

Original article

Eosinophil traffic in the circulation following allergen challenge

Background: Eosinophils contribute to the pathogenesis of asthma and localize to the lung after allergen exposure by uncertain mechanisms.

Methods: We used intrabronchial instillation of allergen to model the interaction between inhaled allergen and the lung. We measured the number of peripheral blood leukocytes and the expression of VLA-4 (CD49d), Mac-1 (CD11b) and PSGL-1 (CD162) up to 4 h after instillation of allergen into a bronchus of eight atopic asthmatics. For controls, we instilled normal saline into a subset of the asthmatic subjects, and allergen into nonatopic, nonasthmatic subjects.

Results: There were changes of total leukocyte number, number of polymorphonuclear leukocytes, lymphocytes, monocytes and eosinophils in all three groups (atopic asthmatics instilled with allergen, atopic asthmatics instilled with saline, nonatopic nonasthmatic subjects instilled with allergen), which were likely related to bronchoscopy. However, the decrease of eosinophils was significant only in the atopic asthmatics instilled with allergen. The remaining eosinophils in the allergen challenged asthmatics were not activated as defined by cell density or change of expression of VLA-4, Mac-1 and PSGL-1.

Conclusions: While eosinophils rapidly and specifically leave the circulation after allergen challenge of atopic asthmatics, the remaining circulating eosinophils are not activated.

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Although there are common symptoms of asthma including wheezing, breathlessness, chest tightness, and coughing, the cause of asthma varies: allergic, occupational, and exercise-induced (1). Dysregulated immunity in asthmatics appears to suppress the T_H1 response in the early stage of life and triggers the T_H2 response (2). Sensitization to allergen is initiated by antigen presenting cells that promote the development of T_H2 cells. These cells secrete interleukin (IL)-4 and IL-13, which promote B cell differentiation into plasma cells that secrete immunoglobulin (Ig)E. Cross-linking of IgE receptors on mast cells causes the release of histamine, prostaglandins, thromboxane, and leukotrienes leading to bronchoconstriction, vasodilation, and mucus secretion (1). A cascade of interactions between cells and soluble molecules may result in chronic bronchial mucosal inflammation and lead to the accumulation of eosinophils and other leukocytes in the lung (3).

Leukocyte, particularly eosinophil localization to the lung is an important event in the pathogenesis of asthma, likely mediated by a series of leukocyte adhesion molecule interactions with endothelium. The accumulation of eosinophils more than neutrophils in the lung after sensitization by allergens has been demonstrated in humans (4). The many toxic components of eosinophils are thought to be important in inducing bronchial mucosal injury and dysfunction (5). However, the mechanisms of

the preferential recruitment of eosinophils has not been fully elucidated although it has been proposed to involve a selective and sequential utilization of leukocyte PSGL-1 for recognition and rolling on endothelium, VLA-4 for rolling or firm attachment on endothelium and β_2 integrin for transmigration with potential contributions from both LFA-1 and Mac-1 (3).

Differences of expression of adhesion molecules on eosinophils compared with neutrophils do not completely explain the selective trafficking to the lung after allergen challenge. For example, PSGL-1, which binds to endothelial P-selectin, is expressed on both neutrophils and eosinophils (6), although PSGL-1 of eosinophils is expressed at a higher site density and has a subtly different structure (7–9). Woltmann demonstrated that IL-13 (a key cytokine in the pathogenesis of asthma) induced P-selectin expression on human umbilical vein endothelial cells and increased binding of eosinophils, but not neutrophils to endothelium (10). VLA-4 is constitutively expressed on eosinophils, but not on neutrophils, and binds to VCAM-1 on endothelium (11). The potential for selective activation of VLA-4 by asthma related chemokines and cytokines such as eotaxin (12) and IL-5 provides one mechanism of more efficient recruitment of eosinophils to the lung. In fact, as we have shown elsewhere, both IL-5 and chemokines induce a change in the affinity of VLA-4 in responding leukocytes (11).

Therefore, the relative importance of changes of eosinophil adhesion marker expression and endothelial expression of ligands for these markers after allergen exposure is unclear.

The goal of this work was to define the early-phase response of eosinophils to an allergen challenge among asthmatics as a prelude to studying molecular trafficking mechanisms of eosinophils in human subjects.

We used intrabronchial instillation of allergen to which asthmatic subjects demonstrated specific IgE as a model of changes that occur after inhalation of allergen. The concentration of allergen used within the lung was determined by skin test titration. This technique causes immediate (within 5 min) substantial bronchial mucosal edema, narrowing of the bronchial lumen, and later migration of eosinophils into both the bronchial mucosa and bronchoalveolar lavage (BAL) fluid (13–16).

We monitored the bronchi of asthmatics and nonasthmatics 5 min after an allergen challenge and analyzed peripheral blood counts preallergen challenge and several times up to 4-h postallergen challenge. We also analyzed the presence of neutrophils and eosinophils in normodense and hypodense layers of Percoll density gradients, and the expression of several adhesion molecules including PSGL-1, VLA-4 and Mac-1 on eosinophils by flow cytometry. Further, we characterized the response of leukocytes in the blood, pre- and postallergen challenge to IL-5.

