

Eosinophils from patients with asthma express higher levels of the pan-leucocyte receptor CD45 and the isoform CD45RO

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Summary

Background Eosinophils and their secreted mediators are heavily implicated as effector cells in asthma and other allergic diseases. Comparisons were made between expression of CD45, CD45RA, CD45RB and CD45RO by eosinophils from asthmatic patients and non-asthmatic atopic and non-atopic, non-asthmatic control subjects.

Methods Twenty-seven patients with asthma and 33 control subjects were recruited for the study. Eosinophil expression of CD45, CD45RA, CD45RB and CD45RO was established by immunostaining and flow cytometry was performed on whole leucocyte samples. Eosinophil apoptosis in response to CD45 and CD45 isoform monoclonal antibody (mAb)-dependent receptor ligation was assessed by binding of annexin V and flow cytometry.

Results Eosinophils from patients with asthma expressed significantly ($P < 0.05$) higher levels of pan-CD45 and CD45RO compared with eosinophils from non-asthmatic, non-atopic subjects. No significant correlations were found between expression of either pan-CD45 or CD45RO and the degree of symptoms in the asthmatic patients as defined by lung function (FEV₁ and FEF_{25–75}) and methacholine PD₂₀. Increased expression of pan-CD45 or CD45RO did not appear to be a consequence of the atopic phenotype. Higher expression of pan-CD45 or CD45RO by eosinophils from asthmatic patients was not associated with greater sensitivity to CD45 and CD45RO mAb receptor ligation-induced eosinophil apoptosis.

Conclusion Higher expression of CD45 and CD45RO by eosinophils from asthmatic patients appeared to be a consequence of asthma rather than atopy and further supports a role for activated eosinophils in asthma.

Keywords apoptosis, asthma, atopy, CD45, eosinophils, membrane receptors

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Introduction

Eosinophils and their secreted mediators are heavily implicated as effector cells in asthma and other allergic diseases. In asthma, eosinophil derived pro-inflammatory products are thought to make a major contribution to the inflammation underlying the pathophysiological changes in the airways [1]. They are also markers of the chronic inflammation characteristic of this disease with asthma severity correlating with the increased numbers of eosinophils found in patients' blood, sputum or bronchoalveolar lavage fluid [2]. The cell surface membrane receptor CD45, the common leucocyte antigen, is a member of a family of structurally related, membrane-spanning, glycoprotein tyrosine phosphatases with an important role in intracellular signal transduction in the immune system [3]. CD45 is expressed by all leucocytes,

including eosinophils [4]. Although encoded by a single gene, alternative splicing of primary mRNA transcripts causes CD45 to be expressed in characteristic cell type-specific patterns as a protein with multiple isoforms that include CD45RA, CD45RB and CD45RO [5]. We have previously demonstrated that monoclonal antibody (mAb)-dependent ligation of CD45 and the isoforms CD45RA and CD45RB but not CD45RO accelerated the constitutive rate of apoptosis in human eosinophils [6]. This may represent an important *in vivo* mechanism for the removal of eosinophils and the abrogation of their detrimental pro-inflammatory effects.

The purpose of this study was to examine the specific expression patterns of several CD45 isoforms in human peripheral blood eosinophils from asthmatic and control subjects. Comparisons were made between expression of CD45, CD45RA, CD45RB and CD45RO by eosinophils from asthmatic patients, atopic, non-asthmatic control subjects and non-asthmatic, non-atopic control subjects. Any correlations between CD45 isoform expression and the

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degree of asthma symptoms were investigated. The functional relevance of any increase in the expression of CD45 by eosinophils from asthmatics was addressed by assessing whether CD45 ligation-dependent apoptosis in eosinophils from asthmatic patients was more rapid than CD45-dependent apoptosis induction in eosinophils from control subjects.

Methods

Antibodies

The following mAbs were used in these studies: IgG1 mouse negative control (Dako, Dak-G01, Angle Drove, UK); IgG2a mouse negative control (Dako, Dak-G05); IgG1 mouse negative control PE conjugate (Sigma) Sigma, Poole, UK; CD16-PE (Pharmingen, Cowley, UK); CD45 RB (Sigma, BRA 11); CD45 (Sigma, BRA 55); CD45 RB (Serotec, MEM55, Oxford, UK); CD45 RA (Serotec, F/8/11/13); CD45 (Serotec, F/10/89/4); CD45 RA (Immuno Quality Products, MB1, Groningen, the Netherlands); CD45 RB (Immuno Quality Products, MT4); and CD45 RO (Dako, UCHL1).

