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Upregulation and activation of eosinophil integrins in blood and airway after segmental lung antigen challenge[1]

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Abstract

We hypothesized that there are clinically relevant differences in eosinophil integrin expression and activation in patients with asthma. To evaluate this, surface densities and activation states of integrins on eosinophils in blood and bronchoalveolar lavage (BAL) of 19 asthmatic subjects were studied before and 48 h after segmental Ag challenge. At 48 h, there was increased expression of α_D and the N29 epitope of activated β_1 integrins on blood eosinophils and of α_M, β_2, and the mAb24 epitope of activated β_2 integrins on airway eosinophils. Changes correlated with the late-phase fall in forced expiratory volume in 1 s (FEV_1) after whole-lung inhalation of the Ag that was subsequently used in segmental challenge and were greater in subjects defined as dual responders. Increased surface densities of α_M and β_2 and activation of β_2 on airway eosinophils correlated with the concentration of IL-5 in BAL fluid. Activation of β_1 and β_2 on airway eosinophils correlated with eosinophil percentage in BAL. Thus, eosinophils respond to an allergic stimulus by activation of integrins in a sequence that likely promotes eosinophilic inflammation of the airway. Before challenge, β_1 and β_2 integrins of circulating eosinophils are in low-activation conformations, and $\alpha_D\beta_2$ surface expression is low. After Ag challenge, circulating eosinophils adopt a phenotype with activated β_1 integrins and upregulated $\alpha_D\beta_2$, changes that are predicted to facilitate eosinophil arrest on VCAM-1 in bronchial vessels. Finally, eosinophils present in IL-5-rich airway fluid have a hyperadhesive phenotype associated with increased surface expression of $\alpha_M\beta_2$ and activation of β_2 integrins.

Keywords

adhesion molecules; eosinophils; cell trafficking; inflammation; lung; integrins

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Introduction

The contribution of eosinophils to certain aspects of asthma, such as airway hyperreponsiveness, remains controversial; but nevertheless there is evidence that eosinophil recruitment to the airway contributes to asthma exacerbations and the chronic character of asthma, by regulating airway inflammation and remodeling (1–15). Thus, the study of how eosinophils traffic from blood to airway is of considerable importance. Integrins, which are versatile cellular adhesion receptors (16,17), are likely determinants of how eosinophils roll and arrest on lung endothelium; extravasate and migrate through endothelium, underlying basement membrane and tissue to the airway wall; and traverse the bronchial epithelium to the airway lumen (18–20).

Human eosinophils express seven integrin heterodimers, $\alpha_4\beta_1$ (CD49d/CD29), $\alpha_6\beta_1$ (CD49f/CD29), $\alpha_L\beta_2$ (CD11a/CD18), $\alpha_M\beta_2$ (CD11b/CD18), $\alpha_X\beta_2$ (CD11c/CD18), $\alpha_D\beta_2$ (CD11d/CD18), and $\alpha_4\beta_7$ (CD49d/β_7)(13,21,22). Each integrin heterodimer interacts with its own set of ligands, which are counter-receptors on other cells or extracellular matrix (ECM) components (17,23). The functions of an integrin on a given cell is regulated by expression level and activation state (24–26).

We hypothesized that there are clinically relevant differences in eosinophil integrin expression levels and activation state in patients with asthma. The purpose of the present study was to define changes in the expression levels and activation states of blood and airway eosinophil integrins 48 h following segmental allergen challenge in asthmatic subjects, thus reflecting the development of allergic inflammation. Segmental Ag challenge induces a strong local recruitment of eosinophils (27). We found characteristic integrin changes on blood and airway eosinophils; differences between single and dual responder asthmatics; and associations among integrin changes, magnitude of eosinophil recruitment to airway, and IL-5 concentration in bronchoalveolar lavage (BAL)[3] fluid. We propose a scenario in which 1) Ag challenge leads to activation of β_1 integrins and increased surface expression of $\alpha_D\beta_2$ on blood eosinophils, 2) such activated eosinophils are more prone to arrest on VCAM-1 (CD106)-bearing endothelium in challenged segments, and 3) IL-5 and other cytokines trigger activation of β_2 integrins, contributing to the hyperadhesive phenotype of airway eosinophils.

