

Modulation by interleukin-4 of cytokine release from mononuclear phagocytes in asthma

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Background: Interleukin (IL)-4 is involved in IgE upregulation and downregulates cytokine release by mononuclear phagocytes. Mononuclear cells release greater amounts of IL-1, tumor necrosis factor- α , and IL-6 in patients with asthma than in control subjects, but the effect of IL-4 on cells from patients with asthma is unknown. The effects of IL-4 on the release of IL-1, tumor necrosis factor- α , and IL-6 by monocytes and alveolar macrophages were compared in 19 patients with asthma and 18 control subjects.

Methods: The release of IL-1, tumor necrosis factor- α , and IL-6 from unstimulated and lipopolysaccharide stimulated monocytes and alveolar macrophages was measured by ELISA. The effect of 30 U of IL-4 on the release of these cytokines was studied.

Results: Lipopolysaccharide-stimulated monocytes released significantly fewer cytokines in patients with asthma than in control subjects. IL-4 significantly inhibited cytokine release by monocytes of both groups. Unstimulated alveolar macrophages from patients with asthma released more cytokines than those of control subjects. Lipopolysaccharide induced a significantly greater increase in cytokine release in alveolar macrophages of control subjects in comparison with asthmatic subjects. IL-4 abolished the release of cytokines in alveolar macrophages from control subjects and had a minimal inhibitory effect on alveolar macrophages from patients with asthma.

Conclusions: Alveolar macrophages from patients with asthma are hyperreactive but less prone to lipopolysaccharide stimulation and IL-4-downregulation than those from normal subjects. (*J ALLERGY CLIN IMMUNOL* 1994;94:997-1005.)

Key words: Monocytes, alveolar macrophages, asthma, IL-1, TNF- α , IL-6, IL-4

Mononuclear phagocytes are involved in the inflammatory processes underlying asthma.¹ Monocytes and airway macrophages can be activated through an IgE-specific mechanism and can release numerous mediators promoting and perpetuating the bronchial inflammatory response.^{2, 3} They are increased in numbers in asthmatic bronchi⁴; are mainly of low density⁵; and are able to release free oxygen radicals,⁶ leukotrienes, and prostaglandins on specific or nonspecific chal-

Abbreviations used

AMs: Alveolar macrophages
BAL: Bronchoalveolar lavage
FCS: Fetal calf serum
IL: Interleukin
LPS: Lipopolysaccharide
TNF: Tumor necrosis factor

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lenge.⁷⁻⁹ In asthmatic subjects pathologic studies have shown that in airways, macrophages are in close contact with other inflammatory cells, particularly mast cells and lymphocytes.¹⁰ Moreover, macrophages have been shown to engulf mast cell granules. Many studies have evaluated the potential deleterious effects of these interactions on the bronchi, but little is known about the in situ regulation of macrophages.

Interleukin (IL)-4 is a cytokine derived from T

cells and mast cells, which promotes the synthesis of IgE.¹¹ Moreover, IL-4 has been found to be an inhibitor of mononuclear phagocyte functions *in vitro* and *ex vivo*.¹¹⁻¹⁴ The inhibitory effect of IL-4 on the generation of proinflammatory cytokines by alveolar macrophages (AMs) has also been reported in normal subjects.¹⁵ However, there is no study of the effects of IL-4 on mononuclear cells from patients with asthma.

Proinflammatory cytokines such as IL-1, tumor necrosis factor- α (TNF- α), and IL-6 produce several effects in common. They are pyrogenic, can induce the synthesis of acute-phase proteins, and amplify the immune and inflammatory reactions by the activation of lymphocytes, granulocytes, and structural cells.¹⁶⁻¹⁸ These cytokines can be released by AMs from normal subjects after lipopolysaccharide (LPS) stimulation.¹⁹ Moreover, it has been shown that TNF- α and IL-6 are released by AMs stimulated by anti-IgE obtained from patients with asthma. Patients in whom a late-phase reaction developed after allergen challenge demonstrated a greater cytokine release.^{20, 21}

To better understand the cell-to-cell relationships in asthmatic and normal subjects, we studied the effects of IL-4 on the spontaneous and LPS-induced release of IL-1 β , TNF- α , and IL-6 from peripheral blood monocytes and AMs obtained from 19 patients with asthma and 18 normal volunteers. We also attempted to correlate the release of cytokines by monocytes or AMs from patients with asthma with the severity and causes of asthma.