Materials and methods

Subjects

The subjects were classified as either atopic asthmatic, or control nonatopic nonasthmatic (Table 1). Atopy is defined as immediate type skin test reactivity to aeroallergens common in the Albuquerque area. The skin test allergens are: fremont cottonwood, Juniper-Mountain Cedar, Bermuda grass, Bluegrass, Frescue, Russian thistle, Kochia, western ragweed, cat dander, and house dust mite (Greer Lab, Lenoir, NC). A subject was considered atopic if he or she had two or more positive skin prick tests (3 mm or greater wheal diameter and no wheal in response to normal saline).

The asthmatic subjects had either 15% increase of FEV₁ in response to 200 µg albuterol or increased sensitivity to inhaled methacholine, using methods standard in our laboratory (17, 18). The subjects' current medications did not include systemic or inhaled glucocorticosteroids or cromolyn-like medications but did include inhaled occasional (<1 inhalation/day) β₂ agonists. None

had experienced symptomatic asthma or upper respiratory tract infection within 4 weeks of bronchoscopy.

Normal subjects had no history of asthma or upper respiratory tract atopy. They had negative immediate type skin tests to the allergens listed above, and normal spirometry. Nonsmoking status in all subjects was confirmed with a serum cotinine level of <14 ng/ml.

Three separate groups were studied. The first group was eight asthmatic subjects challenged with allergen; the second group five of the eight asthmatic subjects who were later challenged with saline; and the third group five nonasthmatic subjects challenged with Juniper 1:100 (the allergen and the concentration that was most commonly used in the allergen challenge of the asthmatics).

Allergen preparation and skin test titration

Allergen was purchased from Greer Lab and dialyzed against sterile normal saline to remove glycerol. All preparations used had endotoxin levels of <7 EU/ml (19). Within 1 week before intrabronchial allergen instillation, subjects underwent skin prick test titration with 10-fold dilutions of Juniper or Bermuda grass allergen (diluted in sterile nonpyrogenic normal saline). Skin test sensitivity was expressed as the most dilute allergen solution that produced a positive skin test as defined above.

Bronchoscopy and allergen instillation

All bronchoscopies were performed in the UNM General Clinical Research Center. After premedication with 1 mg atropine, lidocaine was used for local anesthesia and midazolam (up to 2.5 mg i.v.) was administered as needed. Supplemental oxygen was provided and pulse oximetry monitored.

A fiberoptic bronchoscope (Olympus BF 20D, Olympus Corp, Melville, NY, USA) was introduced into the right middle lobe orifice and a subsegment occluded and 20 ml of diluted allergen was introduced using a concentration that produced a positive skin test (Table 1). After 5 min, the allergen was aspirated. Peripheral blood was obtained 30, 60, 120, and 240 min after allergen instillation.

Complete blood count

Between 0.25 to 0.5 ml of drawn blood was analyzed by automated cell counting (Sysmex 9500; Toa Corp., Kobe, Japan).

Cell isolation from patient samples

One milliliters of blood was overlaid on the Percoll density gradient (Amersham Pharmacia Biotech, Piscataway, NJ) that was diluted by 10× Hank's balanced salt solution (HBSS) (Gibco BRL, Rockville, MD) and sterile water (B. Braun Medical Inc, Irvine, CA) to achieve a density of 1.08 g/l. Blood was centrifuged at 20°C for 30 min at 400 *g* and the resulting two layers of cells were extracted.

Table 1. Group characteristics

Group (<i>n</i>)	Age (mean ± SEM)	Sex (M : F)	Number of skin tests positive (mean, range)	Baseline eosinophil number/mm ³	Allergen (dilution) used for challenge
Atopic asthmatic (eight)	27.4 ± 2.8	4 : 4	8.5 (3–11)	346 ± 55	Juniper 1 : 100–1 : 1000 (six subjects) Bermuda Grass 1:10–1:100 (two subjects)
Control (five)	30.8 ± 4.1	4 : 1	0 (0)	150 ± 36	Juniper 1:100 (five subjects)

Skin tests positive = immediate type skin test reactivity to 11 common aeroallergens.

Same dilution as the most dilute concentration of Juniper or Bermuda grass extract diluted allergen that produced a positive skin test (wheal = 3 mm).

To reflect the cell fractions typically recovered, the low-density cell layer at the top of the gradient was designated the lymphocyte layer and the high-density cell layer in the middle of the gradient was designated the granulocyte layer.

Staining

Cells were resuspended in phosphate-buffered saline supplemented with 0.5% of heat inactivated fetal calf serum (HyClone, Logan, UT). Monoclonal antibodies (mAb) CD11b FITC, CD 16 FITC and PE (Caltag, Burlingame, CA), CD49d FITC (Biosource, Camrillo, CA), and CD162 PE (Ancell, Bayport, MI) were purchased. To detect eosinophils and to characterize their adhesion molecules, dual color staining was carried out in three conditions: CD16 PE and CD11b FITC, CD16 PE and CD49d FITC, CD16 FITC and CD162 PE. The final concentration of each mAb was 10 µg/ml. Cells were incubated on ice for 30 min and then analyzed using a FACScan flow cytometer and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ).

IL-5 treatment of blood

One milliliters of blood was incubated with 3.3 nM of rhIL-5 (BD PharMingen, San Diego, CA) for 30 min at 37°C and kept on ice until the staining by fluorescence labeled mAb.

