

Phenotypic features of alveolar monocytes/macrophages and IL-8 gene activation by IL-1 and TNF- α in asthmatic patients

The alveolar macrophage (AM), a major defense cell in the lung, participates in immune and inflammatory reactions through the release of several regulatory and chemotactic cytokines. In particular, macrophages are considered to play a pivotal proinflammatory role in the production and maintenance of airway inflammation and bronchial hyperreactivity. To assess the phenotypic pattern of AM from asthmatic subjects, we performed the following experiments:

- 1) cytofluorometric analysis of specific phenotypic features (CD11b, CD14, CD16, CD45, HLA-DR, CD71, CD95, and CD44)
- 2) assessment of the production of tumor necrosis factor (TNF)- α , interleukin (IL)-1, and the chemotactic regulatory cytokine IL-8 by unstimulated and lipopolysaccharide-stimulated AM.

In these patients, we phenotypically characterized the AM, showing their strong proinflammatory activity also in patients with mild asthma. Their activity has been clarified by our biomolecular data that showed a constitutive basal IL-8 production by AM, and also indicated that IL-1 and TNF- α were able to upregulate the ability of activated human AM to produce IL-8 at the protein and messenger ribonucleic acid (mRNA) levels.

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Macrophages are considered to play a pivotal proinflammatory role in the production and maintenance of airway inflammation and bronchial hyperreactivity. Cytokine-mediated interactions between macrophages, lymphocytes, and eosinophils are important in the inflammatory reaction of the asthmatic airways. In particular, it has recently been postulated that interleukin (IL)-8 plays a key role in the pathogenesis of asthma. There is a growing body of evidence that IL-8 is a potent eosinophil chemoattractant (1), and, therefore, directly involved in the pathogenesis of respiratory allergic reactions, whereas eosinophils are the principal inflammatory cells mediating tissue damage (2).

The modulation of IL-8 production by alveolar macrophages (AM) is not well known, but various molecules have been identified as their potential stimulators. IL-1 and tumor necrosis factor (TNF)- α were found to affect IL-8 production in peripheral blood monocytes from healthy donors (3). However, several reports show how these two cytokines could regulate the synthesis of IL-8 in human AM in response to activation stimuli, as cytokine synthesis was shown

to be regulated differentially in monocytes and macrophages (4).

Identification of phenotypic features of AM

Since activated macrophages may secrete many immunologic factors, it is important to characterize the AM in terms of both phenotype and secretory pattern (5, 6). In this study, we report results on the activation of unstimulated and (LPS)-stimulated AM from patients with mild asthma. Monocyte/macrophage differentiation and their phenotypic features were assessed with monoclonal antibodies against specific antigens present on the plasma membrane (CD11b, CD14, CD16, CD45, HLA-DR, CD71, CD95, and CD44) by cytofluorometry.

To establish the secretory pattern of AM, we evaluated the secretion and release time of specific cytokines (TNF- α , IL-1 α , IL-1 β , IL-6, and IL-8) in conditioned culture medium of unstimulated and lipopolysaccharide (LPS)-stimulated AM obtained from asthmatic subjects; moreover, using specific