METHODS

Patients

Nineteen patients with asthma, ranging in age from 18 to 70 years (mean $\pm$ SD: 34.6 $\pm$ 16.7 years) were selected as previously described²² according to the criteria of the American Thoracic Society.²³ All had reversible airway obstruction assessed either by an increase of 15% of forced expiratory volume in 1 second (FEV₁) after inhalation of 200 μ g of salbutamol or a positive response to carbachol inhalation challenge. The severity of asthma was graded according to the score of Aas.²⁴ All patients had the same clinical evaluation including pulmonary function tests, allergy skin prick tests, RAST, and lung and sinus x ray studies.

Eighteen normal volunteers, ranging in age from 17 to 70 years (mean $\pm$ SD: 41 $\pm$ 10.2 years) were enrolled in the study. None of them had any history of lung or allergic disease. All subjects had normal lung function test results and negative allergy skin test results.

None of the subjects tested were smokers or former

smokers, and all drugs that might affect the interpretation of the study were carefully avoided. In particular, patients with asthma were excluded from the study if they had been treated with inhaled or systemic corticosteroids for a month; with sodium cromoglycate, nedocromil sodium, antihistamines, or theophylline for the past 2 weeks; or with β_2 -agonists for 48 hours before testing.

All subjects gave informed consent for the procedure, and this study fulfilled the criteria of the ethics committee of our hospital.

Recovery of monocytes

Blood was layered on a Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden) and the mononuclear cells were recovered by centrifugation according to the method of Boyum.²⁵ The monocytes were subsequently resuspended in RPMI 1640 medium (Gibco, Grand Island, N.Y.) at a concentration of 5×10^5 /ml and purified by adherence on plastic dishes for 2 hours. Then, the purity of the monocytes was assessed by immunohistochemistry with the panmacrophage monoclonal antibody anti-CD-14 (Dako, Versailles, France).

Recovery of AMs

The pulmonary cells were obtained by bronchoalveolar lavage (BAL) as previously described.⁵ Five to seven aliquots of 50 ml of sterile and isotonic saline solution were injected and gently aspirated with a syringe. The BAL fluid was at once centrifuged (400 g at 4° C for 20 minutes), and the cell pellet was used for subsequent study. Total and differential cell counts were done with a hemocytometer and Kimura staining. Viability was assessed with the trypan blue exclusion dye test. After cytocentrifugation and May-Giemsa-Grünwald staining, the differential cell populations were characterized. The BAL cells were resuspended at a concentration of 5×10^5 per milliliter in RPMI medium with 10% fetal calf serum and were allowed to adhere on plastic dishes for 2 hours in an incubator gassed with 5% CO₂ in a moist atmosphere. After adherence was achieved, the purity of the cells was assessed by immunohistochemistry with the panmacrophage monoclonal antibody anti-CD-68 (Dako).

Activation of the cells

The nonadherent cells from BAL or blood were removed by three gentle washings, and the remaining cells were maintained in the same environmental conditions overnight. In the first experiment unstimulated cells were incubated for 24 hours in the presence or absence of IL-4 (a kind gift of Dr. Banchereau, Schering-Plough Research, France). In a second experiment cells were stimulated for 24 hours with 10 μ g/ml LPS^{26, 27} in the presence or absence of 30 U of IL-4.¹⁵ The supernatants were recovered after 24 hours of culture and stored at -20° C for cytokine measurements. After IL-4 stimulation, the viability of the cells was assessed by trypan blue exclusion.