Materials and Methods

Subjects and screening

Nineteen subjects with mild asthma as diagnosed by an allergist were studied (Table I). These subjects had a history of asthma exacerbation to aeroallergen, PC_{20} (provocative concentration of methacholine producing a 20% fall in forced expiratory volume in 1 s [FEV_1]) < 8 mg/ml, and/or reversibility to β-agonist > 12%. Subjects were screened as described previously with allergen skin prick tests, determination of airway hyperresponsiveness, and spirometry (28). All subjects had a positive skin prick test to one or more aeroallergens, were nonsmokers, did not have a respiratory infection within 30 days of study, and had not received anti-histamines within seven days or corticosteroids within 30 days of study enrollment. The studies were reviewed and approved by the University of Wisconsin-Madison Health Sciences Human Subjects Committee. Informed written consent was obtained from each subject before participation.

[3]Abbreviations used in this paper: $AgPD_{20}$, provocative dose of Ag producing a 20% fall in FEV_1; BAL, bronchoalveolar lavage; CBU, cumulative breath unit; CD, cat dander; ECM, extracellular matrix; F, female; FEV_1, forced expiratory volume in 1 s; gMCF, geometric mean channel fluorescence; H, homogeneous (distribution); HDM, house dust mite; ICS, inhaled corticosteroid; M, male; PC_{20}, provocative concentration of methacholine producing a 20% fall in FEV_1; r_S, Spearman rank correlation coefficient; RW, ragweed; T, total; % pos., percentage of positive cells; % pred., percentage of predicted value.

Determination of provocative allergen dose and early- and late-phase response to whole-lung allergen challenge

At least four weeks before bronchoscopy, a graded whole-lung inhaled Ag challenge was performed as described (28) to determine the provocative dose of Ag producing a 20% fall in FEV_1 ($AgPD_{20}$) and the magnitude of early- and late-phase responses. Briefly, baseline spirometry was performed and repeated after five breaths of saline diluent. If FEV_1 remained within 10% of baseline, five breaths of allergen were inhaled and spirometry was repeated 10 min later. Consecutively greater concentrations of allergen were given until FEV_1 fell by ≥ 20% from baseline. The maximum immediate or early-phase (within 15–30 min) fall in FEV_1 was determined, and subjects were then monitored every 15 min until FEV_1 returned to within 10% of baseline. Thereafter, the subjects were monitored at 1 h intervals for 8 h to determine whether a late-phase response was present (28,29). Subjects having a FEV_1 fall ≥ 15% 3–8 h after the whole-lung Ag challenge were considered to have a dual response phenotype; the other subjects were considered single responders (28).

Segmental bronchoprovocation with allergen and BAL

Bronchoscopy, segmental bronchoprovocation with allergen, and subsequent BAL were performed in two different bronchopulmonary segments as described (27,28,30). Baseline BAL at 0 h immediately before segmental bronchoprovocation was performed in two segments. Then, a total dose of 30% of the subject's $AgPD_{20}$ was administered incrementally to enhance subject safety: 10% of the $AgPD_{20}$ in the first segment and, when this dose was well tolerated, 20% in the second segment. Forty-eight hours later, a second bronchoscopy was performed by instilling 160 ml of sterile 0.9% NaCl warmed to 37ºC in each segment. BAL fluid recovered from the two segments was pooled for analysis, and the volume of recovered fluid measured.

Antibodies for flow cytometry and ELISA, and recombinant protein standards

Anti-α_D integrin mAb 240I (31) was obtained as a gift from ICOS (Bothell, WA). Activation-sensitive β_2 integrin mAb24 (32,33) was a gift from Nancy Hogg (Cancer Research UK London Research Institute, London, UK). Anti-β_1 mAb MAR4; anti-β_2 L130, anti-β_7 Fib504; anti-α_4 9F10; anti-α_6 GoH3; anti-α_L AI111; anti-α_X Bly6; PE-conjugated goat anti-mouse and anti-rat IgG; FITC-conjugated anti-CD14 and anti-CD16; isotype controls mouse IgG_1, κ (clone A112-2) and rat IgG_{2a}, κ (A110–2); and unlabeled and biotinylated anti-IL-3, anti-IL-5, and anti-IFN-γ mAbs and corresponding recombinant protein standards for ELISA were from BD Biosciences (San Diego, CA). Anti-α_M LM11 and activation-sensitive anti-β_1 N29 (34) were from Chemicon (Temecula, CA). Unlabeled and biotinylated anti-GM-CSF mAb and recombinant protein standard for ELISA were from R&D Systems (Minneapolis, MN).

