

Original article

In allergic asthma experimental exposure to allergens is associated with depletion of blood eosinophils overexpressing LFA-1

Background: In atopic individuals, exposure to allergens is followed by recruitment of blood eosinophils in the target tissue. We investigated whether allergen inhalation challenge could result in depletion of blood eosinophils overexpressing adhesion molecules involved in eosinophil migration.

Methods: Blood eosinophils were isolated from seven atopic asthmatic patients and seven control subjects and the “at baseline” expression of lymphocyte function-associated antigen-1 (LFA-1), macrophage antigen-1 (Mac-1) and very late antigen-4 (VLA-4) was assessed by monoclonal antibody staining and flow cytometry analysis. Asthmatic patients underwent allergen challenge and the expression of LFA-1, Mac-1 and VLA-4 by blood eosinophils was again evaluated 3 h and 24 h after allergen challenge.

Results: As compared to controls, eosinophils from atopics showed at baseline enhanced LFA-1 expression ($P = 0.0012$), but similar Mac-1 or VLA-4 expression ($P > 0.1$, each comparison). In atopics, the percentage and absolute number of blood eosinophils were significantly decreased 3 h after allergen challenge ($P = 0.001$ and $P = 0.022$, respectively) but returned to similar values to prechallenge values after an additional 21 h ($P > 0.1$). Allergen challenge was also followed by a significant decrease in LFA-1 expression by eosinophils, at 3 h ($P = 0.002$) and at 24 h ($P = 0.038$), while no changes in Mac-1 and VLA-4 were observed. A significant correlation between postchallenge decrease in LFA-1 expression and in blood eosinophilia, both expressed as percentage ($r = 0.88$; $P < 0.01$) or absolute number ($r = 0.87$; $P < 0.01$) was demonstrated at 3 h ($r = 0.88$; $P < 0.01$) but not at 24 h ($r = 0.64$, $P > 0.05$ and $r = 0.11$; $P > 0.05$, respectively).

Conclusion: In allergic asthma, an early recruitment of blood eosinophils overexpressing LFA-1 occurs in the first hours after allergen challenge.

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Airway inflammation is an important feature of allergic asthma and is thought to contribute to the development of allergen-induced bronchoconstriction and airway hyperresponsiveness (1, 2). Among the cells characterizing the inflammatory changes associated with asthma, eosinophils are thought to play a major role (1–3). Eosinophils, produced in the bone marrow under the influence of a variety of cytokines, are recruited and activated at sites of disease activity where they release a variety of products, able to enhance the inflammatory reaction, induce bronchoconstriction and damage airway tissues (3–6).

Eosinophil recruitment from the circulation to the lung is a complex mechanism, which includes response to chemotactic signals and interaction between surface adhesion molecules, expressed by circulating cells, and counterreceptors, expressed by vascular endothelial cells

(7–9). Indeed, chemotactic signals, such as Th2 cytokines [interleukin (IL)-3, IL-5 and granulocyte/macrophage-colony stimulating factor (GM-CSF)] and C-C chemokines [eotaxin, monocyte chemotactic protein-3 (MCP-3) and RANTES (regulated upon activation, normal T expressed and presumably secreted)], besides priming and activating eosinophils, increase cell surface receptor and adhesion molecule expression on these cells (10–12). Eosinophils express a variety of adhesion molecules, which include the β_2 -integrins LFA-1 (CD11a/CD18: $\alpha_1\beta_2$) and Mac-1 (CD11b/CD18: $\alpha_M\beta_2$), and the α_4 -integrin VLA-4 (CD49d/CD29: $\alpha_4\beta_7$) (8, 9). VLA-4 (very late antigen-4) and its endothelial cell counterreceptor, vascular cell adhesion molecule-1 (VCAM-1), appear to play an essential role in the initial adherence of activated eosinophils (13), while LFA-1 (lymphocyte function-associated antigen-1), Mac-1 (macrophage

antigen-1) and their counterreceptor, intercellular adhesion molecule (ICAM)-1, are important for eosinophil extravasation (14,15).

In atopic asthma, a condition characterized by increased secretion of Th2 cytokines and C-C chemokines, the expression of adhesion molecules on eosinophil cell membrane is increased and appears to be involved in their migration (16).

The observation that eosinophil migration and LFA-1 expression by these cells are increased in atopic asthmatic subjects is consistent with the concept that "preactivation" of circulating polymorphonuclear leukocytes is present in allergic asthma (17–19) and may explain the large migration of these cells into the target tissues that occurs during allergen-induced acute asthma attacks and shortly after allergen challenge (20–22).