TABLE 1. Demographic data and BAL fluid analysis

Sex	Age (yr)	Allergy	AAS score	FEV ₁ (% pred values)	BAL fluid (recovery %)	Cells (103/ml)	Macrophages (%)	Lymphocytes (%)	Eos (%)	PMNS (%)	Epithelial cells (%)
M	61	0	3	83	36	310	64	22	4	10	0
F	22	+	3	69	40	160	86	8	1	5	0
F	20	+	1	100	44	248	96	3	0	0	1
M	29	+	3	50	42	152	78	19	0	0	3
F	52	0	4	75	56	416	65	21	0	14	0
M	70	0	2	100	25	192	76	5	4	2	13
M	29	+	3	98	52	336	75	4	16	2	3
F	29	+	2	50	40	211	82	13	3	2	0
F	52	0	4	84	44	102	92	8	0	0	0
F	19	+	2	74	53	236	74	17	2	4	3
F	17	0	4	50	20	451	78	19	0	2	1
M	30	0	1	100	90	336	97	2	0	0	0
F	27	0	3	34	40	130	69	24	2	4	1
F	43	0	2	114	40	153	69	10	2	4	15
M	26	+	4	33	60	152	71	9	2	2	16
M	31	0	1	96	46	80	70	11	0	7	12
F	51	0	3	43	40	136	88	7	0	1	0
F	21	+	4	47	32	128	44	26	5	20	5
F	23	0	2	64	60	280	96	2	0	1	3

FEV₁, Forced expiratory volume in 1 second; Eos, eosinophils; PMNs, polymorphonuclear neutrophils.

Cytokine measurement

IL-1 β , TNF- α , and IL-6 were measured in the cell supernatants with a specific ELISA from Medgenix (Belgium). The ELISA was highly specific for each cytokine, and no cross reactivity with other cytokines was detected. The limit of detection of these assays was 10 pg/ml.

Limitations of the study

The number of cells recovered did not allow the performance of the whole study for each subject. For the monocytes, spontaneous and LPS-induced release was studied in 10 patients with asthma and 10 normal subjects, and the modulation by IL-4 was studied in six subjects in both groups. For the AMs, the spontaneous and LPS-induced release was studied in 16 patients with asthma and eight normal subjects, and the modulation by IL-4 was studied in six subjects in both groups.

The release of cytokines by monocytes or AMs from patients with asthma was correlated with the severity of asthma as determined by the clinical Aas score or FEV₁. We also determined whether there was a difference in cytokine release between allergic and nonallergic asthmatic subjects. Allergic asthma was characterized by a typical history of allergic disease and the presence of positive skin test results and specific serum IgE.

Nonparametric tests were used with the Mann-Whitney U test and the Wilcoxon W test. Correlations were performed with the Spearman rank test. Results are expressed as means $\pm$ SD.

RESULTS

Demographic characteristics of the patients

The demographic data concerning the patients and the BAL fluid analysis are summarized in Table 1. The procedure was well tolerated by all of the subjects, and no bronchospasm occurred at the end of the fiberoptic bronchoscopy.

Release of cytokines

Monocytes. The purity of the cells was very high because 99% (mean $\pm$ SD: 98 $\pm$ 1) were CD-14-positive. The spontaneous release of cytokines by peripheral blood monocytes was undetectable in the supernatants from both groups of subjects. The LPS-induced release of IL-1 β was not significantly different in both groups of cells, whereas TNF- α and IL-6 were significantly increased in cell supernatants obtained from normal subjects in comparison with asthmatic patients (5321 $\pm$ 1065 pg/5 $\times$ 10⁵ viable cells vs 3544 $\pm$ 2829 pg/5 $\times$ 10⁵ viable cells for IL-1 β , NS; 9718 $\pm$ 2110 pg/5 $\times$ 10⁵ viable cells vs 5670 $\pm$ 1903 pg/5 $\times$ 10⁵ viable cells for TNF α ; 13708 $\pm$ 2625 pg/5 $\times$ 10⁵ viable cells vs 8298 $\pm$ 3381 pg/5 $\times$ 10⁵ viable cells for IL-6; p < 0.001 for TNF- α and IL-6, Mann Whitney U test) (Fig. 1).

There was no correlation between cytokine release by monocytes and the severity of asthma, as determined by the clinical score of Aas or FEV₁.