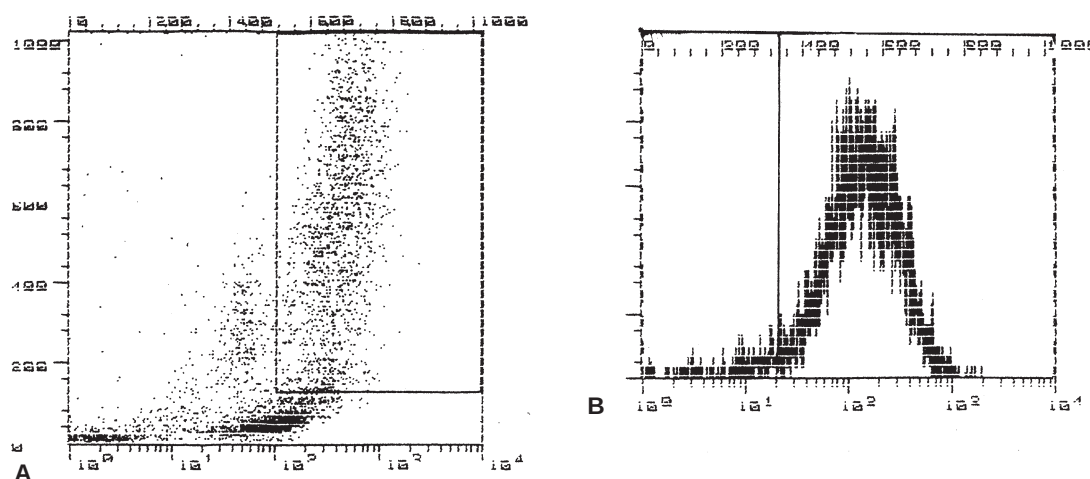


Figure 1. Flow cytometric identification of alveolar macrophages (AM). A) Dot-plot side scatter vs FL3 (CD45 PERCP); gate opened on CD45^{bright} cells to exclude lymphocytes. B) Histogram of CD11b⁺ cells.

antibodies against TNF- α and IL-1, we studied how the release of these inflammatory cytokines upregulates the ability of activated AM to modulate IL-8 synthesis at the protein and messenger ribonucleic acid (mRNA) levels (7).

Experimental study

Sixteen atopic, nonsmoking patients with mild asthma were selected according to the criteria of the American Thoracic Society (8). All patients underwent pulmonary function tests, allergy skin prick test, and chest radiography. Subjects with diseases other than allergic asthma and rhinitis were excluded from this study.

The alveolar cells were obtained by the bronchoalveolar lavage (BAL) technique (9). The BAL cells were resuspended at a concentration of 5×10^5 /ml in RPMI 1640 medium supplemented with L-glutamine, sodium-pyruvate, antibiotics, and 10% heat-inactivated human serum, and were allowed to adhere on plastic dishes in an incubator with 5% CO₂ in air at 37°C. When adherence was achieved, the purity of the cells was assessed by immunohistochemistry with the panmacrophage monoclonal antibody anti-CD68 (Dako).

The nonadherent cells from BAL were removed by three gentle washings. For *in vitro* activation, monolayers of AM were incubated for 24 h in triplicate cultures with or without various amounts of LPS, in the presence or absence of anti-TNF- α and/or anti-IL-1 antibodies. Cytokine concentrations in the cell-free supernatants were measured by commercial enzyme-linked immunosorbent assay (ELISA) (Genzyme, Milan, Italy) in triplicates for each of the experiments, with recombinant cytokines as controls.

Phenotypic features

Macrophages recovered from BAL were centrifuged and resuspended at 1.5×10^6 cells/ml in serum-free medium (RPMI1640/Dulbecco-Vogt, 1:1 v/v) containing 0.01 mg/ml bovine serum albumin and lactalbumin hydrolysate.

The supernatant obtained by centrifugation of BAL was concentrated, filtered through membranes capturing molecules over 5 kDa, and stored at -80°C to be tested by radioimmunoassay (RIA).

Cytofluorometric analysis of BAL cell population was performed according to the following criteria. To define the macrophage cluster, we used physical parameters (forward and side scatter), as well as phenotypic features (CD45^{bright} + and CD11b +) (Fig. 1). CD45 allowed us to distinguish these cells from nonleukocyte cells (CD45-negative) and from polymorphonuclear cells (CD45^{dim}). To determine the percent positivity and the affinity level of the above AM population, we tested with specific mAbs (CD14, CD16, CD45, HLA-DR, CD71, CD95, and CD44).

In our group of patients, a high percentage of AM positive for HLA-DR was detected, the major histocompatibility complex (MHC) II type (Fig. 2). The significant presence of this cell-wall antigen, essential for the antigen recognition by CD4 + lymphocytes, demonstrated macrophage activation in mild asthma, due to their antigen-presenting function. Although showing a different antigenic affinity, a high percentage of AM bound CD71 (mAb for the transferrin receptor), demonstrating an active proliferation of these cells (Fig. 3).

