

## Original article

# Increased CD69 expression on peripheral blood eosinophils after specific inhalation challenge

**Background:** CD69 is a molecule expressed on human eosinophils after cytokine-activation. Different studies have described the eosinophil activation, evaluated by CD69 expression, at the site of an allergic inflammation. In this study we evaluated the expression of CD69 on peripheral blood eosinophils after a specific inhalation challenge (SIC), in order to better define the state of activation of peripheral blood eosinophils after exposure to sensitizers.

**Methods:** CD69 expression was evaluated by flow cytometry in nine asthmatic patients before and after a positive SIC with high or low molecular weight agents (pollens, house dust mites, Penicillia, isocyanates) and in 11 asthmatic patients who underwent an inhalation challenge with placebo. CD69 expression was evaluated at baseline, 120 min, and 240 min after the SIC or the placebo.

**Results:** Baseline (before challenge) CD69 expression was comparable between the group of SIC positive patients and the placebo group. CD69 expression on peripheral eosinophils significantly increased 240 min after the challenge in positive SIC patients compared to placebo. In patients with a positive SIC the percentage of peripheral blood eosinophils significantly decreased at 120 and 240 min after the inhalation challenge with respect to the baseline.

**Conclusion:** CD69 expression on peripheral blood eosinophils is significantly increased in asthmatic patients after exposure to the sensitizing agent. These data show that the effects of a bronchial stimulation are also detectable on peripheral blood eosinophils.

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CD69, a type II integral protein with a C-type lectin binding domain, is an early activation marker expressed on different cell types, with a not well understood function and an unknown natural ligand (1). CD69 is expressed on: activated T cells (2), B and natural killer (NK) cells (3, 4), and several data have shown that CD69 is also expressed on activated eosinophils (5, 6). The expression of CD69 on eosinophils has been induced *in vitro* with interleukin (IL)-3, GM-CSF or IL-5 and it has been detected *in vivo* on eosinophils from the bronchoalveolar lavage (BAL) fluid of patients with mild asthma (7). The triggering of CD69 molecule with monoclonal antibodies can induce apoptosis in eosinophils from normal or allergic donors, suggesting a role for this molecule in the regulation of eosinophil activation (8). Nopp et al. (9) reported that CD69 exists as preformed granules in the cytoplasm of eosinophils and neutrophils and that the activation with different stimuli induces its expression only on eosinophils. The same study also showed that the upregulation of CD69 on eosinophils surface follows the initiation of the degranulation process and the increase of the EG2 epitope expression in the cytoplasm of eosinophils (9).

It is well known that eosinophils play a pivotal role in

allergic inflammation, and several studies have evaluated eosinophil activation assessing the expression of specific markers on their surface *in vivo* and *in vitro* (10). The expression of CD69 on eosinophils has been evaluated in allergic asthmatic patients by Julius et al. (11) in the site of allergic inflammation after segmental allergen provocation. These authors reported an increase in CD69 expression on BAL eosinophils 18 h after the allergen challenge.

The aim of the present study was to evaluate, in a group of asthmatics with hypersensitivity to low or high molecular weight agents (12), the expression of CD69 on peripheral blood eosinophils after SIC. Evaluating the activation of peripheral blood eosinophils after specific stimuli could help us to better understand the complex network of signals between local inflammation and peripheral blood and the kinetic of this co-operation.

## Patients and methods

### Patients

Nine subjects with a diagnosis of asthma made previously according to NIH (13), and with a positive inhalation challenge (SIC) were enrolled. Two subjects

out of these and another nine asthmatic subjects undergoing inhalation challenge with placebo were studied as control group. In order to be eligible for the study, the subjects did not take inhaled or systemic steroids in the 4 weeks before or during the study. After SIC, nobody received inhaled steroids, and three patients received inhaled salbutamol (200 µg) soon after the maximal decrease. Placebo and SIC groups were comparable with regard to age ( $36.6 \pm 13.6$  and  $31.6 \pm 8.4$  years, respectively), FEV<sub>1</sub> and Mch PD20FEV<sub>1</sub>. The characteristics of the subjects are shown in Table 1. The study conformed to the Helsinki Declaration and informed consent was obtained from each subject.