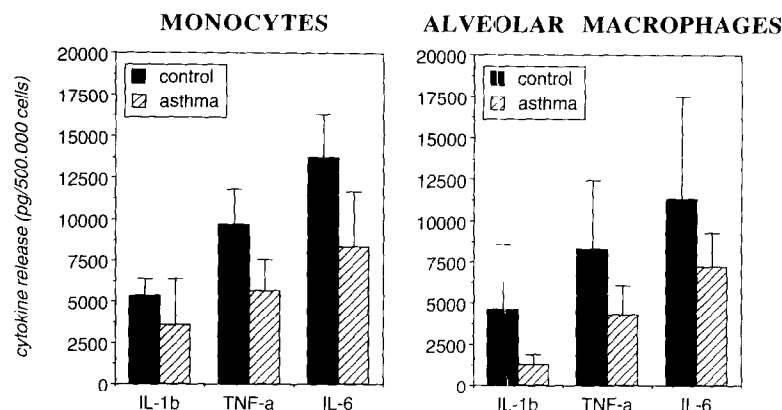


FIG. 1. LPS-induced release of cytokines.

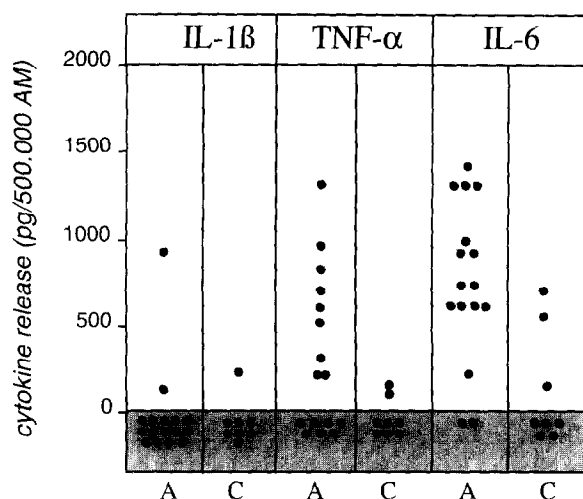


FIG. 2. Spontaneous release of cytokines by AMs. A, Patients with asthma; C, control subjects.

No difference in cytokine release was observed between allergic and nonallergic asthmatic subjects.

AMs. The purity of the cells was very high because 98% (mean $\pm$ SD: 98.5 ± 0.5) were CD-68-positive. AMs from normal subjects spontaneously released IL- β in 1 of 8 experiments, TNF- α in 2 of 8 experiments, and IL-6 in 3 of 8 experiments. AMs from asthmatic subjects spontaneously released IL-1- β in 2 of 16 experiments, TNF- α in 9 of 16 experiments, and IL-6 in 14 of 16 experiments. There was a significant difference in IL-6 release between patients with asthma and normal subjects (Fig. 2).

The number of cytokines released after LPS stimulation was significantly higher in the supernatants of AMs obtained from normal subjects as

compared with those from patients with asthma (4611 ± 3971 pg/ 5×10^5 viable cells vs 1236 ± 601 pg/ 5×10^5 viable cells for IL-1 β ; 8269 ± 4155 pg/ 5×10^5 viable cells vs 4283 ± 1804 pg/ 5×10^5 viable cells for TNF α ; 11291 ± 6217 pg/ 5×10^5 viable cells vs 7187 ± 2157 pg/ 5×10^5 viable cells for IL-6; $p < 0.001$ for all three tests, Mann Whitney U test) (Fig. 1). There was a significant correlation between the LPS-induced release of IL-6 and TNF- α by AMs from patients with asthma ($p = 0.80$, $p < 0.01$, Spearman rank test). There was no correlation between cytokine release by AMs and the severity of asthma assessed by the clinical score of Aas or FEV $_1$. No difference in cytokine release was observed between allergic and nonallergic asthmatic subjects.

Modulation of cytokine release by IL-4

The addition of IL-4 to monocytes or AMs did not modify the spontaneous release of cytokines. The addition of IL-4 to LPS almost totally abolished the generation of cytokines from monocytes and AMs in normal subjects ($p < 0.001$ and $p < 0.03$, respectively, Wilcoxon W test) and significantly reduced the generation of cytokines from monocytes in asthmatic subjects ($p < 0.03$, Wilcoxon W test) (Fig. 3). On the other hand, IL-4 decreased minimally and nonsignificantly the release of cytokines by LPS-stimulated AMs from patients with asthma (Fig. 4). The viability of the cells was not affected by cell adherence or by IL-4 incubation as determined by the trypan blue exclusion test.