Statistical analysis

The blood count data were not skewed, so that logarithmic transformation was not required. Baseline values were compared using one-way ANOVA with Fisher's lsd method of pair-wise comparison when appropriate. Differences between groups in overall level and in time course were examined using repeated measure ANOVA with the three groups (asthmatics with allergen, asthmatics with saline, and nonasthmatics with allergen) as a grouping factor and time as the repeated factor. Statistical significance was determined by $P < 0.05$. Changes of Mac-1, PSGL-1 and VLA-4 were analyzed by one tail t -test with statistical significance of $P < 0.05$.

Cells for the adhesion assay

Eosinophils were prepared as previously described (9). Eosinophils were then purified from the granulocytes by negative selection in the presence of magnetic beads bearing mAb to CD16 and CD3 and consisted of 95% or more eosinophils. Eosinophils were labeled with green fluorescent fluo3 (7.5 µM, 15 min 37°C) and CHO cells were labeled with red fluorescent hydroethidine (250 µM, 15 min, 37°C). Labeled cells were washed, resuspended in buffer and stored on ice until used in assays. The CHO cells transfected with the gene for human VCAM-1 were a gift from Dr J.M. Oliver, University of New Mexico.

Adhesion assay

To provide an adhesion environment in which fluid shear forces were uniform and subject to precise control, cell suspension adhesion assays were performed in a cone-plate viscometer (CPV) (Brookfield DV-III, Brookfield Corp, Middleboro, MA, USA) as previously described (9, 20). A suspension of eosinophils plus control parental untransfected CHO cells was analyzed to determine the number of input eosinophils recovered as nonadherent singlet cells

(green nonred fluorescent events) in the absence of VCAM-1 (I). This was compared with the observed number of eosinophils in the singlet populations when eosinophils were subjected to comparable shear in the presence of VCAM-1 transfected CHO cells (O). The percentage of adherent eosinophils was then calculated as $100 \times (I-O)/I$. Antibodies directed against CD18 (TS1/18; Endogen, Woburn, MA) were present in all adhesion assays to prevent β_2 integrin dependent adhesion of eosinophils to unidentified CHO cell membrane counter-structures observed to occur under some experimental conditions.

Results

Baseline values

The baseline peripheral eosinophil count (PEC) was lower (One way ANOVA, $P = 0.003$) for nonasthmatics compared with asthmatics. Although the baseline number of eosinophils between asthmatics challenged with allergen was lower than the number of eosinophils of these same asthmatics challenged with saline, the difference was not statistically significant (ANOVA). The white blood cells (WBC), polymorphonuclear leukocytes (PMN), lymphocyte, monocyte, and basophil counts were not different between the groups.

Bronchi

Bronchial mucosal edema and luminal narrowing were observed 5 min after the allergen challenge among asthmatics (not shown). This response was specific only for the bronchi of asthmatics when they were challenged by allergens. In contrast when asthmatics were challenged by saline or when nonasthmatics were challenged by allergen, this response was not observed. These results are similar to those previously reported (13–16).

WBC, PMN, lymphocytes, monocytes, basophils

Using repeated measures (RM) ANOVA, we found a group (asthmatics challenged with allergen, asthmatics challenged with saline, and control subjects challenged with allergen) difference in level for PMN ($P = 0.02$); but no other differences over time because of groups. There were differences over time for WBC ($P < 0.001$); PMN ($P < 0.001$); lymphocytes ($P < 0.001$); eosinophils ($P < 0.001$); monocytes ($P < 0.001$); but not basophils ($P = 0.10$).

Eosinophils

We found a group difference in eosinophil number (RM ANOVA, main effect, $P = 0.003$) and over time (RM ANOVA, interaction, $P = 0.004$). The interaction effect is due to the time course of asthmatics challenged with allergen being different than the other groups.

Therefore, the only change of peripheral blood cells specific to allergen challenged asthmatics was a decrease of eosinophils.

Complete blood count results

The results from experiments on eight asthmatics treated with allergen (five subsequently treated with saline) and five nonasthmatics are summarized in Fig. 1. All subjects showed a characteristic increase in total white blood cells between 120 and 240 min that was accounted for by an elevation of PMN and appears to be a response to the bronchoscopy procedure.