Patient characteristics

Twenty-seven patients with asthma and 33 healthy control subjects were recruited for the study. Eligible asthmatic patients were stable for at least 3 months prior to the study and had not altered their medications nor received a course of oral corticosteroids or antibiotics during this period. Nineteen of the asthmatic patients and 16 non-asthmatic subjects were atopic as defined by positive skin prick testing to standard allergens. Skin prick testing was performed following a standard protocol (Bencard Testing Solutions, Welwyn Garden City, UK) using extracts including house dust mite, cat, dog, grass, tree and weed pollen in addition to a negative control. Results were read after 15 min, a positive reaction being defined as a weal diameter of at least 2 mm greater than the negative control. Skin prick testing has been shown to be

strongly associated with other markers of atopy as well as atopic disease expression [7, 8].

All subjects had lung function assessed by spirometry with recording of forced expiratory volume in 1 s (FEV₁) and forced expiratory flow (FEF)_{25–75}. Spirometry was performed according to the American Thoracic Society criteria [9] using a Vitalograph[®] compact spirometer (Vitalograph Ltd., Buckingham, UK) with a computer-assisted pneumotachograph head and pressure transducer. The spirometer was calibrated daily with a Vitalograph[®] 1-L precision syringe. In addition, patients with asthma underwent methacholine bronchial challenge testing, with measurement of the provocative dose of methacholine causing a 20% fall in FEV₁ from baseline (PD₂₀). Methacholine bronchial challenge was performed using a standardized computer-assisted dosimetric method as previously described by Beach et al. [10]. In brief, methacholine was administered at 5-min intervals in doubling cumulative doses from 3.125 to 6400 µg until a 20% reduction in FEV₁ was recorded.

Flow cytometric staining of eosinophil cell surface antigens

The cell phenotype of eosinophils was established by flow cytometry performed on whole leucocyte samples. Blood was obtained from asthmatic patients or control subjects and red cells were removed by dextran sedimentation. Hundred microlitres of the cell suspension (2×10^6 /mL) was incubated with experimental mAbs at saturating concentrations for 30 min on ice followed by incubation with an FITC-conjugated anti-mouse for 30 min on ice. The cell suspensions were washed and incubated with anti-CD16 PE mAb for 30 min on ice. Contaminating erythrocytes were removed by hypotonic lysis with FACSTM lyse (Becton Dickinson, Cowley, UK). Non-specific fluorescence was controlled by incubation with isotype-specific control antibodies. Washed cells were analysed on a fluorescent activated cell analyser (FACScan; Becton Dickinson). Granulocytes were identified on the basis of their forward and side scatter (Fig. 1a). Eosinophils were distinguished from neutrophils in the