Flow cytometry of blood and BAL cell samples

Because purification of blood eosinophils has been found to cause activation of β_1 (35), flow cytometry was done on unfractionated blood and BAL cells. Surface expressions of α_M, α_L, α_X, α_D, α_4, α_6, β_1, β_2, β_7, activated β_1, and activated β_2 were determined. Blood was drawn routinely into lavender-top standard tubes (giving a final EDTA concentration of 1.8 mg/ml) (BD Vacutainer Systems, Franklin Lake, NJ). For determination of mAb24 reactivity, blood was drawn into green-top tubes (giving a final heparin concentration of 14 USP units/ml). Control experiments revealed that the anticoagulant had no effect on the results, except, as reported, for mAb24, whose epitope is not exposed in the presence of EDTA (32). Not all samples were subjected to complete analysis, due to changes in the mAb panel that were made based on ongoing analysis of results from this study and other studies. Originally, we focused on differences between blood and BAL fluid 48 h after segmental challenge. Blood from before segmental challenge was included as a routine later, when we had indications of interesting differences between blood after versus before challenge. Similarly, we originally focused on

subunits of the integrins known to be able to bind VCAM-1, $\alpha_4\beta_1$, $\alpha_4\beta_7$, and $\alpha_D\beta_2$ (13,21), and added α_M later when we had evidence that $\alpha_M\beta_2$ is involved in the adhesion of purified airway eosinophils to VCAM-1 and other ligands (22,36). BAL fluid cells were recovered, cytospun, and stained for differential counts as described (29,30).

EDTA-treated blood (100 μl) was incubated with 0.5 μg primary antibody or isotype control in 100 μl FACS buffer (PBS with 2% BSA and 0.2% NaN_3) for 30 min. For mAb24 (and its isotype control), heparin-treated blood was used, since the mAb24 epitope is not exposed in EDTA (32), and was incubated with primary antibody in RPMI 1640 with 10% FBS at 37ºC following the protocol especially designed for mAb24 (37). After primary antibody incubation, samples were washed with 1 ml PBS, washed with 250 μl FACS buffer, and resuspended in 250 μl FACS buffer containing PE-conjugated goat anti-mouse or anti-rat IgG (2 μg/ml). After incubation for 30 min, samples were washed again with PBS, resuspended in 100 μl FACS buffer with a mixture of FITC-conjugated anti-CD14 (0.125 μg) and anti-CD16 (0.625 μg) and incubated for 30 min. Red blood cells were lysed by incubation with 2 ml FACS lysing solution (BD Biosciences) for 10 min, followed by centrifugation. Incubations were at room temperature until after red blood cell lysis and then at 4ºC. Samples were washed with 500 μl FACS buffer, resuspended in 250 μl FACS fixative (1% paraformaldehyde, 67.5 mM sodium cacodylate, 113 mM NaCl, pH 7.2), stored at 4ºC in the dark, and washed with 1 ml PBS and resuspended in 250 μl FACS buffer just prior to data collection. Fixation did not decrease signals (not shown). Data were collected from 30,000 - 170,000 events, using a FACS Calibur (BD Biosciences; available through the Flow Cytometry Facility, Comprehensive Cancer Center, University of Wisconsin-Madison) and Cellquest software (BD Biosciences). Rainbow calibration fluorescent beads (Spherotech, Libertyville, IL) were run each day to calibrate and check the performance of the instrument and channels. Data were analyzed using Cellquest and FlowJo (TreeStar, Ashland, OR). Eosinophils were gated based both on scattering (high side scatter) and lack of staining with anti-CD14 and anti-CD16 (30,35). Thus, the cells that were analyzed for PE signal fit two criteria for eosinophils by being gated inside both characteristic regions in a plot of side scatter versus FITC staining and a plot of side versus forward scatter. When cells gated on side versus forward scatter were collected by cell sorting followed by cytospin, ≥96% stained for the eosinophil marker eosinophil major basic protein. To analyze circulating neutrophils and monocytes in the same data sets, these leukocyte types were gated inside characteristic regions in the two plots, neutrophils having intermediate side scatter and FITC positivity and monocytes having relatively low side scatter and FITC positivity. Data are expressed as specific geometric mean channel fluorescence (gMCF; specific gMCF = gMCF with a specific integrin mAb – gMCF with isotype control) and as percentage of positive cells (isotype control set with a marker to 2% positive cells) as before (35). For unfractionated BAL cells, flow cytometry was performed with 2×10^5 cells (in 100 μl) and the protocol was as described for blood samples, except that all incubations were at 4ºC and the erythrocyte lysis step was omitted and replaced by a wash with 1 ml PBS.