We also found a low percentage of CD95 + cells, a marker of FAS-mediated apoptosis (Fig. 4). These data confirmed their preferential differentiation to a proliferative rather than apoptotic condition, even if other apoptotic pathways could not be excluded.

The AM tested showed a low percentage of CD14 and CD16 expression (Fig. 5), specific markers of

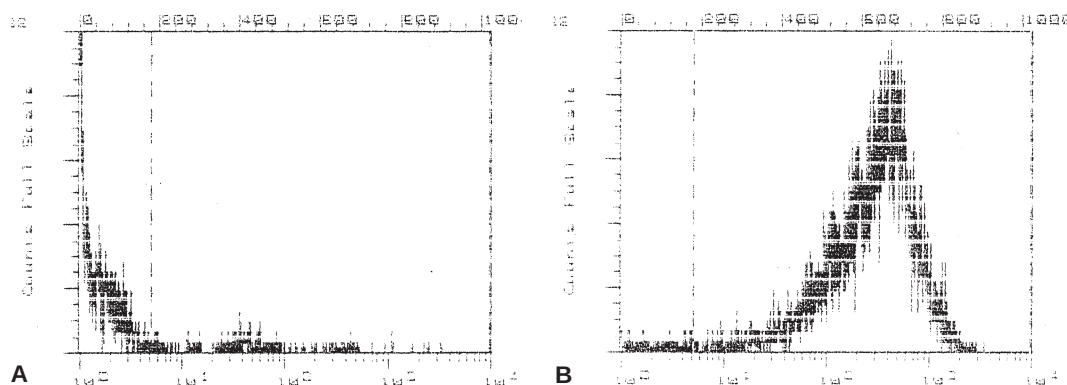


Figure 2. Histogram referring to region identifying AM in Fig. 1. A) Negative control. B) Cells HLA-DR+.

monocyte cells; these cells probably exhibit selective differentiation growth, explaining the lack of recognition of their origin.

Finally, AM from asthmatic patients exhibited an enhanced expression of CD44 (Fig. 6), the receptor for hyaluronic acid, a molecule involved both in the interaction with endothelial cells and with the macrophage migration from peripheral blood to the tissues (13).

Time kinetics of cytokine protein induction in AM

The purity of the AM was very high because 97% were CD68-positive. We examined the time kinetics of cytokine release by LPS- (1 μ g/ml) stimulated AM over 24 h. Results for TNF- α , IL-1, IL-6, and IL-8 are summarized in Fig. 7. Accumulation of TNF- α induced by LPS seemed to reach of plateau after approximately 12 h, following a strong increase beginning 4 h after stimulation. The next measured cytokine to appear was IL-1. Concentrations rose rapidly from background levels at 4 h, and reached a plateau at 12 h, the values not changing significantly throughout the remainder of the time course.

Large amounts of IL-6 could be detected after 12 h, while the maximum level of IL-6 in the supernatant was reached at 24 h. Although the IL-6 levels in the

conditioned media increased very rapidly until 15 h, they continued to rise at slower rate up to 24 h.

In contrast, IL-8 revealed a quite different kinetic pattern of expression. Levels increased slowly during the first 12 h, and then rose rapidly, still apparently escalating at 24 h. AM viability was also evaluated throughout the time course and was found not to change significantly over the 24-h incubation period.

LPS induction of cytokine mRNA transcription

In previous experiments, we found that cytokine mRNA levels reflect the profile of protein release, generally increasing with the magnitude of the LPS stimulus (12). In this study, the pattern of IL-1 β , IL-6, and IL-8 mRNA expression in response to 1 μ g/ml LPS was also examined at the 4- and 24-h time points. Moreover, IL-1 β and IL-6 mRNA expression was transient, returning to near background levels at 24 h, whereas IL-8 mRNA levels continued to rise throughout the 24-h period (Fig. 8).