#### Study design

Before SIC, all patients underwent spirometry, bronchodilator test, methacholine challenge, skin-prick tests (SPT) with common allergens, blood sampling for measurement of total serum immunoglobulin (Ig)E, and serum eosinophil cationic protein (ECP). Then subjects underwent SIC with the sensitizing agent or with placebo. Challenges with seasonal allergens were performed out of the pollination period. CD69 expression on peripheral blood eosinophils was evaluated before and 120, 240 min after the specific challenge. In two subjects the expression of CD69 was evaluated also at 24 h after the SIC.

#### Specific inhalation challenge

Subjects were challenged with agents suspected to be responsible of their asthma according to patient history. For high molecular weight agents (pollens, mites, and penicillia) the possible hypersensitivity was previously confirmed by SPT. SIC was performed with the method of intermittent delivery of the allergen with standardized allergenic extracts (Stallergenes S.A., Antony, Cedex, France) by means of a MEFAR dosimeter. Doses were increased starting from a concentration of 0.015 RI (Reactivity Index) or 0.015 CI (Concentration Index) and doses were applied two-fold. Spirometry was performed immediately after each inhalation and 15 min after inhalation. The inhalations were stopped after a FEV<sub>1</sub> fall  $\geq 15\%$  (14).

As concerns TDI, the exposure took place in an inhalation chamber for an increasing duration of exposure up to a 30 min cumulative exposure as previously described (15). Spirometry was performed every 10 min for the first h, every 30 min for the second h, and then hourly for a total of 7 hours. Positive SIC was defined by a FEV<sub>1</sub> fall greater than 20% from baseline. During the placebo challenge no significant change of FEV<sub>1</sub> (variation within 10% of baseline) took place.

#### Cell staining and flow cytometric analysis

Fifty µl of EDTA anticoagulated peripheral blood were stained with 5 µl of anti-CD16-PE (Pharmingen, Becton Dickinson, San Jose, CA, USA) and 5 µl of anti CD69-FITC (Pharmingen) and incubated for 30 min at 4°C. Blood samples were then incubated for 15 min at room temperature with a lysing solution (Ortho, Raritan, NJ, USA), the peripheral blood leukocytes were then washed with phosphate-buffered saline (PBS) and acquired with the Cyturon flow cytometer (Ortho) and analyzed with the Immunocount program (Ortho). In order to select eosinophils among leukocytes, total granulocytes were gated on the base of forward scatter/side scatter characteristics. Among gated granulocytes, eosinophils were detected as CD16 negative cells as previously described (16). The percentage of eosinophils (CD16<sup>-</sup> expressing the CD69 surface marker (CD69 positive eosinophils) was determined in comparison with the level of IgG1-FITC isotype negative control expression (Pharmingen). The cytofluorimetric analysis was performed on live cells, assessed by propidium iodide staining.

Data were expressed as the percentage of CD69 positive eosinophils and not as the mean intensity fluorescence (MFI). We chose this way to represent data because the local stimulation, as occurs in asthmatic patients after SIC, induced, as expected, an increase of CD69 expression only in a small number of peripheral blood eosinophils. This increase was not sufficient to cause a significant modification in the MFI of all the peripheral eosinophils.

#### Evaluation of other laboratory parameters

Serum was analyzed for total IgE and for specific IgE towards common pneumoallergens (CAP system IgE-RIA and RAST-RIA, Pharmacia Diagnostics, Uppsala, Sweden). For sECP determination, sera were collected and handled according to the manufacturer's instructions and stored at  $-20^{\circ}\text{C}$ . The sECP levels were evaluated with a commercially double antibody radioimmunoassay (Pharmacia Diagnostics, Uppsala, Sweden).