The present study was designed to evaluate whether experimental exposure to allergens in asthmatics could result in detectable depletion of blood eosinophils overexpressing adhesion molecules.

Material and methods

Subjects

Seven men, aged 17–27 years (mean 18.8 ± 2.3), with history of mild intermittent asthma (23, 24) were screened for the study. They were all sensitized only to house-dust mites as demonstrated by a standardized skin prick test and RAST (Pharmacia Diagnostics AB; Uppsala, Sweden) and were studied in the winter. None of them were taking antiasthmatic treatments other than short-acting β_2 -agonists "as-necessary," and these were avoided for 12 h before the study.

Seven age-matched healthy men (mean age 19.4 ± 2.6 years), served as a control group to compare blood eosinophilia and adhesion molecule expression at baseline. They had normal pulmonary function parameters, were negative to the standardized skin prick test and had normal IgE serum levels (< 100 IU/ml). All the participants were told about the scope of the study and the procedures involved, and they gave informed consent. The study protocol was approved by the Ethics Committee of the Department of Movement Science (DISM).

Lung function and bronchial challenge

All the subjects were able to perform forced expiratory manoeuvres. Forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV_1) were measured by spirometry (Medical Graphics, Pulmonary Function System 1070 series 2, Medical Graphics Corporation; ST. Paul, MN). On each occasion, three forced expiratory manoeuvres were obtained and the best values were retained. Both atopics and controls had baseline $FEV_1 > 70\%$ of predicted (25). Circadian variation of lung function and unexpected response to the diluent were assessed by monitoring FEV_1 for 8 h after inhalation of control solution with 50 breaths on a separate day. No subjects had FEV_1 variation from baseline values exceeding 5%.

Bronchial inhalation challenge with the sensitizing allergen, was performed on allergic asthmatic individuals as previously described (2) by using lyophilized allergens, assayed in RAST arbitrary units (AU) by RAST-inhibition technique on an in-house (Lofarma, Milan, Italy) standard sera pool with a 5000 AU of *Derma-*

phagoides pteronyssinus (0.16 mg/ml of protein). The following dilutions were extemporaneously prepared in physiologic solution: 1000, 500, 250, 125, 62.5, 31.25, ~ 16 , ~ 8 , ~ 4 AU. The allergen bronchial challenge was started with an initial dose of 4 AU, with two-fold increments until the FEV_1 fell below 80% of control values or the maximum dose of 1000 AU was reached. Aerosolization was performed by a dosimetric technique using an SM1 (Sensor Medics) French-Rosenthal dosimeter and a 646 ampule. Each incremental concentration was inhaled with 20 quiet breaths. The allergen dose causing a 20% decrease in FEV_1 (PD_{20} allergen) was calculated by interpolation of the dose-response curves and the non-cumulative PD_{20} was logarithmically transformed before statistical analysis.

FEV_1 was monitored every hour up to 8 h and LAR (late asthmatic response) was defined as the maximum of FEV_1 fall between 3 and 8 h.

Eosinophil purification

Isolation of blood eosinophils was performed on discontinuous Percoll gradients, as previously described (16), at baseline (T0) and, only in allergic asthmatic individuals, 3 h (T3) and 24 h (T24) after allergen inhalation challenge. Briefly, 10 ml of heparinized blood was added to an equal amount of 6% dextran in 0.9% sodium chloride, mixed gently and incubated at 37°C. After 30–40 min the upper phase was collected, washed once in phosphate-buffered saline (PBS), resuspended in 1.5 ml of 1.070 g/ml Percoll plus 5% foetal calf serum (FCS) and layered on a discontinuous Percoll gradient with the following volumes (ml) and densities (g/ml), respectively: 1.5, 1.100; 3, 1.090; 3, 1.085; 3, 1.080. The Percoll gradient densities were obtained by mixing nine parts of Percoll with one part of $10 \times$ Hank's balanced salt solution (HBSS) (90% Percoll) and then diluting the 90% Percoll solution with $1 \times$ HBSS plus 5% FCS. The normodense eosinophils recovered between the layers containing, respectively, 1.090 and 1.095 g/ml of Percoll were washed twice in PBS and resuspended at 2×10^6 eosinophils/ml in 100 μ l of PBS + 2% FCS + 0.5 μ g/ml sodium azide (NaN₃) (staining medium). Recovered cells were $> 85\%$ of eosinophils with neutrophils as contaminants (as established by Diff-Quick staining) and 90% were viable, as determined by Trypan-blue exclusion test (16).