Anti-TNF- α and anti-IL-1 antibody effect on IL-8 production

IL-1 and TNF- α have been shown to be potent inducers of IL-8 gene expression in cells cultured from a number of different tissues (7). Accordingly, experiments were

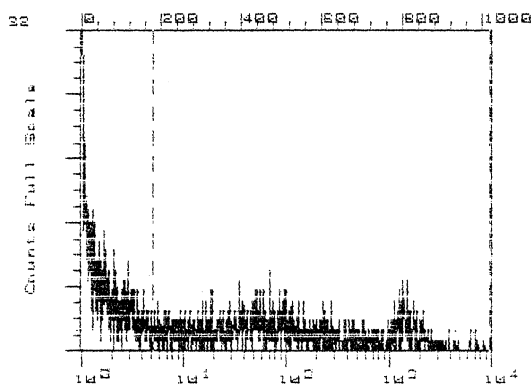


Figure 3. Histogram of CD71+ cells, referring to region identifying AM in Fig. 1.

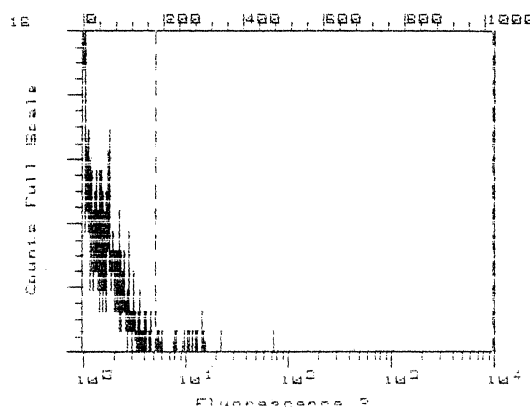


Figure 4. Histogram of CD95+ cells, referring to region identifying AM in Fig. 1.

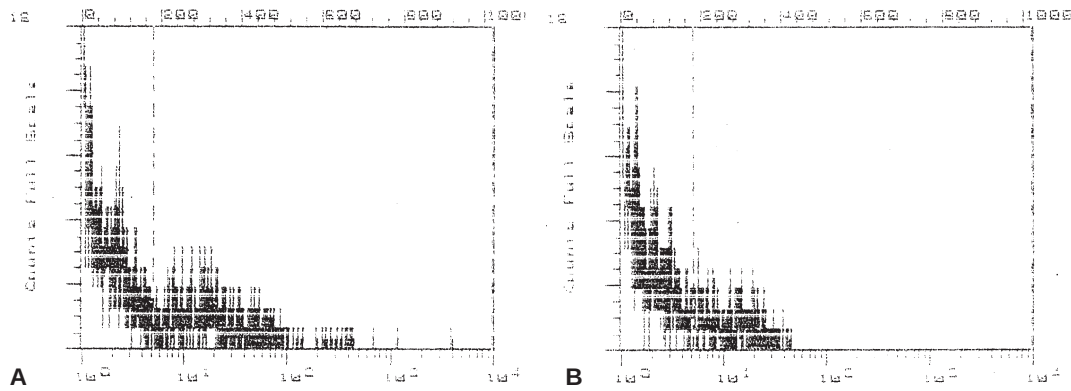


Figure 5. A) Histogram of CD14+ cells, referring to region identifying AM in Fig. 1. B) Histogram of CD16+ cells, referring to region identifying AM in Fig. 1.