#### Analysis of the results

Comparison of CD69 expression at different time points between the positive SIC group and the placebo group was made by using analysis of variance for repeated measures. Considering that the subjects with positive SIC had different kinetics of expression of CD69, for each subject we calculated the CD69 increase as the ratio between the percentage of CD69 positive eosinophils at 120 or 240 min and the basal percentage of CD69: the maximal CD69 increase was considered for each subject, independently from the different time points. The

Table 1. Characteristics of the subjects

| SIC + subjects   | Sex | Age (years) | VC (%) | Baseline FEV <sub>1</sub> (% predicted) | PD <sub>20</sub> FEV <sub>1</sub> mch µg/ml | Specific inhalation challenge | Pattern of response | Fall in FEV <sub>1</sub> after SIC (%) |
|------------------|-----|-------------|--------|-----------------------------------------|---------------------------------------------|-------------------------------|---------------------|----------------------------------------|
| 1*               | F   | 26          | 87     | 88                                      | Buffer                                      | Penicillia                    | Early               | −58                                    |
| 2                | M   | 47          | 95     | 89                                      | 272                                         | TDI†                          | Early               | −32                                    |
| 3*               | M   | 33          | 123    | 120                                     | 1480                                        | TDI†                          | Early               | −52                                    |
| 4                | M   | 23          | 110    | 100                                     | > 2560                                      | Pollen                        | Early               | −38                                    |
| 5                | M   | 23          | 90     | 91                                      | > 2560                                      | Pollen                        | Early               | −26                                    |
| 6                | F   | 29          | 106    | 98                                      | > 2560                                      | Mite                          | Early               | −40                                    |
| 7                | F   | 41          | 104    | 110                                     | > 2560                                      | Pollen                        | Dual                | −26                                    |
| 8                | M   | 26          | 81     | 93                                      | > 2560                                      | Mite                          | Early               | −32                                    |
| 9                | F   | 37          | 110    | 118                                     | 1128                                        | Mite                          | Early               | −27                                    |
| Placebo subjects | Sex | Age (years) | VC (%) | Baseline FEV <sub>1</sub> (% predicted) | PD <sub>20</sub> FEV <sub>1</sub> mch µg/ml | Placebo challenge             |                     |                                        |
| 1                | F   | 29          | 97     | 99                                      | 974                                         | Saline                        |                     |                                        |
| 2                | M   | 30          | 122    | 134                                     | > 2560                                      | Saline                        |                     |                                        |
| 3                | F   | 49          | 104    | 115                                     | > 2560                                      | Saline                        |                     |                                        |
| 4                | F   | 25          | 93     | 87                                      | > 2560                                      | Saline                        |                     |                                        |
| 5                | F   | 35          | 102    | 100                                     | 1707                                        | Saline                        |                     |                                        |
| 6                | M   | 69          | 81     | 76                                      | 1640                                        | Saline                        |                     |                                        |
| 7                | M   | 22          | 98     | 102                                     | > 2560                                      | Saline                        |                     |                                        |
| 8                | F   | 47          | 130    | 136                                     | 1227                                        | Saline                        |                     |                                        |
| 9*               | F   | 26          | 87     | 86                                      | Buffer                                      | Saline                        |                     |                                        |
| 10*              | M   | 33          | 123    | 117                                     | 1480                                        | Saline                        |                     |                                        |
| 11               | M   | 47          | 92     | 88                                      | 280                                         | Saline                        |                     |                                        |

\*Subjects undergoing inhalation challenge with placebo and 2 day later with specific agent.

† TDI = toluene diisocyanate.

comparison between the maximal CD69 increase in positive specific inhalation challenge versus placebo was made by the Mann–Whitney *U*-test.

As FEV<sub>1</sub> and CD69 positive cells were assessed at different intervals after the challenge, the area under the curve relating the time interval on the abscissa values on the ordinate was also calculated. Mann–Whitney *U*-test was used to compare the area under the curves of CD69 in SIC positive subjects versus placebo subjects.