ELISA for cytokines in BAL fluid

To measure cytokine concentrations, BAL fluid was concentrated ten-fold at 4ºC using a low protein-binding Centriprep® centrifugal filter unit (Millipore, Billerica, MA) with a molecular weight cut-off limit of 3 kDa. A sensitive two-step sandwich ELISA was used as described (38). The assay sensitivities were below 3 pg/ml for IL-5 and GM-CSF, 12 pg/ml for IL-3, and 25 pg/ml for IFN-γ. Values are presented as the concentration in recovered BAL fluid prior to the concentration step.

Statistics

The Mann-Whitney *U* test was used to compare data between groups. The Spearman rank correlation test was used to analyze correlations. A level of $p \leq 0.05$ was considered significant. Analyses were performed using Prism 3.0 (GraphPad, San Diego, CA).

Results

Response to whole-lung allergen challenge

All subjects responded to whole-lung inhaled Ag challenge with a drop in FEV_1 within 15–30 min to a median value of 31% early FEV_1 fall (Table I). Nine of the 19 subjects had a late-phase response (39), as defined by a FEV_1 fall ≥ 15% from baseline 3–8 h after challenge (Table I). Using this criterion (28), the nine subjects were classified as having a dual response phenotype. The other ten subjects were considered single responders (Table I). There were no significant differences between the single and dual responders in regards to age, sex, airway responsiveness to methacholine, FEV_1 at the screening visit, or early-phase fall in response to whole-lung Ag challenge (Table I). FEV_1 48 h after segmental Ag challenge was decreased minimally compared to before segmental Ag challenge and was not significantly different between single and dual responders (medians 98% and 96% of the value before segmental challenge in single and dual responders, respectively).

Median number of BAL eosinophils after segmental challenge was > tenfold higher in the dual responders than in the single responders (Table I). Median numbers of BAL neutrophils and macrophages, in contrast, were about twofold higher and not significantly different in dual versus single responders (medians in single and dual responders were 3.1×10^6 and 7.5×10^6 neutrophils, and 43×10^6 and 93×10^6 macrophages; respectively). Neutrophil percentage was not significantly different and macrophage percentage was significantly lower in dual responders, due to the increased proportion of eosinophils (medians in single and dual responders were 2.3% and 3.0% neutrophils, and 47% and 22% macrophages; respectively).

Integrin expression on blood and BAL eosinophils after segmental allergen challenge

Sample flow cytometry histograms for eosinophil expression of total β_1 (*A*), the activation-sensitive β_1 epitope for mAb N29 (*B–D*), total β_2 (*E*), the activation-sensitive β_2 epitope for mAb24 (*F*), α_M (*G*), and α_D (*H*) are shown in Fig. 1. Expression distributions were homogeneous in some samples (Fig. 1*A,B,F*) and heterogeneous and asymmetric with one or more “shoulders” in others (Fig. *1C,D,G,H*). β_1 distributions were mostly heterogeneous on blood eosinophils and uniformly homogeneous on BAL eosinophils (Fig. 1*A*, Table II). N29 reactivity of blood eosinophils was variable (Fig. 1*B–D*, Table II). Some samples had two peaks (Fig. 1*C*) or a “shoulder” of reactivity (Fig. 1*D*). N29 reactivity of BAL eosinophils was uniformly homogeneous (Fig. 1*B–D*, Table II). β_2 was heterogeneous on blood eosinophils, often with two or several distinct populations; whereas the peak of BAL eosinophils was mostly homogeneous and shifted to the right with higher fluorescence intensity compared to the most positive blood eosinophil population (Fig. 1*E*, Table II). MAb24 reactivity was homogeneous and low on blood eosinophils and mostly homogeneous and shifted to the right on BAL eosinophils (Fig. 1*F*, Table II). α_M was mostly heterogeneous on blood eosinophils and homogeneous and shifted to the right on BAL eosinophils (Fig. 1*G*, Table II). α_D was mostly heterogeneous on blood eosinophils and mostly homogeneous on BAL eosinophils (Fig. 1*H*, Table II). Thus, distributions on BAL eosinophils were typically homogeneous; whereas distributions of β_1, β_2, α_M, and α_D on blood eosinophils were mostly heterogeneous.