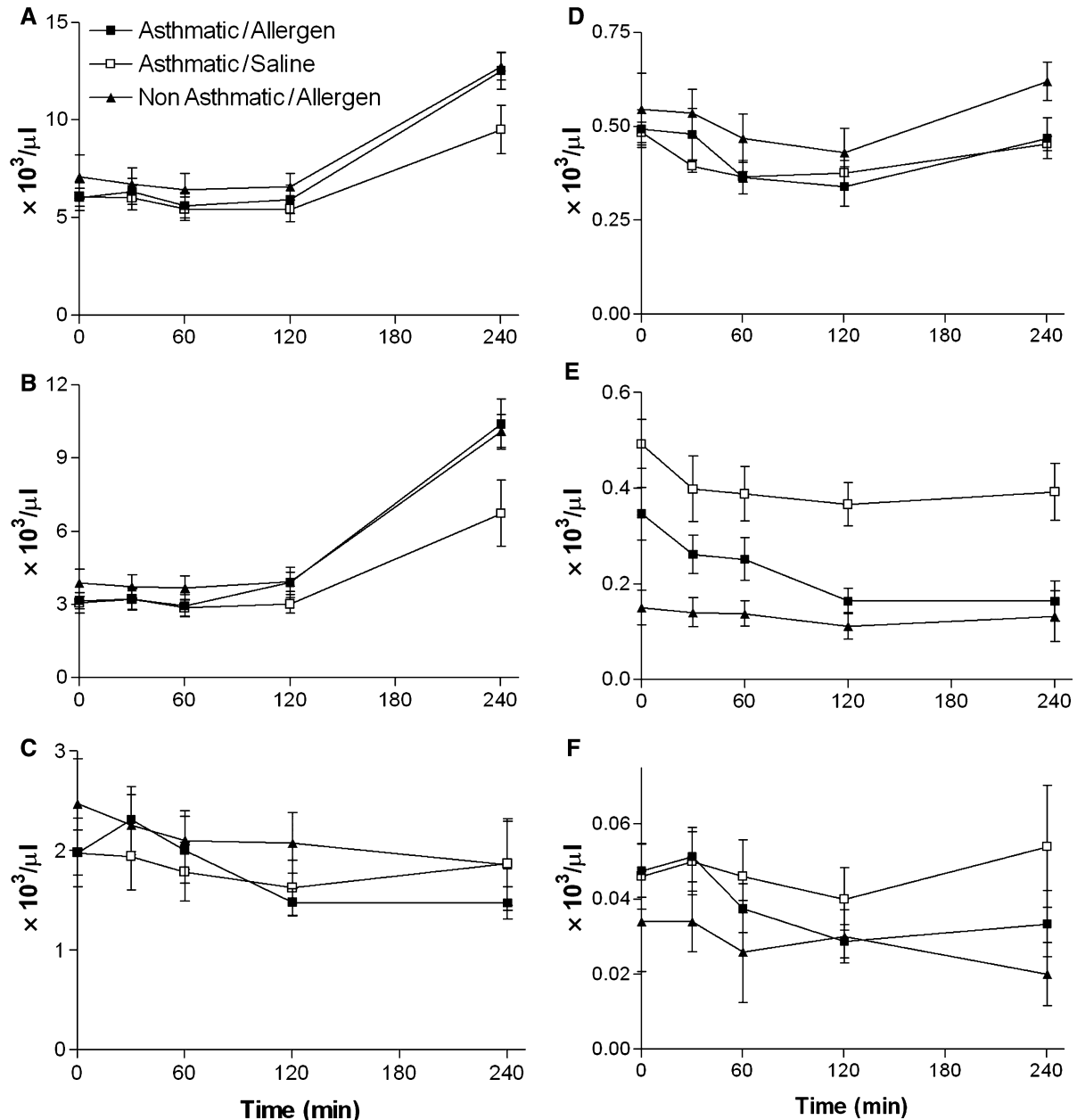


Figure 1. Time course of peripheral complete blood count for all donors. (A) Total leukocytes. (B) Polymorphonuclear leukocytes. (C) Lymphocytes. (D) Monocytes. (E) Eosinophils. (F) Basophils. Blood was collected from eight asthmatic and five nonasthmatic volunteers: before the challenge, 30, 60, 120, and 240 min after the challenge. Five asthmatics were given the allergen or saline at different studies. Nonasthmatics were given saline only. ■: allergen challenged asthmatics (eight). □: saline challenged asthmatics (five). ▲: allergen challenged nonasthmatics (five).

Figure 1E shows the eosinophil response of the asthmatics to allergen challenge. The eosinophil count dropped progressively reaching 50% of the initial level after 240 min (RM ANOVA, $P = 0.004$). When nonasthmatics were given allergen, and asthmatics were challenged with saline, peripheral blood eosinophils did not change from baseline (Repeated measures ANOVA, $P > 0.20$). Taken together, these results show the specific effect of the allergen to the bronchi and peripheral blood

eosinophils of asthmatics. Additionally, these results support the increased eosinophil influx to bronchial mucosa and BAL fluid (14, 16) – observed by others using similar challenges (13–16), although we did not directly sample bronchial mucosa or BALF.

Experiments were performed to analyze other cellular characteristics in these subjects by flow cytometry. A representative set of flow cytometry results from an asthmatic subject given allergen is shown in Fig. 2. Before challenge, eosinophils are detected primarily in the