Evaluation of adhesion molecule expression on eosinophils

LFA-1 (CD11a/CD18: $\alpha_1\beta_2$), Mac-1 (CD11b/CD18: $\alpha_M\beta_2$) and VLA-4 (CD49d/CD29: $\alpha_4\beta_7$) expression on eosinophils from healthy subjects and allergic asthmatics were evaluated by staining with specific monoclonal antibodies (mAbs) and flow cytometry analysis (16). Cells resuspended in staining medium were stained with 10 μ g/ml of mouse IgG₁ antihuman (ah) CD16b-fluoresceinisothiocyanate (FITC) conjugated mAb (green fluorescence, to distinguish by two-color flow cytometry the contaminating neutrophils, CD16b⁺, from the eosinophils, CD16b[−]) (Ortho Diagnostic System, Milan, Italy) (16) and 10 μ g/ml of the following mouse IgG1 R-phycoerythrin (RPE) conjugated (red fluorescence) mAbs: ahCD11a/RPE, ahCD11b/RPE, ahCD49d/RPE, ahCD69/RPE or ahCD3/RPE (as mouse IgG isotype control) (Serotec, Oxford, UK). The cells were then incubated for 1 h at 4°C, washed twice in staining medium, and resuspended in 200 μ l of PBS. The red fluorescence intensity of CD16b[−] cells (eosinophils) was read on 10 000 acquired cells with flow cytometry (FACScan; Becton Dickinson, Milan, Italy) and expressed as mean fluorescence channel (MFC). To compare the fluorescence intensity of different samples, the cells were acquired with identical settings of the logarithmic amplifier and analyzed with Lysis II software (Becton

Dickinson, Milan, Italy). After conversion to linear fluorescence intensity units, to obtain a linear function of fluorescence intensity over a wide range, the average background obtained with the control antibody was subtracted from the average fluorescence intensity of the specifically stained cells (16).

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean. Statistical comparisons between healthy and asthmatic subjects were made by unpaired *t*-test. Changes of parameters over time in asthmatic subjects were assessed by analysis of variance and post hoc comparisons (Duncan test). Relationships between independent variables were assessed by Pearson correlation test. Statistical significance was considered for *P* < 0.05.

Results

Pulmonary function studies

The asthmatic participants had spirometric values not significantly different from those of controls (*P* > 0.05 for both FVC and FEV₁). In asthmatic individuals, analysis of the degree of bronchial reactivity to house-dust mite allergens, showed an allergen log PD₂₀ ranging from 1.30 to 2.85. Three subjects had definite LAR (FEV₁ fall > 20%), three borderline LAR (FEV₁ fall between < 20% and $\geq$ 10%) and one negative LAR (Table 1).

Blood eosinophilia and adhesion molecule expression

As compared to controls, asthmatics showed a significantly higher degree of blood eosinophilia (controls: $2.2 \pm 0.3\%$, asthmatics: $5.9 \pm 1.1\%$; *P* = 0.007). Evaluating adhesion molecule expression by flow cytometry, we found that LFA-1 (CD11a/CD18) expression was significantly higher on eosinophils isolated from allergic asthmatic subjects than on those from controls (respectively 158.9 ± 29.2 and 32.14 ± 6.6 MFC, *P* = 0.0012), while there were no differences in Mac-1 (CD11b/CD18) or VLA-4 (CD49d/CD29) expression (*P* > 0.1) (Fig. 1). Adhesion molecule expression was not modified by the process of eosinophil separation, since changes in