Expression data were scored either as percentage positive cells or as expression level (specific geometric mean channel fluorescence [gMCF])(Table II, Fig. 2). Fig. 3 shows a comparison between the results with these two scoring methods on blood eosinophils for N29 (Fig. 3*A*)

and α_D (Fig. 3*B*). At lower fluorescence intensity, percentage positive cells and expression level correlate; whereas at higher intensity the percentage positive cells plateaus (Fig. 3*A,B*). Because of its greater dynamic range, specific gMCF was chosen as the more informative measure of integrin expression and activation (Fig. 2). It should be emphasized that for the samples displaying heterogeneous bimodal or multimodal distributions of the β_2 integrin subunits, neither gMCF nor percentage positive cells fully captures the complexity of the distributions.

At 48 h after segmental Ag challenge, on circulating eosinophils of dual responders the expression level of the epitope for activation-sensitive anti-β_1 mAb N29 (34,40,41) was higher 48 h after segmental challenge as compared to before challenge (Fig. 2*B*). Circulating eosinophils also had significantly higher expression of the α_D integrin subunit compared to before segmental challenge (Fig. 2*F*). α_D and N29 epitope expression on blood eosinophils 48 h after segmental challenge correlated with each other (r_s [Spearman rank correlation coefficient] = 0.73, $p = 0.002$). Thus, the results demonstrate that segmental Ag challenge causes significantly increased surface expression of the α_D subunit of β_2 integrins and, in dual responders, activation of β_1 integrins at 48 h on blood eosinophils.

Eosinophils in BAL 48 h after segmental Ag challenge had significantly higher β_2 and α_M subunit expression than eosinophils in blood obtained before or 48 h after segmental challenge (Fig. 2*C,E*). The expression of the epitope for the activation-sensitive anti-β_2 mAb24 (32,33) was significantly increased on BAL eosinophils; binding of mAb24 to blood eosinophils was low in blood both before and after segmental challenge (Fig. 2*D*). α_D expression was significantly increased on BAL eosinophils compared to blood eosinophils before challenge but not compared to blood also sampled at 48 h (Fig. 2*F*). In dual responders, BAL eosinophils had significantly more total and activated β_1 (as ascertained by N29) than blood eosinophils before challenge (Fig. 2*A,B*). α_6 expression was lower on BAL eosinophils than on blood eosinophils after challenge (specific gMCF = 560 ± 140 [mean ± SEM] versus 920 ± 110, $p \leq 0.05$)(not shown). No differences were found in expression of α_L, α_X, α_4, or β_7 (not shown). Overall, the results indicate that $\alpha_M\beta_2$ is upregulated on BAL eosinophils and BAL eosinophil β_2 integrins are in a conformationally altered and activated state compared to on blood eosinophils.

To examine the specificity of the changes on blood eosinophils, circulating neutrophils and monocytes were also analyzed. Before segmental challenge, neutrophils had higher N29 reactivity than eosinophils and monocytes even higher (Figs. 4*B* and 5*B*, compare to Fig. 2*B*). At 48 h after segmental Ag challenge, circulating neutrophils and monocytes, like eosinophils, had significantly higher α_D expression compared to before challenge (Figs. 4*F* and 5*F*). Monocytes from dual responders had higher α_D after segmental challenge than those from single responders (Fig. 5*F*). N29 reactivity of neutrophils (as with eosinophils) increased significantly in dual responders upon segmental challenge, although to a lesser degree (1.4-fold for neutrophils, 1.7-fold for eosinophils)(Fig. 4*B*, compare to Fig. 2*B*); whereas N29 reactivity of monocytes did not increase significantly (Fig. 5*B*). Further, α_M on blood neutrophils and monocytes and β_2 on blood monocytes increased significantly upon segmental challenge (Figs. 4*E* and 5*C,E*); in contrast to blood eosinophil α_M and β_2, which did not (Fig. 2*C,E*).