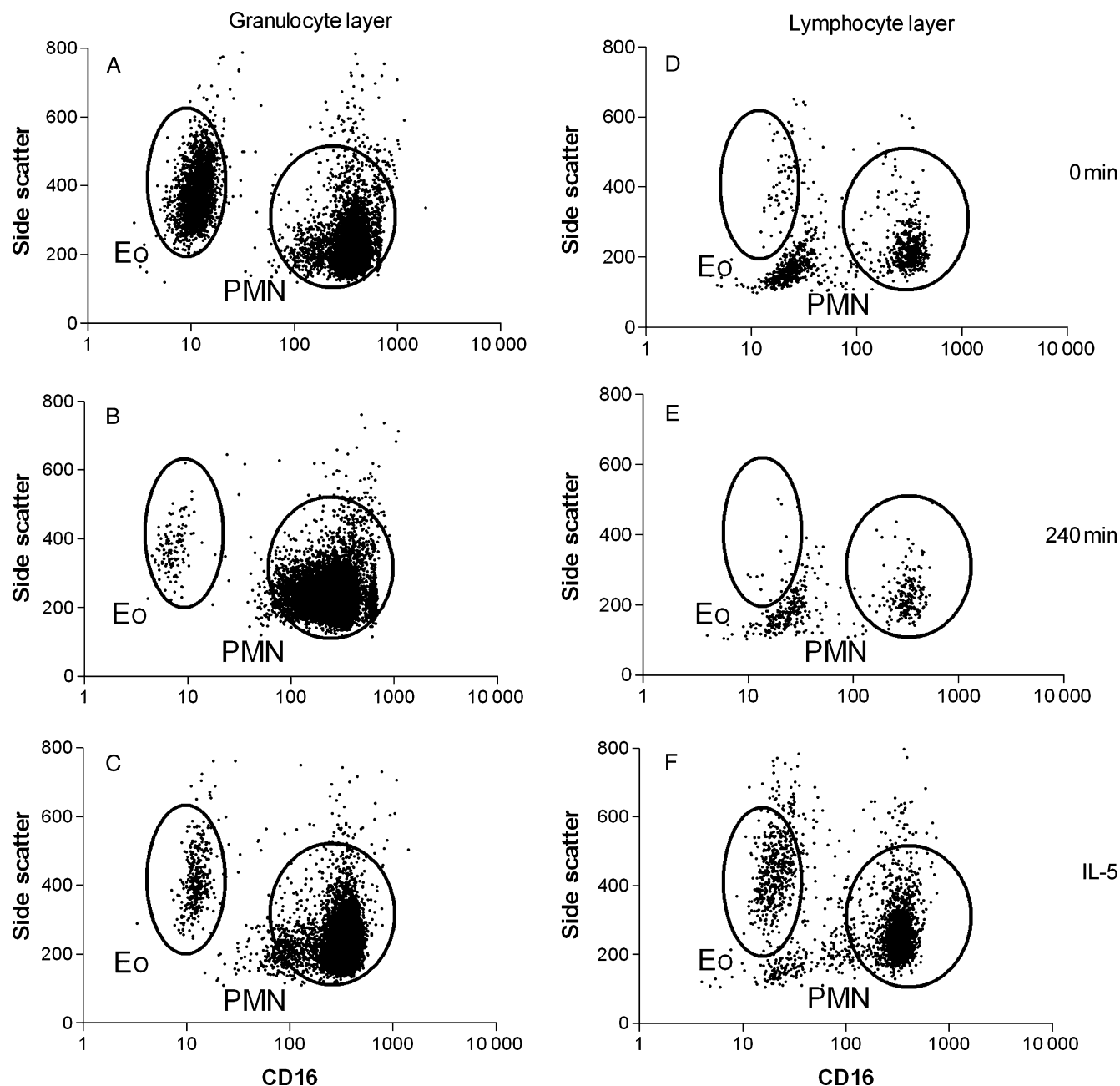


Figure 2. Flow cytometric dot plots (side scatter vs CD16 – PE fluorescence) of granulocytes detected in granulocyte and lymphocyte Percoll gradient layers by flow cytometric analysis. Granulocytes are identified by characteristically high side scatter signals relative to mononuclear cells. Eosinophils are identified as granulocytes with low CD16 fluorescence intensity and neutrophils as granulocytes with high CD16 fluorescence intensity (enclosed within left and right elliptical gates in each panel, respectively). Left panels show profiles of eosinophils and neutrophils recovered from granulocyte layers. (A) Before the allergen challenge. (B) 240 min after the challenge. (C) Before the allergen challenge and treated with interleukin (IL)-5. Right panels show profiles of eosinophils and neutrophils recovered from lymphocyte layers. (D) Before the allergen challenge. (E) 240 min after the challenge. (F) Before the allergen challenge and treated with IL-5. Results are from one donor representative of the asthmatic response to allergen.

granulocyte layer and only sparsely in the lymphocyte layer (Fig. 2A, D, respectively). Characteristically, 240 min after allergen challenge, there is a decrease of the number of eosinophils associated with the granulocyte layer, but no corresponding systematic increase in the number of eosinophils detected in the lymphocyte layer (Fig. 2B, E). Because of the importance of IL-5 in asthma, we wished to determine if the changes that we observed *in vivo* after allergen challenge could be reproduced by exposure of eosinophils to this cytokine *in vitro*. We found that exposure of blood from a representative asthmatic before allergen challenge to IL-5, caused the eosinophils to become hypodense and shift from the normodense granulocyte layer to the hypodense lymphocyte layer (Fig. 2C, F). It is interesting to note that cells with the light scatter characteristics of PMNs also shift to the lymphocyte layer following IL-5 treatment of the blood samples.