DISCUSSION

This study shows that mononuclear phagocytes obtained from patients with asthma differ from

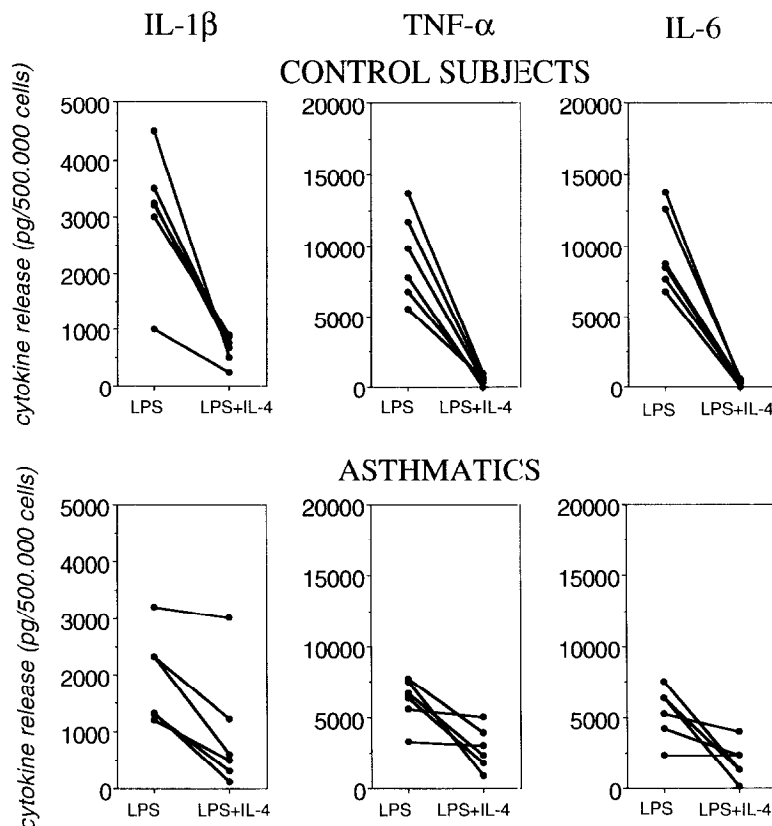


FIG. 3. Inhibitory effects of IL-4 on the release of cytokines by LPS-stimulated monocytes.

those obtained from normal subjects in their ability to release inflammatory cytokines, spontaneously or after stimulation. Furthermore, the cell regulation is different in the two groups of subjects. AMs from asthmatic subjects spontaneously release more cytokines than AMs from normal subjects, but they have a decreased response to a nonspecific *in vitro* LPS stimulation. IL-4 blocks the release of cytokines by monocytes and AMs stimulated by LPS in normal subjects. In patients with asthma IL-4 downregulates moderately LPS-stimulated monocytes and has no effect on LPS-stimulated AMs.

This study was carried out because: (1) mononuclear phagocytes are important in airway inflammation, (2) they can release several cytokines including IL-1 β , TNF- α , and IL-6^{16, 18} that may be relevant to the regulation of inflammation of the airways, and (3) IL-4 released in the airways of patients with asthma^{28, 29} has been shown to exert immunoregulatory effects on mononuclear phagocytes.¹²⁻¹⁴ This study is original in many aspects including the activation of mononuclear phagocytes from patients with asthma by LPS and the

regulation of cell activation in patients with asthma by IL-4.

The release of cytokines has been shown to be triggered by several stimuli. Among these, LPS appears to be an important stimulus and is clinically relevant in infectious diseases and possibly in asthma.³⁰ We did not test our culture medium for endotoxin, and it is possible that some might be present, but such a possibility does not change the significance of our results because we used the same culture medium throughout the study. Furthermore, it is also possible that the airways themselves may contain some endotoxins. Moreover, because there was a high spontaneous release of cytokines in asthma and a minor effect of LPS on the same cells, the contamination by endotoxins does not appear to be relevant. We did not select pneumallergens as stimuli because none of the normal subjects and only some of the patients with asthma were allergic to them. The use of IgE-anti-IgE has been previously shown to stimulate the production of cytokines, but this requires a first step of *in vitro* passive sensitization with serum, which may contain priming cytokines