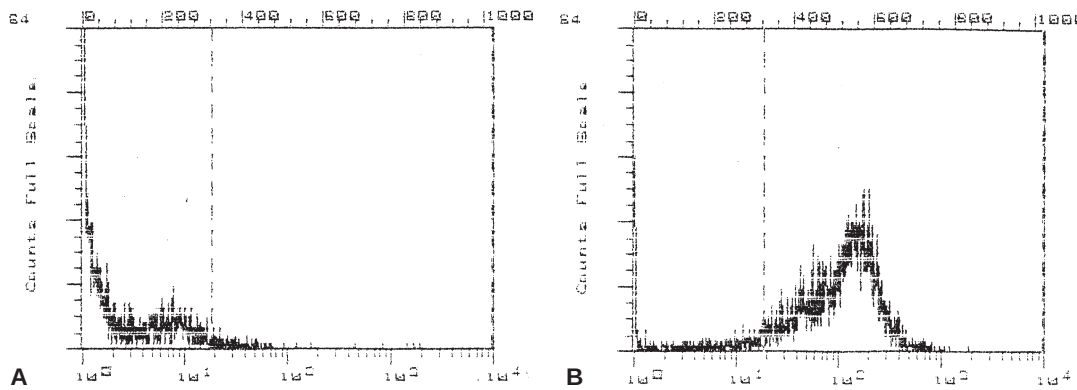


Figure 6. Histogram referring to region identifying AM in Fig. 1. A) Negative control. B) CD44+ cells.

performed to determine whether LPS-stimulated AM could synthesize IL-8 after exposure to anti-TNF- α and/or anti-IL-1 antibodies. The neutralizing antibodies were added singly and in combination. Fig. 8 shows the IL-8 release in the presence of LPS plus control antibody and LPS combined with both the anti-TNF- α and anti-IL-1 antibodies. IL-8 levels in AM incubated with LPS

plus control antibody increased significantly ($P < 0.01$) if compared with those present in the cultures incubated with LPS plus the anti-TNF- α or anti-IL-1 antibodies. The combination of the two antibodies caused a significant ($P < 0.001$) further reduction of IL-8 secretion.

IL-8 mRNA expression was also examined in the presence of LPS alone and LPS with the combined anti-TNF- α and anti-IL-1 antibodies. Consistent with the results for the levels of secreted IL-8 protein as measured in the ELISA, the presence of the antibodies anti-TNF- α and anti-IL-1 produced a marked abrogation of mRNA expression (Fig. 7). The anti-TNF- α and anti-IL-1 antibodies alone were found to have a similar but slightly less substantial ability to block IL-8 mRNA production.

Conclusion

In this study, the AM from mildly asthmatic patients were detected and characterized for their phenotypic pattern of activation and differentiation (14). This new method of AM recognition considered the various affinities of these cells to specific mAb (HLA-DR, CD14, CD16, CD71, CD44, and CD95). The elevated expression of HLA-DR, CD71, and CD44 and the

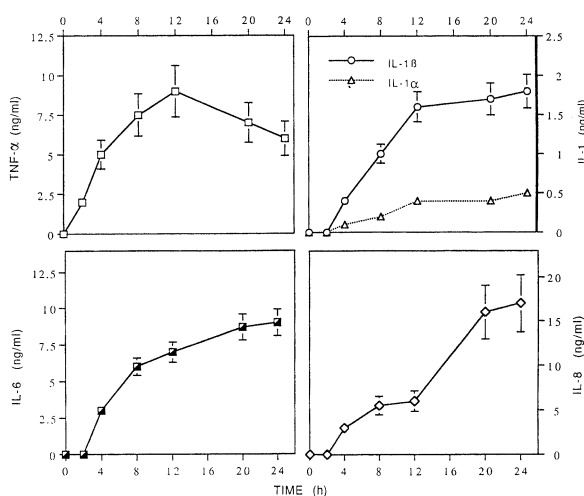


Figure 7. Time kinetics of cytokine protein induction in AM.