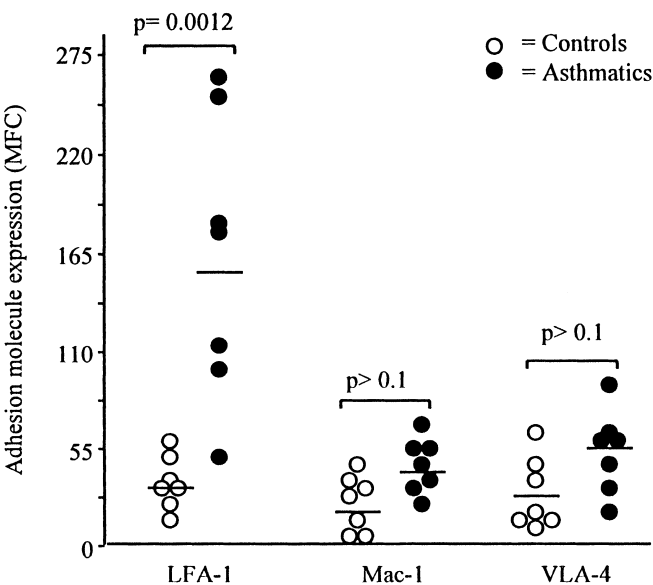


Figure 1. Constitutive expression of lymphocyte function-associated antigen-1 (LFA-1), macrophage antigen-1 (Mac-1) and very late antigen-4 (VLA-4) by circulating eosinophils from seven control subjects (○) and seven allergic asthmatic patients (●). The adhesion molecule expression is indicated as mean fluorescence channel (MFC) on the ordinate, and the different adhesion molecules are shown on the abscissa. The horizontal lines represent mean values.

LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and VLA-4 (CD49d/CD29) levels were neither found in allergic asthmatic subjects nor in controls when eosinophils from unseparated whole-blood samples or from samples obtained after discontinuous Percoll gradient separation were evaluated (*P* > 0.1, not shown).

Changes in eosinophil counts and in adhesion molecule expression after challenge

As compared with baseline values, asthmatic patients showed a significant decrease in blood eosinophils

Table 1. Patient characteristics

Patient	Gender	Age (years)	RAST class	FEV ₁ (% predicted)	Log PD ₂₀ All (AU)	LAR (max % fall)	Blood eosinophils			LFA-1 (MFC)
							% leukocytes	number ($\times 10^3/\mu\text{l}$)		
1 MA	Male	17	4	70	2.27	63	4.5	0.18		103
2 LP	Male	25	3	93	1.30	29	8.9	0.42		247
3 SC	Male	27	4	100	2.85	30	6.1	0.35		176
4 PP	Male	26	3	82	2.45	10	4.9	0.20		110
5 PM	Male	21	4	100	2.55	18	10.1	0.52		255
6 FC	Male	26	3	96	2.40	5	5.3	0.21		175
7 FM	Male	21	3	100	2.70	16	1.4	0.07		46

AU, arbitrary units; FEV₁, forced expiratory volume in 1 sec; LAR, late (3–8 h) asthmatic response; LFA-1, lymphocyte function-associated antigen-1; MFC, mean fluorescence channel; RAST, radioallergosorbent test.