We also examined levels of adhesion molecules expressed on eosinophil membranes before and after allergen challenge. VLA-4, Mac-1 and PSGL-1 are each expressed on 100% of eosinophils. Therefore, to quantify changes in expression we measured the mean fluorescence intensity (MFI) of eosinophil staining with antibodies directed against each adhesion molecule before and after allergen challenge. The MFI measurements made subse-

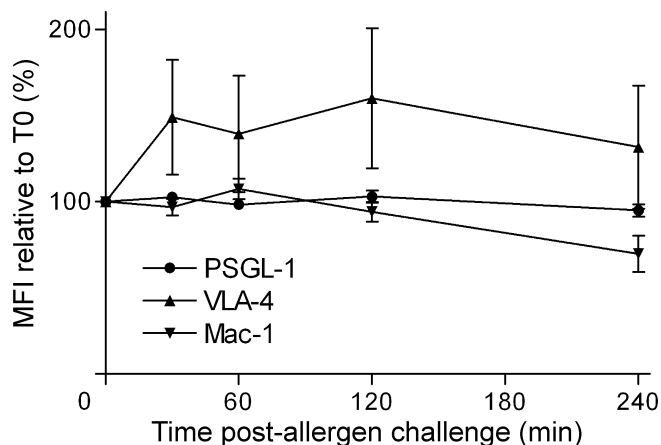


Figure 3. Flow cytometric analysis of adhesion molecule expression levels on circulating eosinophils of asthmatics. Eosinophils recovered from the Percoll gradient granulocyte layer were gated for flow cytometric analysis of adhesion molecule expression on the basis of low CD16 fluorescence intensity and high side scatter intensity as in Fig. 1. To analyze Mac-1 and VLA-4 expression, cells were stained with CD16-PE in combination with CD11b-FITC and CD49d-FITC, respectively. To analyze PSGL-1 expression, cells were stained with CD16-FITC in combination with CD162-PE. The mean fluorescence intensity of PSGL-1 (circles, $n = 3$), VLA-4 (upright triangles, $n = 3$), and Mac-1 (inverted triangles, $n = 5$) were measured at each time point and normalized relative to values measured at the 0 min time point (preallergen challenge). Represented are mean and SD for each time point.

quent to allergen challenge were then normalized relative to the prechallenge values. VLA-4 expression was highly variable and, despite a trend towards increased expression at each time point postchallenge, there were no statistically significant changes (Fig. 3). Likewise, Mac-1 and PSGL-1 expression levels were invariant in response to allergen challenge. Similarly, challenge of asthmatics with saline or nonasthmatics with allergen did not produce a change of VLA-4, Mac-1 and PSGL-1 (Table 2). When prechallenge granulocytes from these patients were treated with IL-5, eosinophils subsequently recovered from both the granulocyte and lymphocyte Percoll gradient layers expressed 133 ± 16 and 143 ± 21 levels of Mac-1, respectively, relative to normalized 100% levels in control untreated eosinophils recovered from the granulocyte layer (mean $\pm$ SD, $P < 0.05$ for each via one-tailed *t*-test). This was consistent with previous reports of Mac-1 integrin upregulation in eosinophils by IL-5 (21, 22). These results suggested that if Mac-1 upregulation was likewise induced *in vivo* by allergen treatment, eosinophils with this phenotype must have been among those exiting the circulation. Eosinophil expression levels of PSGL-1 and VLA-4 were unperturbed by IL-5 treatment (data not shown).

IL-5 stimulates VLA-4 dependent eosinophil adhesive interactions with VCAM-1

To test a possible mechanism by which eosinophils leave the circulation after allergen challenge (Fig. 1), we examined the adhesive properties of eosinophils treated with IL-5 *in vitro*. After a brief 5-min exposure to IL-5, purified eosinophils were combined with CHO cell VCAM-1 transfectants and subjected to fluid shear in a CPV for

Table 2. Changes of mean fluorescent intensity (MFI) after challenge

CD	Time post-treatment (min)	MFI relative to T0 (% $\pm$ SD)*	
		Asthmatic saline	Nonasthmatic allergen
11b	0	100	100
	30	93 $\pm$ 16	91 $\pm$ 10
	60	99 $\pm$ 18	86 $\pm$ 15
	120	94 $\pm$ 11	86 $\pm$ 19
	240	81 $\pm$ 16	86 $\pm$ 47
49d	0	100	100
	30	96 $\pm$ 1	127 $\pm$ 57
	60	98 $\pm$ 13	99 $\pm$ 10
	120	104 $\pm$ 16	92 $\pm$ 5
	240	93 $\pm$ 7	117 $\pm$ 39
162	0	100	100
	30	102 $\pm$ 2	100 $\pm$ 3
	60	104 $\pm$ 5	100 $\pm$ 6
	120	104 $\pm$ 7	99 $\pm$ 10
	240	102 $\pm$ 8	106 $\pm$ 12

$n = 5$ in each group.

There are no differences between the groups or between the times.