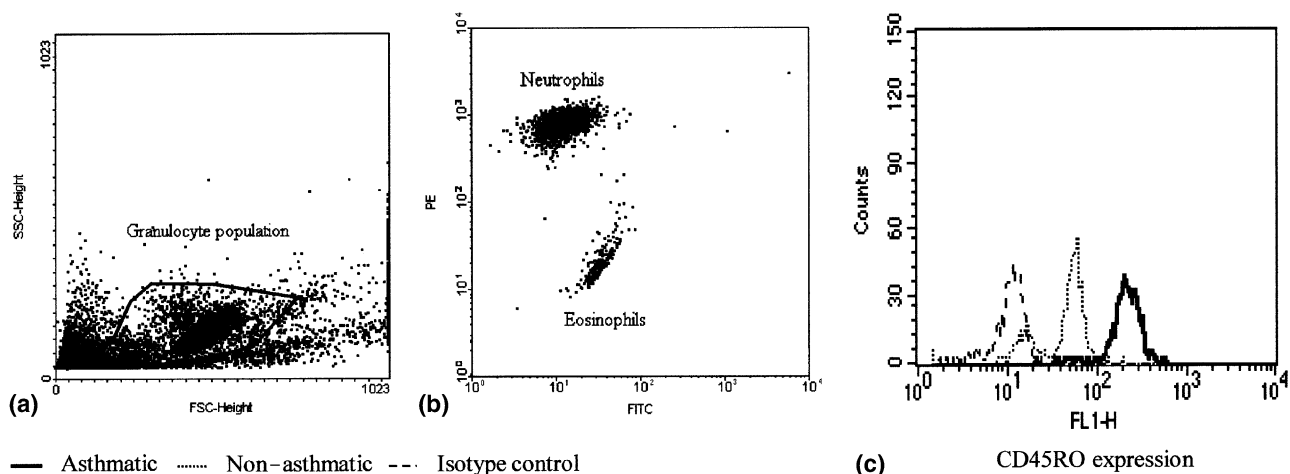


Fig. 1. Representative experiment showing (a) the granulocyte population in a mixed leucocyte population based on forward and side scatter, (b) differential determination of eosinophil CD45RO (FITC) expression by eosinophils in a mixed granulocyte population based on CD16 PE expression by neutrophils but not eosinophils, and (c) CD45RO expression by eosinophils from an asthmatic patient (heavy line) compared with eosinophils from a normal control subject (dotted line). The negative isotype control is represented by the heavy dotted line.

granulocyte population by their inability to bind CD16. Test mAb were labelled with FITC that gives a green fluorescence (FL-1) while neutrophils were labelled with CD16-PE that gives a red fluorescence (FL-2), allowing the two cell populations to be clearly distinguished. Thus, only the CD16-PE-negative eosinophil population was analysed for CD45 and CD45 isoform expression (Fig. 1b). A representative experiment showing CD45RO expression by eosinophils from an asthmatic and control subject in a mixed leucocyte population is shown in Fig. 1c.

Determination of eosinophil apoptosis by flow cytometry

Eosinophils were purified under sterile conditions using our standard immunomagnetic-negative selection method [6]. Briefly, following removal of red cells by dextran sedimentation, the leucocytes were centrifuged on Percoll gradients. Contaminating red cells were removed by hypotonic shock and the granulocytes incubated on ice with micromagnetic beads coated with anti-CD16 for 40 min before passage through the magnetic-activated separation column to remove neutrophils. This method consistently yields eosinophils with a purity of 99% and greater than 99% viability.

Eosinophils (2×10^5) were cultured in RPMI 1640 culture medium supplemented with 10% fetal calf serum in the presence of saturating concentrations of the CD45 and CD45 isoform mAb and suitable controls as listed above for various time-points as previously described [6]. The degree of apoptosis in these cells was measured using our established technique as described [11]. Briefly, control and ligated eosinophils were washed in cold phosphate-buffered saline (PBS) and resuspended in binding buffer (HEPES-buffered PBS supplemented with $2.5 \times \text{mM}$ CaCl_2) prior to the addition of FITC-conjugated annexin V for 10 min at room temperature. Cells were then washed, resuspended in binding buffer and propidium (PI) iodide added to give a final concentration of 1 mg/mL. They were then analysed immediately by flow cytometry performed on the basis of eosinophils gated for their forward and side scatter profiles, with any cell debris excluded from the analysis. The use of annexin V and PI staining for the determination of eosinophil apoptosis has been previously validated as being both accurate and highly reproducible [6,11]. In addition, this methodology allows the distinction to be made between viable, early apoptotic, late apoptotic (i.e. with evidence of secondary necrosis) and necrotic eosinophils.

Statistics

Data were analysed using either Friedman or Kruskal–Wallis ANOVA, and Wilcoxon signed ranks or Mann–Whitney *U*-test as appropriate, with a *P*-value of less than 0.05 being considered statistically significant.

Results

The details of asthmatic patients and control subjects are shown in Table 1. Comparison of CD45 expression levels showed that eosinophils from asthmatic patients expressed significantly higher levels of the pan-CD45 receptor and the

Table 1. Characteristics of study population

	Non-asthmatic non-atopic	Atopic non-asthmatic	Asthmatic non-atopic	Asthmatic atopic
<i>N</i>	17	16	8	19
Age (years)				
Median	24	28	52	27
Range	18–39	18–47	20–63	18–64
Gender (male/Female)	5/12	6/10	5/3	11/8
FEV ₁ (%)				
Mean (SEM)	104.2 (2.6)	96.6 (2.3)	88 (5.8)	86.3 (3.4)
FEF _{25–75} (%)				
Mean (SEM)	93 (5.1)	78 (4.6)	59 (6.6)	57 (4.4)
Methacholine PD ₂₀ (μg)				
Mean (SEM)	—	—	161 (41.3)	83 (18.1)
Number of Positive Skin Prick				
Mean (SEM)	—	3.3 (0.4)	—	3.5 (0.4)
Subjects taking inhaled corticosteroids (daily dose in μg)	None	None	7 (400–2000)	9 (400–1200)