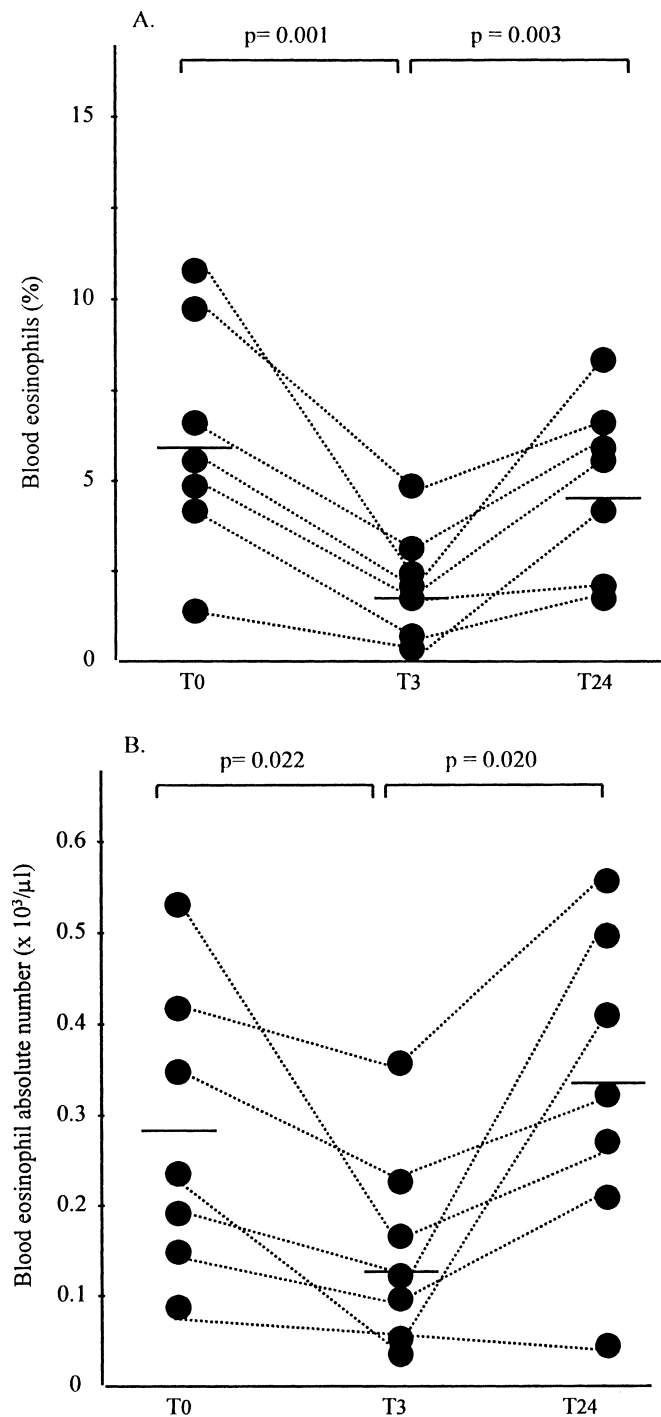


Figure 2. Time-dependent modification in blood eosinophilia at baseline (T0) and 3 h (T3) and 24 h (T24) after allergen challenge in allergic asthmatics. Blood eosinophilia, expressed as percentage (A) or absolute number (B), is shown on the ordinate. The different time points (T0, T3, T24) are reported on the abscissa. The horizontal lines represent mean values.

expressed as percentage ($T0 = 5.9 \pm 1.1\%$, $T3 = 2.0 \pm 0.5\%$; $P = 0.001$) (Fig. 2A) or absolute value ($T0 = 0.28 \pm 0.06 \times 10^3/\mu\text{l}$, $T3 = 0.13 \pm 0.05 \times 10^3/\mu\text{l}$;

$P = 0.022$) (Fig. 2B) 3 h after allergen inhalation challenge. After this early fall, blood eosinophil percentage increased 24 h after challenge ($T24 = 4.7 \pm 0.8\%$; $P = 0.003$, T3 vs. T24), returning to values similar to baseline ($P > 0.1$, T0 vs. T24) (Fig. 2A). Similarly, the absolute number of blood eosinophils increased 24 h after challenge [$T24 = 0.34 \pm 0.07 (\times 10^3/\mu\text{l})$; $P = 0.020$, T3 vs. T24], reaching baseline values ($P > 0.1$, T0 vs. T24) (Fig. 2B). Allergen inhalation challenge was also followed by a significant decrease in LFA-1 (CD11a/CD18) expression by blood eosinophils at 3 h ($T0 = 158.9 \pm 29.2$ MFC, $T3 = 46.9 \pm 16.8$ MFC; $P = 0.002$) (Figs 3A and 3B). At 24 h, LFA-1 (CD11a/CD18) expression showed a clear tendency to increase, with respect to T3 values ($T24 = 105.3 \pm 22.7$ MFC; $P = 0.026$) but without reaching baseline levels ($P = 0.038$, T0 vs. T24).

At 3 h a significant correlation between postchallenge reduction in LFA-1 (CD11a/CD18) expression and decrease in blood eosinophilia, both expressed as a percentage ($r = 0.88$; $P < 0.01$) (Fig. 4A) or an absolute number ($r = 0.87$; $P < 0.01$) (Fig. 4B), was observed. This correlation became not statistically significant 24 h after challenge ($r = 0.64$; $P > 0.05$ and $r = 0.11$; $P > 0.05$, respectively) (Figs 4C and 4D). No significant changes in Mac-1 (CD11b/CD18) and VLA-4 (CD49d/CD29) expression were observed after allergen challenge in asthmatic patients ($P > 0.1$, each comparison).

In asthmatics, at baseline, no correlations were found between eosinophil counts or adhesion molecule expression and pulmonary function parameters ($P > 0.1$, each comparison). In addition, no correlations between allergen PD_{20} and postchallenge variation in blood eosinophilia or adhesion molecule expression were observed at 3 h or 24 h after allergen challenge ($P > 0.1$, each correlation). Similarly, no correlations between the magnitude of FEV_1 fall and postchallenge variation in blood eosinophilia or adhesion molecule expression were observed at 3 h or 24 h after allergen challenge ($P > 0.1$, each correlation). A similar lack of correlation between the magnitude of FEV_1 fall and postchallenge variation in blood eosinophilia or adhesion molecule expression was found limiting the analysis to the subjects with definitive and borderline LAR ($P > 0.1$, each correlation).