Spearman's correlation test was used to evaluate the associations between CD69 and FEV<sub>1</sub> at each time point, maximal CD69 increase and max-FEV<sub>1</sub> fall, the area under the curve of CD69 and the area under the curve of FEV<sub>1</sub>. *P*-values < 0.05 were considered as significant.

## Results

### CD69 expression on eosinophils after specific inhalation challenge

The percentage of CD69 positive eosinophils at the baseline was comparable between positive SIC and placebo group (Table 2). At 120 and 240 min after the SIC there was an increase in the percentage of CD69 positive eosinophils in patients with positive SIC with respect to the placebo being the difference statistically significant 240 min after the challenge (*P*=0.02) (Table 2). Changes of the CD69 expression in two subjects who underwent SIC and placebo are represented in Fig. 1. In these subjects the expression of CD69 evaluated 24 h after the SIC was found similar to baseline levels.

Subjects with a positive SIC (*n*=9) had a maximal

CD69 increase significantly higher (*P*<0.03) than placebo (*n*=11,  $3.75 \pm 3.01$  and  $1.56 \pm 0.81$ , respectively) see Fig. 2. Moreover, when we analyzed the area under the curve of the CD69 expression on peripheral blood eosinophils, we found a statistically significant higher area under the curve in positive SIC subjects with respect to placebo (*P*<0.04).

### CD69 expression and correlation with clinical and laboratory parameters

The mean max-FEV<sub>1</sub> fall of positive SIC subjects was  $-36.7\% \pm 11.5\%$ . The fall in FEV<sub>1</sub> at each time point did not correlate with the percentage of CD69 expression on eosinophils. Moreover, the area under the curve of CD69 expression did not correlate with the area under the curve of FEV<sub>1</sub>.

In order to evaluate whether subjects with an earlier increase in CD69% expression on eosinophils were the ones with a more severe respiratory response, we correlated the max-FEV<sub>1</sub> fall with the time of the occurrence

Table 2. CD69 Expression on peripheral blood eosinophils before, 120 and 240 min after placebo or positive specific inhalation challenge (SIC). Results are expressed as percentage of eosinophils expressing CD69 (mean  $\pm$  standard deviation)

|                             | Expression of CD69 |                   |                     |
|-----------------------------|--------------------|-------------------|---------------------|
|                             | 0 min              | 120 min           | 240 min             |
| Placebo ( <i>n</i> =11)     | $2.61 \pm 2.15\%$  | $2.53 \pm 2.21\%$ | $2.89 \pm 1.24\%$   |
| Positive SIC ( <i>n</i> =9) | $2.68 \pm 3.08\%$  | $7.27 \pm 8.53\%$ | $6.71 \pm 6.10\%^*$ |

\**P* vs. placebo 0.02.