isoform CD45RO than eosinophils from normal control subjects (Fig. 2). No significant increase in the expression of CD45RA or CD45RB was observed in eosinophils from asthmatic patients compared with control subjects. These findings are further detailed for the two pan-CD45 mAb and for CD45RO in Fig. 3, which clearly illustrate the higher expression of these membrane receptors by eosinophils from asthmatic patients. However, there were no significant positive correlations between increased eosinophil pan-CD45 and CD45RO expression and the degree of asthma symptoms as defined by lung function (FEV₁ and FEF_{25–75}) and methacholine PD₂₀. Moreover, no positive correlations were found between eosinophil pan-CD45 or CD45 isoform expression and inhaled corticosteroid usage in the asthmatic group or peripheral blood eosinophil count in any of the subjects studied (data not shown).

We next investigated whether increased expression of pan-CD45 or CD45 was a consequence of atopy in the asthmatic patients. Comparison of the eight patients with non-allergic asthma with 19 patients with allergic asthma and with control subjects revealed that significantly higher expression of pan-CD45 and CD45RO was seen in the non-atopic asthma group (Fig. 4). Increased expression of pan-CD45 or CD45RO was not observed in the non-asthmatic atopic group compared with non-allergic, non-asthmatic control subjects. There were no significant differences in the severity of asthma between the allergic and non-allergic asthmatic patients. These data indicate that the observed increase in CD45 expression was not a consequence of the allergic status of these patients.

We have previously demonstrated that mAb-dependent ligation of pan-CD45, CD45RA and CD45RB results in accelerated apoptosis in human eosinophils [6]. Therefore, we investigated whether the increase in pan-CD45 and CD45RO expression in asthmatics had any functional consequence in terms of enhanced apoptosis in eosinophils isolated

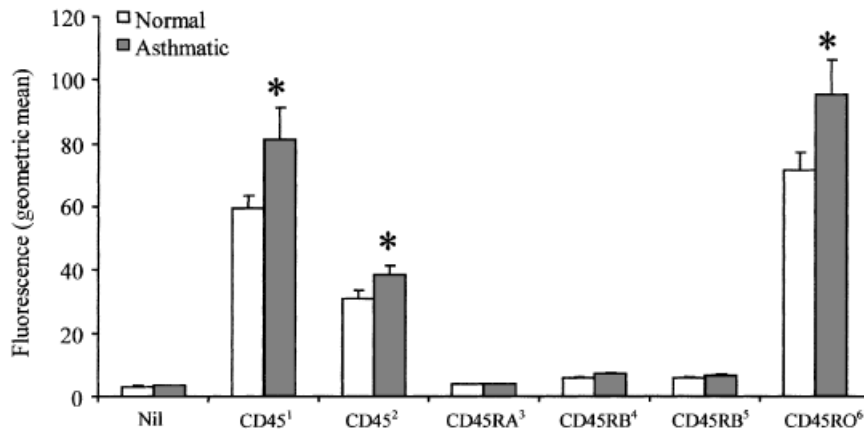


Fig. 2. Comparison of pan-CD45 (¹BRA55; ²F/10/89/4), CD45RA (³F8/11/13), CD45RB (⁴BRA11; ⁵MEM55) and CD45RO (⁶UCHL1) expression levels from asthmatic ($n = 27$) and normal individuals ($n = 33$). * $P < 0.05$ difference between asthmatic and normal individuals. Each bar represents mean $\pm$ SEM.

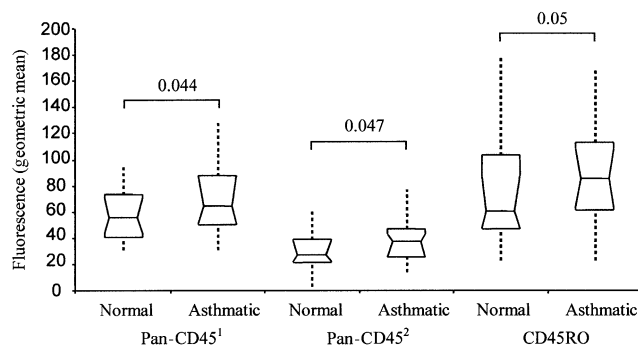


Fig. 3. Comparison of pan-CD45 (¹BRA55; ²F/10/89/4) and CD45RO expression levels by eosinophils in whole leucocyte populations from asthmatic ($n = 27$) and normal individuals ($n = 33$). Box and whisker plots show median, 25th and 75th percentiles, with ranges as vertical lines.