Discussion

This study demonstrates that in atopic asthmatic individuals experimental allergen exposure induces an early recruitment of blood eosinophils overexpressing adhesion molecules, such as LFA-1, involved in the process of eosinophil transendothelial migration. Indeed, 3 h after allergen challenge, the percentage of blood eosinophils and LFA-1 expression were decreased, as compared to baseline values, with a very strong correlation between

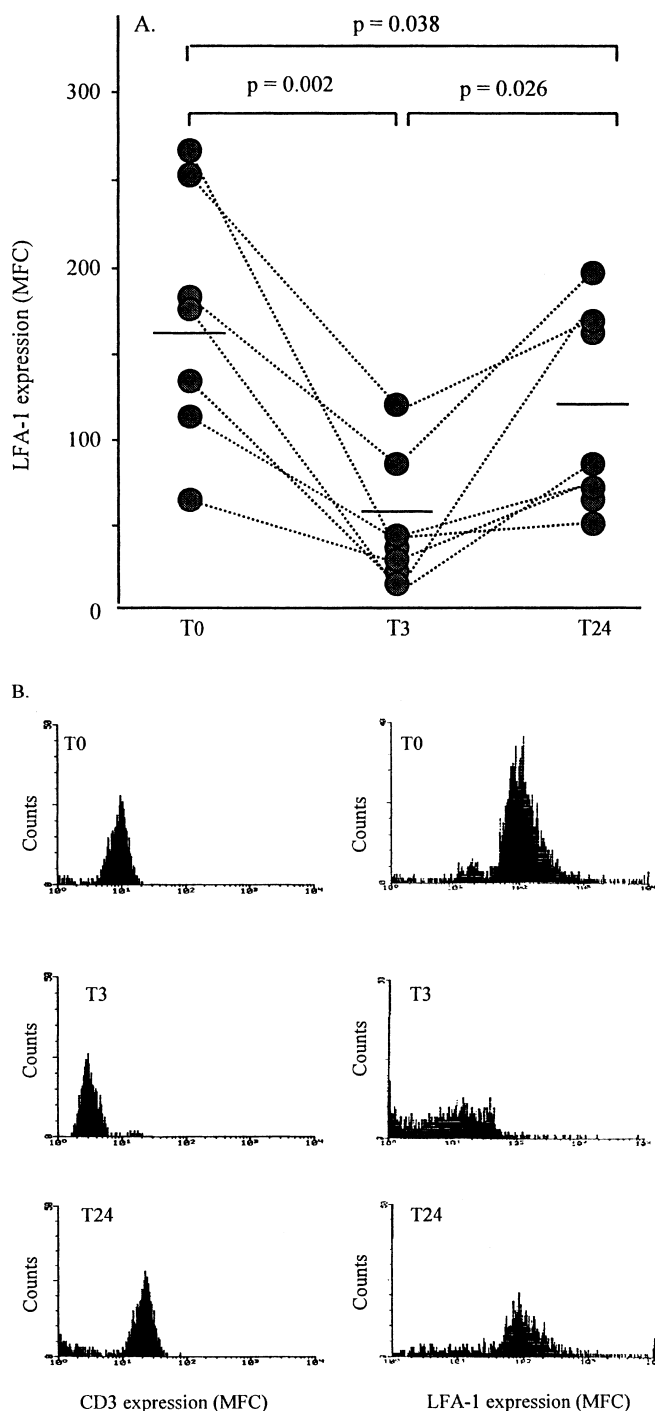


Figure 3. Time-dependent modification in lymphocyte function-associated antigen-1 (LFA-1) expression at baseline (T0) and 3 h (T3) and 24 h (T24) after allergen challenge in allergic asthmatics. (A) LFA-1 expression is indicated as mean fluorescence channel (MFC) on the ordinate and the different time points (T0, T3, T24) are reported on the abscissa. The horizontal lines represent mean values. (B) Flow cytometric histograms of CD3 (mouse IgG isotype control) and LFA-1 expression on eosinophils representative of one allergic asthmatic.

the observed changes in the two parameters. Twenty-four hours after challenge, a relative increase in blood eosinophil counts and LFA-1 expression was observed, as compared to the values at 3 h, but the latter parameter did not reach the prechallenge values.