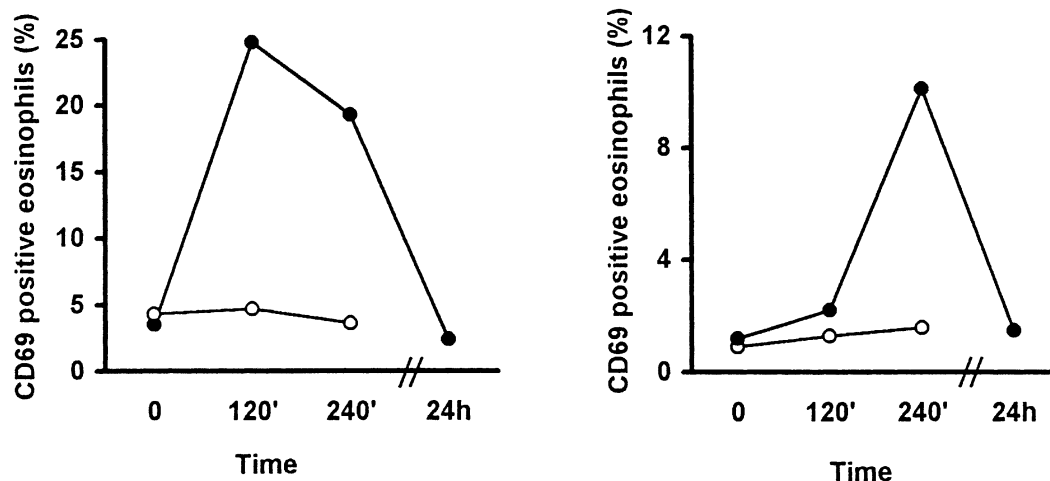


Figure 1. CD69 expression on peripheral blood eosinophils in two representative subjects who underwent the placebo (open circles) and 2 days after the specific inhalation challenge (closed circles). Results are expressed as the percentage of CD69 positive eosinophils at each time point.

of the maximal CD69 increase on eosinophils. The correlation of these data ( $r = -0.71$ ,  $p < 0.03$ ) showed that the rapidity of the CD69 increase is associated with a more severe bronchoconstrictive response. The maximal CD69 increase did not correlate with the concentration of baseline serum ECP (mean  $24.3 \pm 12$   $\mu\text{g/l}$ ) and with serum total IgE levels (mean  $964.8 \pm 784.6$   $\text{kU/l}$ ).

#### Percentage of circulating eosinophils

The percentage of eosinophils with respect to total granulocytes evaluated by flow cytometry, was comparable, at the baseline, between the group of positive SIC subjects with respect to the placebo group (Table 3). At

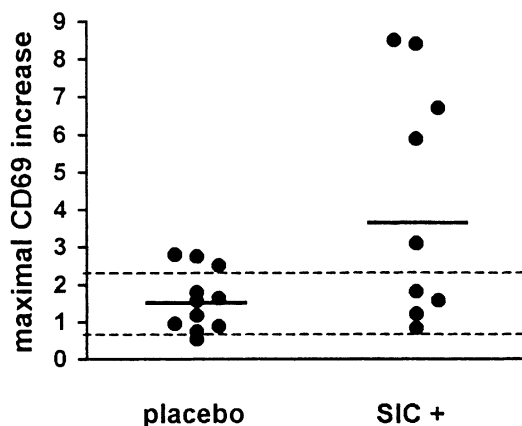


Figure 2. Maximal increase in the CD69 expression in subjects who underwent a placebo challenge (placebo) and in subjects with a positive specific inhalation challenge (SIC+). The CD69 increase was calculated as the ratio between the percentage of CD69 positive eosinophils at 120 or 240 min and the basal percentage of CD69: the maximal CD69 increase was considered for each subject, independently from the different time points. The mean value of each group is represented by the continuous lines. The upper and lower levels of placebo (mean  $\pm$  1 SD of placebo) are shown by the dotted lines.

120 and 240 min after the inhalation challenge, the percentage of peripheral blood eosinophils from subjects with a positive SIC significantly decreased ( $P = 0.01$ ) with respect to placebo (Table 3).

#### Discussion

In this study we have found a significant increase in the CD69 expression on the surface of peripheral blood eosinophils after SIC with low or high molecular weight agents in asthmatic subjects.

It is well known that CD69 is expressed on different cell types, including activated T, B, NK cells monocytes, neutrophils, and platelets (2–4, 17–19). It has also been reported that CD69 is an activation marker for eosinophils (5, 6). Its expression was in fact demonstrated on the surface of lung eosinophils in eosinophilic pneumonia and in peripheral blood eosinophils after an *in vitro* activation with IL-3, GM-CSF, or IL-5 (5). CD69 expression was also found on eosinophils from skin chamber fluids recruited by the major birch allergen, Bet v 1, in birch pollen-allergic patients (20). Julius et al. (11) have previously reported an increase in CD69 expression on BAL eosinophils from allergic asthmatic patients 18 h after segmental allergen provocation. In the same study a slight, but not significant, increase in the CD69