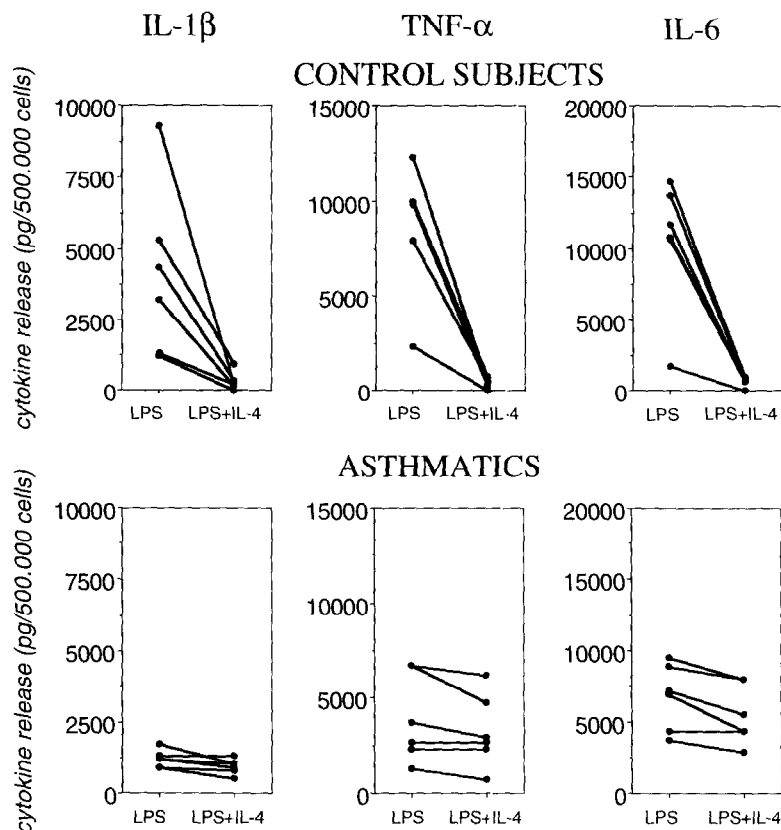


FIG. 4. Inhibitory effects of IL-4 on the release of cytokines by LPS-stimulated AMs.

and could increase the spontaneous response of the mononuclear phagocytes.^{20, 21}

Although the isolation of mononuclear cells by adherence is a routine procedure, the culture of cells with fetal calf serum overnight and adherence on plastic can activate them to spontaneously release mediators and to express mRNA.^{31, 32} However, because the procedure was identical in asthmatic and normal subjects, it should affect cells similarly.

The measurement of cytokines with accurate and sensitive methods is now widespread. The ELISAs used in this study are probably the most suitable and sensitive. They are based on a two-site-method and use several monoclonal antibodies against the distinct epitopes of each cytokine, thus allowing a better sensitivity of detection.³³ However, levels obtained with these tests may be higher than those obtained with other commercially available tests.

Release of cytokines and significance

In accordance with previous results, the spontaneous production of cytokines was low or undetectable in the supernatants from monocytes and

AMs of normal subjects.^{19, 20, 26} On the other hand, AMs from patients with asthma were already stimulated because they produced cytokines as previously shown for IL-1,²⁷ IL-6, and TNF- α .^{20, 21}

The number of cytokines released after stimulation of the mononuclear phagocytes by LPS was significantly greater in normal subjects than in patients with asthma, except for the release of IL-1 β by monocytes. In normal subjects the production of cytokines by stimulated mononuclear phagocytes has been widely studied. AMs release lower amounts of IL- β than monocytes.³³ On the other hand, the release of TNF- α and IL-6 is usually, but not always, higher in AMs than in monocytes.¹⁹ These results are in accordance with those of this study. Using an IgE-anti-IgE stimulus, Gosset et al.²⁰ found an increased release of cytokines in the supernatant of AMs and monocytes obtained from patients with asthma,²¹ but according to the reactivity of the patients after allergen challenge, they observed differences in cytokine release by AMs. On the other hand, no difference was observed in the LPS-induced liberation of IL-1 β in the supernatant of AMs from seven patients with asthma and eight normal

subjects.²⁷ These discrepancies may be explained by the heterogeneity of asthma or by the presence of cytokine inhibitors. An inhibitory factor against IL-1 has been found in the supernatants of AMs from normal subjects and patients with asthma,^{34, 35} which might explain the lack of spontaneous production by AMs, as shown in this study.