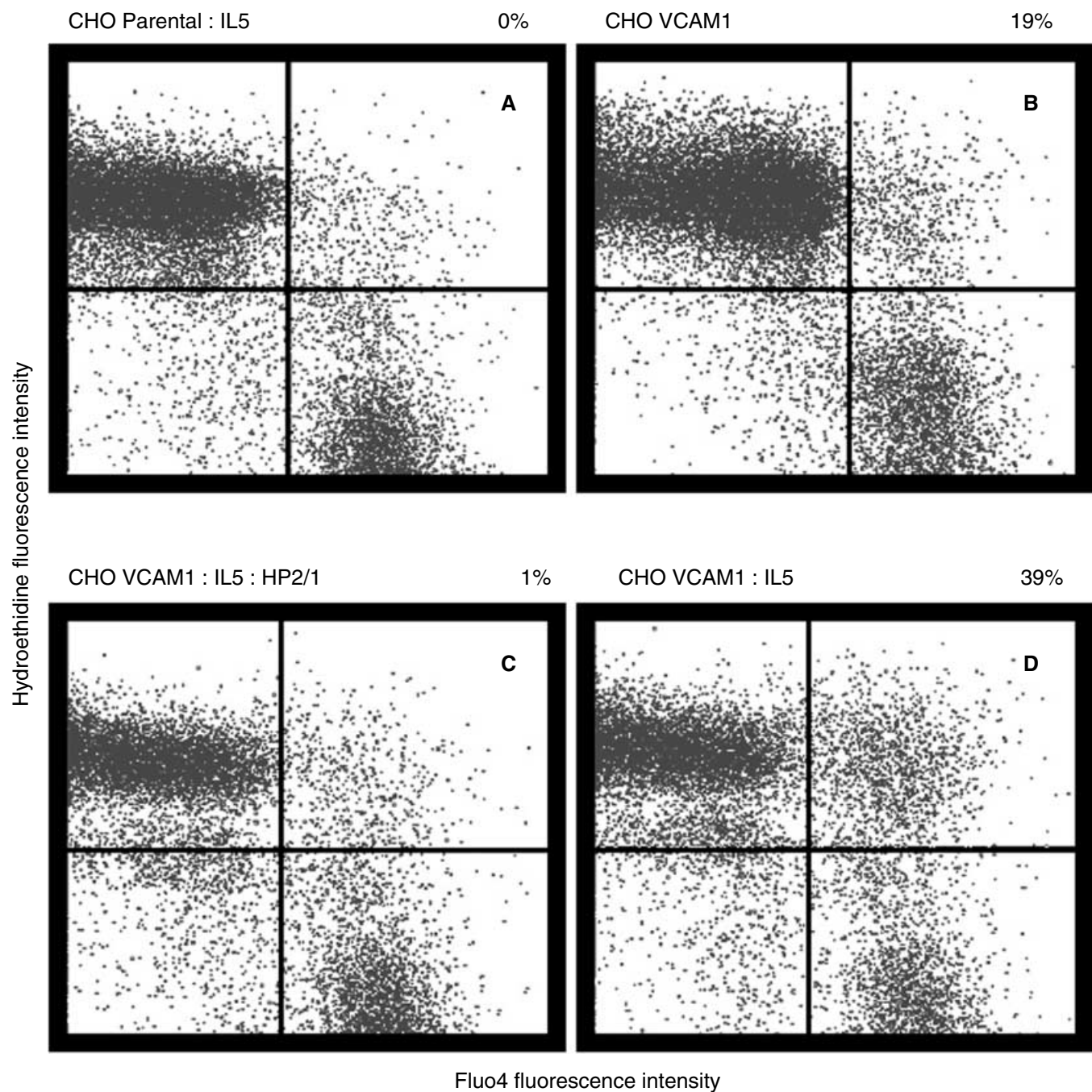


Figure 4. Interleukin (IL)-5 stimulates eosinophil VLA-4 dependent adhesion to VCAM-1. Eosinophils prelabeled with green fluorescent Fluo-4 were incubated 5 min 37°C with (A, C, D) or without (B) 2 nM IL-5, then mixed with red fluorescent hydroethidine prelabeled CHO cells expressing transfected human VCAM-1 (B, C, D) or untransfected control CHO cells (A). In one group, the VLA-4 function-blocking antibody HP2/1 was added to eosinophils during the IL-5 treatment step (C). The cells were subjected to fluid shear for 5 min at a shear rate of 114/s (*c.* 0.9 dynes/cm²) in a cone and plate viscometer, then aspirated directly from the viscometer into a flow cytometer for analysis of eosinophil adhesion to CHO cells. Adhesion was quantified as the depletion of eosinophils from the singlet eosinophil quadrant (lower right in each panel) as they were recruited into the adherent cell conjugates quadrant (upper right in each panel). The numbers of singlet eosinophils observed in groups A–D were 13 570, 11 000, 13 410, and 8350, which corresponded to 0, 19, 1, and 39% adherent eosinophils, respectively. Results are representative of more than 10 experiments with eosinophils from six different nonasthmatic subjects.

an additional 5 min. When subsequently analyzed in the flow cytometer, 19% of control untreated eosinophils were adherent to CHO VCAM-1 cells (Fig. 4B). The IL-5 treatment promoted a doubling of adherent eosinophils to 39% (Fig. 4D). Adhesion of IL-5 treated eosinophils and untreated eosinophils was abrogated in the presence of VLA-4 function blocking antibodies (Fig. 2C and not shown) or in the absence of CHO cell VCAM-1 expression (Fig. 2A and not shown), an indication that adhesion was strictly dependent upon VLA-4 adhesion to VCAM-1. Thus, although membrane expression levels of VLA-4 were unaffected by IL-5, there was a clear IL-5-induced increase in VLA-4 adhesive function.