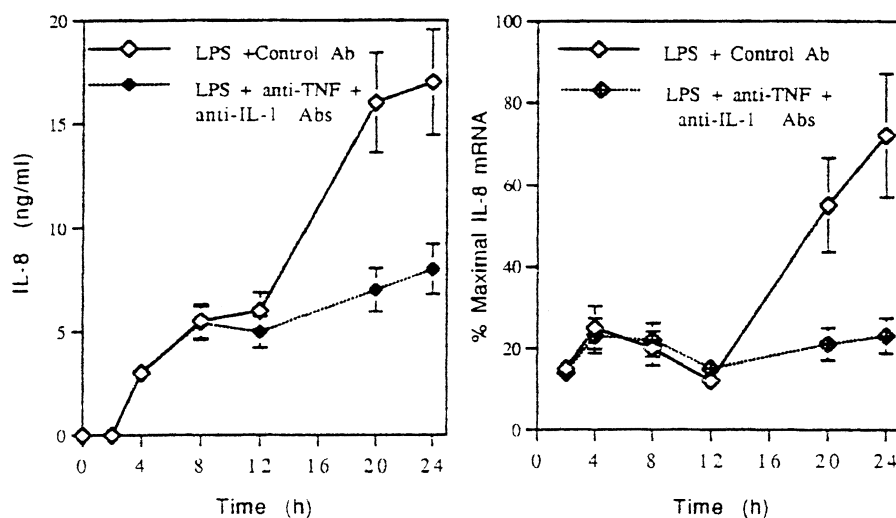


Figure 8. LPS induction of cytokine mRNA transcription.

correspondingly low expression of CD95 indicated a high grade of activation level of AM.

The low expression of CD14 and CD16, specific antigens of monocyte cells, suggested that AM follow a specific differentiation pathway different from the common monocyte differentiation line.

Secondly, the same cells were used as an *ex vivo* model to study the local IL-8 production. Our *in vitro* studies demonstrated that the IL-8 gene was transcriptionally active in unstimulated AM from mildly asthmatic patients, although mRNA levels were low. As previously reported, the basal rate of IL-8 synthesis in freshly isolated AM was considerably higher than that of the other inflammatory cytokines, such as IL-1, IL-6, and TNF- α (14). AM expression of IL-8 is particularly sensitive to induction by LPS, and it is induced and expressed at the highest level under conditions which weakly induce TNF- α and IL-1. Indeed, the kinetics of IL-8 production depicted in Fig. 7 closely followed the time course of IL-1 and TNF- α . The sequential appearance of the cytokines, in the order of these molecules on IL-8 synthesis, is consistent with synergetic positive effects of IL-1 and TNF- α on IL-8 production (15). Indeed, both TNF- α and IL-1 were observed to induce IL-8 secretion, while IL-6 was found to have no effect on IL-8 synthesis at all concentrations used (data not shown). This finding has been validated by other experiments in which anti-TNF- α antibody and a mixture of anti-IL-1 antibodies were added to LPS-stimulated AM

alone and in combination. In fact, only a low percentage of production of IL-8 protein and mRNA occurring after stimulation of LPS was not altered by the presence of the antibodies (16). Furthermore, the addition of the anti-TNF- α and anti-IL-1 antibodies alone showed a less substantial inhibition of IL-8 production than the combination of anti-TNF- α plus anti-IL-1. These data suggest that the stimulus for IL-8 synthesis determined by LPS itself may be self-limiting, while the early, LPS-stimulated production of cytokines such as TNF- α and IL-1 served as the signal for the prolonged, escalating IL-8 secretion. Thus, although the initial phase of production of IL-8 (both protein and mRNA) occurring over 4–12 h after stimulation was not altered by the presence of the antibodies, the secondary prolonged rise observed in the presence of LPS, and anti-TNF- α and anti-IL-1 antibodies was almost completely ablated at the level of both protein and mRNA.

In conclusion, one can describe a possible autocrine/paracrine cytokine network regulating IL-8 release by LPS-stimulated AM, as reported in the literature (17). During the first 12 h of stimulation by LPS, AM initially secreted a certain amount of IL-8, with a concomitant release of IL-1 and TNF- α . The latter cytokines have the potential to exert feedback modulation on the adjacent or the same AM by binding to their respective receptor (IL-1R and TNF- α R), resulting in increased secretion of IL-8 at later (up to 20 h) incubation times.

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