IL-1 β , TNF- α , and IL-6 released by mononuclear phagocytes are involved in the modulation of cellular immunity and in recruitment and activation of inflammatory or structural cells.¹⁶⁻¹⁸ These cytokines may therefore be involved in asthmatic bronchial inflammation. TNF- α is a potent *in vivo* and *in vitro* chemoattractant and cell activator, which is also able to activate AMs in an autocrine pathway, leading to an increase in their phagocytic activities.³⁶ IL-6 is involved in the control of many cell functions including IgE antibody synthesis, T-cell cytotoxicity, stem cell differentiation, and acute-phase protein synthesis.³⁷ IL-6 can inhibit the proliferation of macrophages,³⁸ and their spontaneous release by AMs of patients with asthma may explain the lack of proliferation of AMs we have recently described.³⁹ Moreover, in patients with asthma the release of IL-6 is significantly correlated with release of TNF- α in AMs stimulated by anti-IgE²⁰ or LPS (this study), suggesting common regulatory pathways.³⁷ The spontaneous release of cytokines by AMs in asthma may be related to the increased activation of lymphocytes and epithelial and endothelial cells described in asthma.^{40, 41}

The lack of relationship between the severity of asthma assessed by the Aas score and the release of cytokines is questionable. This score is based on asthma symptoms that occurred during the year preceding the procedure, and we had shown in at least one other study⁵ that the recent ongoing activity of the disease during the last 2 weeks might be more related to the *in vitro* activation of the cells than to the Aas score. The inflammatory processes underlying bronchial asthma involve numerous cells and mediators, which may not differ in allergic or nonallergic subjects.²²

Regulatory effect on IL-4

IL-4 is involved in a variety of immunologic reactions. It is a growth factor for activated T cells and mast cells and stimulates the maturation of blood monocytes.⁴² It can increase the expression of cell membrane markers such as the Fc ϵ RIIb (CD23) and class II major histocompatibility complex antigen on many cells, including monocytes⁴³ and IL-2R (CD25) on B cells.⁴⁴ Furthermore, IL-4 is a key factor in the production of IgE,¹¹ and its

role in the pathophysiology of allergic disease is currently stressed.

The regulation of mononuclear phagocytes by IL-4 has been widely studied in normal subjects. IL-4 was shown to decrease the production of cytokines such as IL-1, TNF- α , IL-6,¹⁵ oxygen free radicals,⁴⁵ and metalloproteinases.⁴⁶ This inhibition was effective at the transcriptional level¹⁵ and was not due to cytotoxic effect because the cell viability remained unchanged.¹⁵ The results of this study are in accordance with previously published data on cells from normal subjects. On the other hand, in patients with asthma there was a significantly decreased effect of IL-4 on monocytes and almost no effect on AMs.

Monocytes and AMs appear to be differentially regulated in normal subjects and patients with asthma. In normal subjects, depending on their patterns of maturation and differentiation, mononuclear phagocytes differ by their peroxidase activity, arachidonic acid derivatives,^{47, 48} and potential antigen-presenting cell activity.⁴⁹ Monocytes and AMs are activated in asthma as shown by their morphologic features⁵ and their ability to release mediators.^{50, 51} The differences in the response of monocytes and AMs from patients with asthma to IL-4 may be due to different mechanisms. The local inflammation surrounding AMs might alter their potential response to the inhibitory effect of IL-4. These findings suggest a constant activation of AMs in asthma and a lower sensitivity of these cells to physiologic suppressive factors. Alternatively, this lack of response may be due to a decreased number or affinity of IL-4 receptors or to the rapid degradation of IL-4 as a result of the high potential proteolytic activity of AMs. In patients with asthma it appears therefore that IL-4 cannot play its major role as a suppressor of immune and inflammatory responses of mononuclear phagocytes, leading to the amplification of the deleterious effects of AMs on the airways.

This study shows that monocytes and AMs of patients with asthma differ in their activation patterns and regulation. It is likely that AMs from patients with asthma are spontaneously upregulated but are less reactive to stimulatory and inhibitory effects.

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