Table 3. Peripheral blood eosinophils before, 120 and 240 min after placebo or positive specific inhalation challenge (SIC). Eosinophils are expressed as percentage with respect to total granulocytes (mean  $\pm$  standard deviation)

|              | Eosinophils (%) |                   |                   |
|--------------|-----------------|-------------------|-------------------|
|              | 0 min           | 120 min           | 240 min           |
| Placebo      | $8.48 \pm 5.05$ | $7.54 \pm 4.59$   | $7.10 \pm 4.44$   |
| Positive SIC | $7.72 \pm 5.20$ | $5.53 \pm 3.72^*$ | $4.78 \pm 2.88^*$ |

\*P vs. placebo 0.01.

expression was found on peripheral blood eosinophils at the same time point. This finding could be related to the type of challenge (segmental), which stimulates only a small portion of the bronchial tree, or to the fact that the expression of CD69 on peripheral blood eosinophils could have occurred earlier than the time-point considered (18 h after the segmental challenge) (11).

In our study we found a significant increase in the CD69 expression on peripheral blood eosinophils of asthmatic subjects 240 min after a positive SIC as compared to a group of asthmatic subjects who underwent a placebo inhalation challenge. In two subjects with a positive SIC, CD69 was also evaluated 24 h after the challenge and its expression was found similar to baseline values.

Because the nine subjects of the SIC positive group had different kinetics of CD69 expression, we also evaluated the maximal CD69 increase for each subject, independently from the different time points considered. The maximal CD69 increase was significantly higher on peripheral blood eosinophils of the subjects with positive SIC than on eosinophils from the group who underwent the placebo inhalation challenge. The increase in the expression of CD69 on peripheral blood eosinophils of asthmatic patients with a positive SIC, suggests that eosinophil activating cytokines IL-3, IL-5, GM-CSF released during the stimulation challenge could spread the activation stimulus from the tissue to the circulation. Therefore, it has been reported that after segmental bronchoprovocation, peripheral blood and airway eosinophils survived longer in culture than did eosinophils isolated from the peripheral circulation of unchallenged allergic patients, suggesting analogue consequences, determined by the segmental bronchoprovocation, for eosinophil survival between circulating and airways cells (21).

The increase in the CD69 expression was found both in patients undergoing SIC with high molecular weight agents and in patients undergoing SIC with low molecular weight agents, but these data were not statistically analyzed because of the small number of the subjects in the two groups.

When we look at CD69 increase after SIC in the single subjects separately, an heterogeneity in the response must be noticed: besides subjects with significant CD69 maximal increase in some patients the increase fell in the range of the placebo group. Such heterogeneity could be the result of many different causes. Subjects with the lowest maximal CD69 increase did not seem to differ from subjects with the highest increase in terms of functional parameter ( $FEV_1$ ), non-specific bronchial hyperactivity, baseline inflammatory parameters (white blood and eosinophil count, and eosinophil basal CD69), type of sensitizing agent and pattern of the response. It is possible that no or minimal peripheral activation occurred after SIC in such patients. However, it is also possible that the heterogeneity in the response may reflect a

different kinetic of activation in different subjects: a maximal CD69 increase could have occurred earlier or later than the time points we have considered.

The percentage of CD69 expressed on peripheral blood eosinophils did not correlate with the maximal fall in  $FEV_1$ . This may be owing to the fact that the CD69 expression on peripheral blood eosinophils and bronchoconstriction occurred in different compartments with different kinetics. On the other hand, we found a significant correlation between the maximal fall in  $FEV_1$  and the time at which subjects presented the maximal expression of CD69, suggesting that patients with a relevant respiratory response had preactivated eosinophils ready to degranulate and to express activation markers. In addition, unlike peripheral blood eosinophils from subjects who underwent placebo, eosinophils from SIC positive subjects might have been exposed to a variety of activating factors *in vivo* which could facilitate a rapid upregulation of CD69. These data agreed with Nopp et al. who have already reported that CD69 exists in preformed granules in the cytoplasm of eosinophils ready to be released after eosinophil activation (9).

