchoalveolar leukocytes from patients with bronchial asthma. *Am Rev Respir Dis* 1993; 147:291–5.
- 23 Moffatt MF, Cookson WO. Tumor necrosis factor haplotypes and asthma. *Hum Mol Genet* 1997; 6:551–4.
- 24 Leung DY, Spahn JD, Nimmagadda SR, Szefer SJ. Induction of corticosteroid resistance in vitro. *Am J Respir Crit Care Med* 1996; 154:S34–8.
- 25 Leung DY, Martin RJ, Szefer SJ, Sher ER, Ying S, Kay AB et al. Dysregulation of interleukin-4, interleukin-5, and interferon- γ gene expression in steroid-resistant asthma. *J Exp Med* 1995; 181:33–40.