from asthmatic individuals compared with normal controls. These results are summarized in Fig. 5. At 20 h post receptor ligation with specific mAb to pan-CD45, CD45RA and CD45RB, we observed significant increases in the constitutive rate of eosinophil apoptosis compared with isotype-matched control mAb. No significant increase was observed following ligation with CD45RO mAb, an observation that confirms our previous findings [6]. However, we observed no significant difference in the CD45 or CD45 isotype mAb-induced increase in the constitutive rate of apoptosis in eosinophils from asthmatic patients compared with cells from non-asthmatic atopic or non-asthmatic, non-atopic controls. Incubation with CD45 mAb for longer time-points did not result in any significant differences in the constitutive rate of apoptosis in asthmatic or control subject eosinophils nor did we observe significant differences in the rates of eosinophil apoptosis in cells from atopic asthmatics compared with non-atopic asthmatics (data not shown).

Discussion

There is a considerable body of evidence demonstrating a relationship between asthma severity and levels of eosinophils

and of their products in blood, induced sputum and in bronchial biopsies or lavage samples [1, 2]. This study has demonstrated that eosinophils in whole-blood samples from asthmatic patients express significantly higher levels of the pan-CD45 receptor and the isoform CD45RO than eosinophils from normal control subjects. These observations support a large body of evidence that eosinophils from asthmatic patients are more activated than eosinophils from healthy individuals. Evidence of activation includes greater secretion of pro-inflammatory mediators, prolonged survival [1, 2] and enhanced expression of membrane receptors [12, 13]. No significant correlations were found between eosinophil expression of pan-CD45 or CD45 and the degree of asthmatic symptoms in these patients, findings that may be partly due to the relatively smaller number of patients recruited for this part of the study. Interestingly, we were unable to show any significant association between increased pan-CD45 or CD45RO expression and atopy. Moreover, no positive correlations were found between pan-CD45 or CD45 isoform expression and corticosteroid usage or peripheral blood eosinophil count. This suggests that the observed higher expression of pan-CD45 and CD45 expression by eosinophils from asthmatic patients is more likely due to inflammatory changes associated with the asthma phenotype rather than the presence of allergy.

Apoptosis or programmed cell death is a tightly controlled, central and essential process in the resolution of inflammation. Eosinophil removal from the tissues is thought to be dependent on their death by apoptosis, followed by their recognition and phagocytosis by macrophages [14] or resident bronchial epithelial cells [15, 16]. Thus it can be envisaged that apoptosis induction in eosinophils followed by their recognition and removal by phagocytes is a rational avenue for the development of novel therapy for asthma [17]. This is supported by a clinical study showing that treatment of asthma exacerbations with corticosteroids resulted in the resolution of eosinophilic inflammation through apoptosis induction in lung eosinophils that were subsequently recognized and phagocytosed by alveolar macrophages [18]. We have also recently demonstrated that reduced apoptosis in eosinophils present in induced sputum significantly correlates with asthma severity as defined by airflow obstruction and symptom scores [19]. Eosinophil apoptosis can be induced by

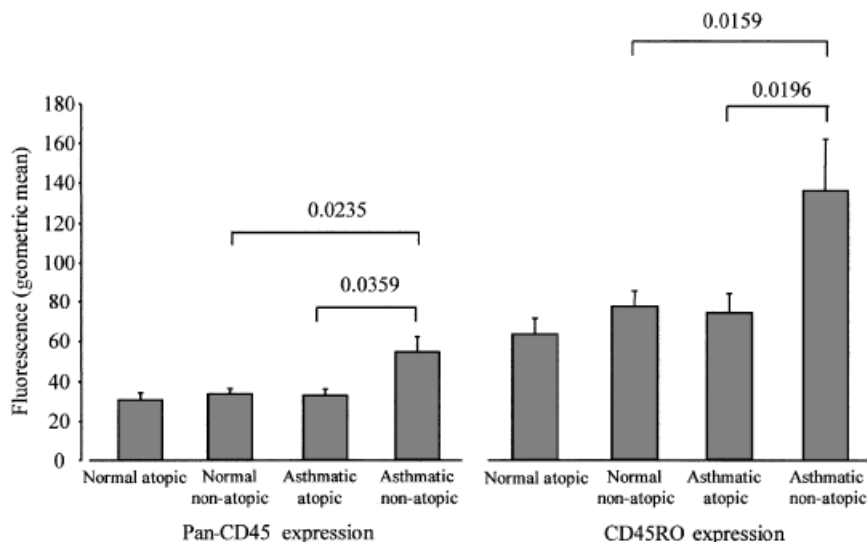


Fig. 4. Comparison of pan-CD45 and CD45 RO expression by eosinophils in whole-leucocyte populations from patients with allergic asthma ($n = 19$), non-allergic asthma ($n = 8$), non-allergic control subjects ($n = 17$) and allergic control subjects ($n = 16$). Each bar represents mean $\pm$ SEM.