Eosinophils in the peripheral circulation represent only a small fraction of the total number of eosinophils in the body, particularly the ones which can be recruited by chemotactic factors to the sites of allergic inflammation. It has already been reported that peripheral blood eosinophils decrease 9 h after a SIC, during a late asthmatic reaction (22), as well as 5 h after an endobronchial allergen challenge (23). In our study, we found that the percentage of peripheral blood eosinophils significantly decreased at 120 and 240 min, after the positive inhalation challenge with respect to the placebo. This decrease could be explained by eosinophil migration into the tissues, driven by different chemotactic factors such as RANTES, eotaxins, MIP-1 $\alpha$ , MCP-3, MCP-4, known to attract cells to the site of allergic inflammation (24–26). The early decrease in peripheral blood eosinophils observed in our study could be related to the early and relatively severe bronchial response after the challenge, as shown by the percentage of  $FEV_1$  fall.

In conclusion, in this study we found a significant increase in the CD69 expression on peripheral blood eosinophils of asthmatic subjects after a positive SIC. This finding shows that during a specific inhalation challenge the inflammatory response, as assessed by the CD69 expression, also involves peripheral blood resulting in the possibility of a less invasive investigation of eosinophilic activation. In the complex network of signals between the local site of inflammation and peripheral blood, the CD69 expression seems to be an intermediate event between the local activation and eosinophils recruitment in the airways. Our results also suggest that the CD69 expression on peripheral blood eosinophils may represent a marker of short-term exposure to sensitizing agents, however, further studies are needed to evaluate this possibility.