Allergic asthma is one of the best models of eosinophil recruitment. In this disorder, allergen exposure results in the activation of inflammatory and immune effector cells with local production and release of eosinophil-priming cytokines (IL-3, IL-5, GM-CSF), endothelial cell-activating cytokines (IL-1 β , TNF- α , IL-4, IL-13) and C-C chemokines (eotaxin, RANTES, MCP-3) (1–7, 26). Most of these products have also marked effects on activation of eosinophils, increasing the expression of surface receptors and converting normal into hypodense (or degranulated) eosinophils, which release granule-associated proteins, proinflammatory substances and cytotoxic molecules (1–7).

Although some of these cytokines also have the biological property of stimulating the bone marrow to increase the production of granulocytes (27–29), natural (21) and experimental (30) exposure to allergen results in a significant decrease in blood eosinophilia. Indeed, evaluation of atopic children during allergen-induced acute asthma episodes showed a fall in circulating eosinophil numbers that correlated with the magnitude of arterial hypoxia (21). Similarly, measurements of eosinophil counts at intervals before and after experimental allergen inhalation challenge showed a fall in eosinophil counts 9 h after. In agreement with these data, in the present study we observed a decrease in circulating eosinophil numbers, detectable 3 h after challenge and tending to return to prechallenge values after additional 21 h. The fate of eosinophils after allergen inhalation challenge is unclear. The observation that allergen exposure results in a significant increase in the number of eosinophils in bronchial lumen (20) and bronchial tissues (20,31) suggests that these cells may be recruited in the lung in response to chemotactic stimuli released by cells in the airways. Transendothelial migration alters several eosinophil characteristics, including cell membrane receptor expression, preventing through surface phenotyping identification of the cells migrated from blood to the airways (9, 25, 32, 33).

In agreement with previous reports (16, 34, 35) and with the concept that “preactivation” of circulating polymorphonuclear leukocytes occurs in asthma (17–19), we demonstrated significantly increased expression of LFA-1, but not of Mac-1 or VLA-4 in our asthmatic subjects.

In atopic patients, LFA-1 expression by eosinophils is related to disease severity and to serum levels of allergen-specific IgE (16, 34) and in-vitro and in-vivo experiments have demonstrated that this is one of the adhesion molecules specifically involved in eosinophil migration through the endothelium (15, 16, 30, 31, 34).