Discussion

The goal of these experiments was to gain insight into the early responses of circulating eosinophils following allergen challenge. To do so, we evaluated circulating cell numbers and expression of several leukocyte adhesion molecules including VLA-4, MAC-1, and PSGL-1 that could contribute to eosinophil traffic. The experiments presented here indicate that eosinophils specifically leave the circulation of asthmatic subjects treated with allergen. We did not find evidence of changes of Mac-1, VLA-4, or PSGL-1 on the remaining peripheral blood eosinophils.

We did not find evidence of the appearance or accumulation of hypodense or activated eosinophils in the circulation, similar to those found in broncho-alveolar lavage fluid after allergen challenge (23). Even during the time at which eosinophils are leaving the granulocyte layer of the Percoll separation gradients, there is no corresponding increase in the number of eosinophils in the lymphocyte layer, suggesting that the eosinophils were leaving the circulating pool and not simply migrating to another region of the Percoll gradient. This dichotomy between the effects of allergen challenge on broncho-alveolar and blood eosinophils are similar to those recently reported by Liu et al. using a similar model of intrabronchial allergen challenge. Despite profound changes of broncho-alveolar lavage eosinophil IL-5 receptor expression, there were no changes of peripheral blood eosinophil IL-5 receptor expression (24).

Lantero et al. recently reported a similar decrease of peripheral blood eosinophils 3 h after allergen inhalation challenge. They also reported a decrease of eosinophils expressing LFA-1, but no change of eosinophils expressing Mac-1 and VLA-4 (25). Therefore, the results reported here correspond to those reported by Lantero, despite a difference of the nature of the allergen challenge (inhalation vs bronchial instillation). Dang et al. (26) reported that eosinophils from allergic asthmatics had increased expression of PSGL-1 and increased adhesion of eosinophils from allergic asthmatics to P-selectin. Our experiments were designed to detect short-term changes

in PSGL-1 expression within individuals and did not permit a quantitative analysis of differences in PSGL-1 expression between asthmatics and nonasthmatics.

There are several mechanisms by which eosinophils could specifically leave the circulation after allergen challenge. These could involve either activation of endothelium or activation of leukocytes, or a combination of both. With respect to the endothelium, on the time scale of minutes, allergen could increase the P-selectin levels on endothelial cells through the induction of histamine release in the lung. Because eosinophils adhere to P-selectin more efficiently than neutrophils via PSGL-1, vascular recognition could potentially be accomplished in significant part by the upregulation of endothelial P-selectin (9). If followed by engagement of VLA-4 to VCAM, firm attachment could ensue. We demonstrated that IL-5 stimulated eosinophil adhesion *in vitro* via a VCAM-1 dependent mechanism (Fig. 4). In an analogous model of intrabronchial allergen challenge, Montefort found an increase of ICAM-1 and E-selectin expression on bronchial mucosal endothelium, but no increase of VCAM-1 6 h after allergen instillation (16). In fact, expression of VCAM-1 on lung endothelial cells stimulated by IL-4 *in vitro* does not change over 24 h (R.S. Larson and H. Tsuji, unpublished data). P-selectin expression on bronchial endothelium is reported not to increase 24 h after allergen instillation (13), but has not been examined at earlier time periods. In addition, other stimuli can also increase bronchial mucosal endothelium P-selectin expression (27).

A second mechanism of eosinophil egress from the circulation after allergen challenge could involve the induction of circulating IL-5 or other soluble mediators such as chemokines by allergen instillation and its sequelae. In this case, there would be an eosinophil VLA-4 response, likely associated with a change in VLA-4 affinity or avidity similar to that demonstrated by Fig. 4. As IL-5 induces changes in eosinophil Mac-1 expression levels, and VLA-4 dependent adhesion, the newly formed hypodense eosinophil population would not accumulate in the circulation nor separate with normodense granulocytes in the Percoll gradient granulocyte layer. Rather, the activated eosinophils would rapidly adhere to the vascular endothelium even with basal levels of VCAM-1 and therefore be removed from the circulation. Either of these mechanisms, working alone or in concert could account for the preferential disappearance of eosinophils on the time frame suggested by our experiments. Neither of these mechanisms would lead to an accumulation of activated cells in the circulation.

It is worth noting the relative increase of neutrophils in the lymphocyte layer of the Percoll gradient following IL-5 treatment. Surprisingly, in whole blood, neutrophils as well as eosinophils appear to become hypodense. This response is not likely to be a direct effect of IL-5, but is presumably through the elaboration of soluble mediators induced by IL-5.

After the patient studies described here were completed, a role for the CCR3 receptor in allergen response was reported in an analogous mouse knockout model (28). CCR3 recognizes eotaxins 1, 2, and 3; regulation upon activation, normal T-cell expressed and secreted (RANTES); MIP-1 α ; and MCP-2, 3 and 4. As soluble mediators these would be expected to contribute to the second mechanism of response, and as surface associated-mediators to the first mechanism. Moreover, it is worth noting that peripheral neutrophils are well-known to exhibit characteristic changes in light scatter and density analogous to those observed here when stimulated by chemoattractants and that under such activation conditions, they upregulate Mac-1 receptor numbers and LFA-1 and Mac-1 avidity. While these current studies do not discriminate between possible mechanisms, they do

provide a framework for further investigation in humans. With inhibitors available for several of the putative steps in the trafficking pathway, it should be possible to use this short-term allergen response model as a means to evaluate the molecules involved in the initial trafficking steps.

Acknowledgments

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