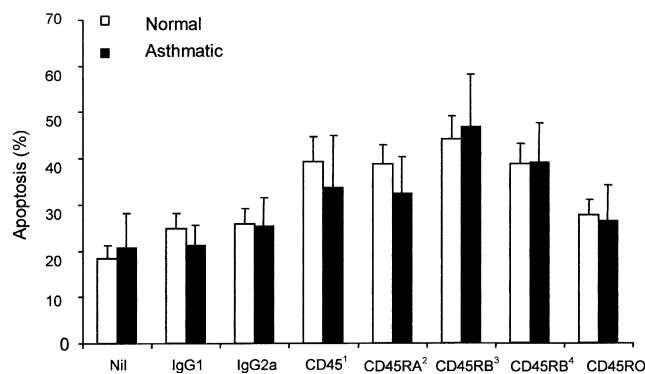


Fig. 5. Effects of saturating concentrations of CD45 and CD45 isoform mAb on the rate of constitutive apoptosis in eosinophils from normal subjects and asthmatic patients at 20 h of stimulation. Compared with controls, significant ($P < 0.01$) increases in % apoptosis were seen with pan-CD45 (¹BRA55), CD45RA (²F8/11/13) and CD45RB (³BRA11; ⁴MEM55) mAb. No significant differences were observed in constitutive apoptosis rates in eosinophils from normal or asthmatic individuals. Each bar represents mean $\pm$ SEM.

treatment with corticosteroids [20] or ligation of membrane receptors by specific mAb for Fas (CD95) [21, 22], CD69 [23] and CD45 [6]. Thus, we examined whether eosinophils from asthmatic patients were more susceptible to CD45-induced apoptosis induction. However, despite significantly higher expression of pan-CD45 and CD45RO by eosinophils from asthmatics, we did not observe significantly higher apoptosis levels in eosinophils isolated from asthmatic patients compared with healthy controls following CD45 and CD45 isoform mAb-dependent receptor ligation. The most likely explanation for this finding is that CD45RO was the only isoform that exhibited significantly increased expression on eosinophils in patients with asthma. The pan-CD45 mAb recognizes all forms of CD45; thus the increase in pan-CD45 expression by eosinophils from asthmatics was most likely due to increased CD45RO expression. Ligation of the CD45RO isoform does not accelerate apoptosis in eosinophils [6]. Thus, it is not surprising that increased CD45RO

expression did not lead to a greater susceptibility to apoptosis in eosinophils from asthmatic patients. This portion of the study was performed using isolated eosinophils, while pan-CD45 and CD45 isoform expression was established using whole-blood eosinophils. The latter approach was used as there is ample evidence that eosinophil purification by density gradient centrifugation followed by immunomagnetic selection can influence eosinophil function [24, 25], including membrane receptor expression [26]. Indeed in our previous study, pan-CD45, CD45RA, CD45RB and CD45RO was higher in eosinophils isolated from 10 normal individuals than reported here [6]. Unfortunately, we were obliged to use isolated eosinophils to examine CD45 mAb-induced apoptosis in the present study, as to our knowledge, there are no reliable methods for apoptosis detection in whole-blood eosinophil samples. We cannot therefore discount the possibility that different results might have been observed if whole-blood eosinophils had been used for this portion of the study.

In conclusion, we have demonstrated that eosinophils from patients with asthma express higher levels of pan-CD45 and the isoform CD45RO compared with eosinophils from normal control subjects. Increased expression did not appear to be a consequence of atopy and elevation in CD45 and some of its isoforms appear to be a marker of non-atopic asthma. These findings provide further evidence that eosinophils from asthmatic patients are more activated than those from normal subjects. Larger and more comprehensive studies would be required to answer the question that monitoring of enhanced expression of CD45 by eosinophils might be more prevalent in non-atopic asthma than in atopic-associated disease.

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