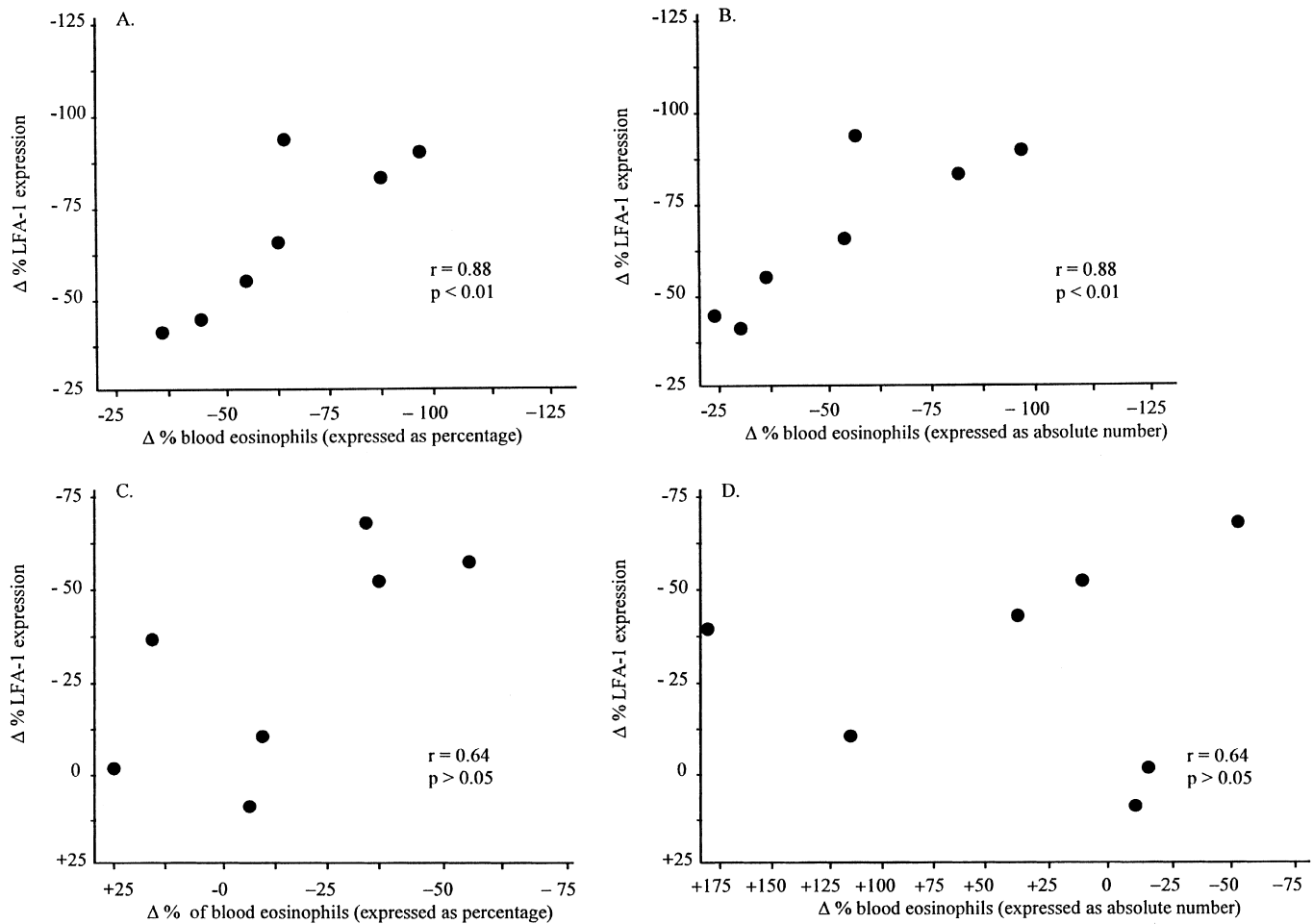


Figure 4. Correlation between changes in lymphocyte function-associated antigen-1 (LFA-1) expression and in blood eosinophil counts: [cell percentage (A,C) or absolute number (B,D)], 3 h (A,B) or 24 h (C,D) after allergen inhalation challenge in allergic asthmatics. Changes are expressed as percent variation between data collected at baseline and 3 h (A) or 24 h (B) after allergen challenge. Changes (% Δ) in LFA-1 expression are expressed on the ordinate; changes in blood eosinophilia are shown on the abscissa.

On the contrary, VLA-4 appears to be determinant only in the initial process of adherence to the vessel wall, allowing the cells to slow down in the bloodstream (7). Mac-1, whose expression is increased in bronchial eosinophils as compared to blood eosinophils from the same subjects (33), seems to be involved in processes that follow transendothelial migration, such as the interaction with other airway cells in the tissues. Different modulation of adhesion molecule expression on the eosinophil surface may be observed in different clinical or experimental conditions (36), or evaluating also hypodense eosinophils, which may differ from normal eosinophils in terms of adhesion molecule expression (37) and which were excluded from our study.

Consistent with the concept that LFA-1 is one of the adhesion molecules involved in eosinophil migration through the endothelium, 3 h after challenge we found a positive correlation between the decrease in blood eosinophilia and the reduction in LFA-1 expression by

blood eosinophils. This suggests that cells overexpressing LFA-1 may preferentially migrate into the allergen-exposed tissues. The decreased LFA-1 expression could also be explained by other phenomena, such as the efflux of eosinophils newly recruited from the bone marrow, or by a down-regulation of adhesion molecule expression on eosinophils remaining in the blood by undefined mediators released during the early-phase reaction to allergen exposure (27–29). However, the tight association between the decreased LFA-1 expression and the reduction in blood eosinophil numbers and percentages, makes these two last possibilities less likely.

Interestingly, LFA-1 expression was still decreased 24 h after challenge, when the percentage of circulating eosinophils returned to baseline values. This finding may reflect the presence in the bloodstream of eosinophils newly recruited from the bone marrow and not yet fully activated by soluble factors up-regulating LFA-1 expression (16, 29, 38). Indeed, in addition to eosinophil-

priming cytokines such as IL-5 and GM-CSF (26,29,39), stimulation of the eosinophil IgE low-affinity receptor has been shown to increased LFA-1 expression and cell migration (34).

Although adhesion mechanisms of circulating cells can also be up-regulated through conversion of the surface molecules from a "low" to a "high" avidity state (38), the observations here reported are in agreement with the

hypothesis of LFA-1 involvement in the in-vivo transendothelial migration of eosinophils.

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