## References

1. TESTI R, D'AMBROSIO D, DE MARIA R, SANTONI A. The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. *Immunol Today* 1994;**15**:479–483.
2. CORTE G, MORETTA L, DAMIANI G, MINGARI MC, BARGELLES A. Surface antigens specifically expressed by activated T cells in humans. *Eur J Immunol* 1981;**11**:162–164.
3. RISSO A, COSULICH ME, RUBARTELLI A, MAZZA MR, BARGELLES A. MLR3 molecule is an activation antigen shared by human B, T lymphocytes and T cell precursors. *Eur J Immunol* 1989;**19**:323–328.
4. LANIER LL, BUCK DW, RHODES L, et al. Interleukin 2 activation of natural killer cells rapidly induces the expression and phosphorylation of the Leu-23 activation antigen. *J Exp Med* 1988;**167**:1572–1585.
5. NISHIKAWA K, MORII T, AKO H, HAMADA K, SAITO S, NARITA N. In vivo expression of CD69 on lung eosinophils in eosinophilic pneumonia. CD69 as a possible activation marker for eosinophils. *J Allergy Clin Immunol* 1992;**90**:169–174.
6. MATSUMOTO K, APPIAH-PIPPIM J, SCHLEIMER RP, BICKEL CA, BECK LA, BOCHNER BS. CD44 and CD69 represent different types of cell-surface activation markers for human eosinophils. *Am J Respir Cell Mol Biol* 1998;**18**:860–866.
7. HARTNELL A, ROBINSON DS, KAY AB, WARDLAW AJ. CD69 is expressed by human eosinophils activated *in vivo* in asthma and *in vitro* by cytokines. *Immunology* 1993;**80**:281–286.
8. WALSH GM, WILLIAMSON ML, SYMON FA, WILLIAMS GB, WARDLAW AJ. Ligation of CD69 induces apoptosis and cell death in human eosinophils cultured with granulocyte-macrophage colony-stimulating factor. *Blood* 1996;**87**:2815–2821.
9. NOPP A, LUNDAHL J, HALLDEN G. Quantitative, rather than qualitative, differences in CD69 upregulation in human blood eosinophils upon activation with selected stimuli. *Allergy* 2000;**55**:148–156.
10. WARDLAW AJ, MOQBEL R, KAY AB. Eosinophils: biology and role in disease. *Adv Immunol* 1995;**60**:151–266.
11. JULIUS P, LUTTMANN W, KNOEHEL B, KROEGEL C, MATTHYS JC, VIRCHOW JC. CD69 surface expression on human lung eosinophils after segmental allergen provocation. *Eur Respir J* 1999;**13**:1253–1259.
12. NATIONAL ASTHMA EDUCATION AND PREVENTION PROGRAM. Guidelines for the Diagnosis and Management of Asthma., Expert Panel Report 2. National Institutes of Health. National Heart, Lung, and Blood Institute 1997.
13. JOHANSSON SGO, O'B HOURIHANE J, et al. A revised nomenclature for allergy. An EAACI position statement from EAACI nomenclature task force. *Allergy* 2001;**56**:813–824.
14. MELILLO G. EAACI provocation tests with allergens. Report prepared by the European Academy of Allergology and Clinical Immunology Subcommittee on provocation tests with allergens. *Allergy* 1997;**52**:1–20.
15. CARTIER A. Definition and diagnosis of occupational asthma. *Eur Respir J* 1994;**7**:153–160.
16. MAWHORTER SD, STEPHANY DA, OTTESEN EA, NUTMAN TB. Identification of surface molecules associate with physiologic activation of eosinophils. *J Immunol* 1996;**156**:4851–4858.
17. DE MARIA R, CIFONE MG, TROTTA R, et al. Triggering of human monocyte activation through CD69, a member of the natural killer cell gene complex family of signal transducing receptors. *J Exp Med* 1994;**180**:1999–2004.
18. GAVIOLI R, RISSO A, SMILOVICH D, et al. CD69 molecule in human neutrophils: its expression and role in signal-transducing mechanisms. *Cell Immunol* 1992;**142**:186–196.
19. TESTI R, PHILLIPS JH, FRATI L, GAZZANGIA PP, SANTONI A. CD69 is expressed on platelets and mediates platelet activation and aggregation. *J Exp Med* 1990;**172**:701–707.
20. NOPP A, HALLDÉN G, LUNDAHL J, et al. Comparison of inflammatory responses to genetically engineered hypoallergenic derivatives of the major birch pollen allergen Bet v 1 and to recombinant Bet v 1 wild type in skin chamber fluids collected from birch pollen-allergic patients. *J Allergy Clin Immunol* 2000;**106**:101–109.
21. SEDGWICK JB, CALHOUN WJ, VRTIS RS, BATES ME, McALLISTAR PK, BUSSE WW. Comparison of airway and blood eosinophil function after in vivo antigen challenge. *J Immunol* 1992;**149**:3710–3718.
22. COOKSON WOCM, CRADDOCK CF, BENSON MK, DURHAM SR. Falls in peripheral eosinophil counts parallel the late asthmatic response. *Am Rev Respir Dis* 1989;**139**:458–462.
23. NDUKWU IM, NAURECKAS ET, MAXWELL C, WALDMAN M, LEFF AR. Relationship of cellular transmigration and airway response after allergen challenge. *Am J Respir Crit Care Med* 1999;**160**:1516–1524.
24. EBISAWA M, YAMADA T, BICKEL C, KLUNK D, SCHLEIMER RP. Eosinophils transendothelial migration induced by cytokines. III. Effects of the chemokine RANTES. *J Immunol* 1994;**153**:2153–2160.
25. TAHA RA, MINSHALL EM, MIOTTO D, et al. Eotaxin and monocyte chemotactic protein-4 mRNA expression in small airways of asthmatic and non asthmatic individuals. *J Allergy Clin Immunol* 1999;**103**:476–483.
26. DAHINDEN CA, GEISER T, BRUNNER T, et al. Monocyte chemotactic protein 3 is a most effective basophil- and eosinophil-activating chemokine. *J Exp Med* 1994;**179**:751–756.