Thus, 1) α_D is upregulated on all three leukocyte populations, not just eosiniophils; 2) β_1 at baseline is more activated on neutrophils and monocytes compared to eosinophils; 3) in dual responders we found increased activation state of β_1 on neutrophils despite the high baseline activation state; and 4) surface expression of α_M is upregulated on monocytes and neutrophils, but not on blood eosinophils.

Correlations between integrin expression and eosinophil numbers in BAL after segmental allergen challenge

As with the variability in eosinophil numbers in BAL after segmental Ag challenge in the 19 subjects (Table I), there was considerable variability in integrin activation or expression on BAL eosinophils (Fig. 2). BAL eosinophils from dual responders had significantly higher N29 epitope and total α_2 expression than BAL eosinophils from single responders (Figs. 2 and 6). Grouping all subjects together, reactivity of BAL eosinophils with the activation-sensitive mAbs, N29 against β_1 and mAb24 against β_2, correlated significantly with percentage of eosinophils in BAL (Fig. 7). Surface expression of other integrin subunits on BAL eosinophils did not correlate with BAL eosinophil percentage (not shown). These results indicate that activation of both β_1 and β_2 integrins are associated with eosinophil recruitment to the airway.

Correlations between integrin expression after segmental allergen challenge and the magnitude of the late-phase fall in FEV_1 after whole-lung allergen challenge

We also analyzed for possible correlations between eosinophil integrins after segmental challenge and the magnitude of the maximum fall in FEV_1 3–8 h after whole-lung allergen challenge (late-phase fall). α_D expression and reactivity with N29 of blood eosinophils 48 h after segmental challenge each correlated with the magnitude of the late-phase fall in FEV_1 after whole-lung challenge (Fig. 8*A*,*B*). β_2 expression and reactivity with mAb24 of BAL eosinophils 48 h after segmental challenge also correlated with the magnitude of the late-phase FEV_1 fall (Fig. 8*C*,*D*), as did α_M expression of BAL eosinophils ($r_s = 0.83$, $p = 0.008$)(not shown). In addition, the late-phase FEV_1 fall correlated with the percentage of eosinophils in BAL 48 h after segmental challenge ($r_s = 0.70$, $p = 0.001$)(not shown). Thus, the results indicate that greater α_D upregulation and β_1 activation on blood eosinophils and greater $\alpha_M\beta_2$ upregulation and β_2 activation on BAL eosinophils after segmental challenge in a subject are associated with a greater late-phase FEV_1 fall in response to whole-lung challenge.

Correlations between integrin expression and cytokine concentrations in BAL fluid after segmental allergen challenge

To identify factor(s) possibly responsible for the changes in integrins observed following segmental Ag challenge, the concentrations of IL-5, GM-CSF, IL-3, and IFN-γ in BAL fluid were measured (Fig. 9). The concentration of all four cytokines was significantly higher 48 h after segmental challenge than in samples obtained immediately before segmental challenge (Fig. 9). There was a highly significant difference in BAL fluid IL-5 after segmental challenge between single and dual responders; median IL-5 concentration in BAL fluid from dual responders was about 40-fold greater than from single responders (Fig. 9). Also IL-3 and IFN-γ were significantly different between single and dual responders; medians from dual responders were about ten- and three-fold those from single responders for IL-3 and IFN-γ, respectively (Fig. 9). GM-CSF was not different between single and dual responders (Fig. 9).

The concentration of BAL fluid IL-5, but not that of the other cytokines, correlated significantly and strongly with the percentage of eosinophils in BAL 48 h after segmental Ag challenge ($r_s = 0.69$, $p = 0.001$ for IL-5; $r_s = 0.23$, $p = 0.36$ for GM-CSF; $r_s = 0.32$, $p = 0.19$ for IL-3; and $r_s = 0.39$, $p = 0.11$ for IFN-γ). It is possible that one or both of the other IL-5 family cytokines would correlate with BAL eosinophil percentage if a higher number of subjects were studied. IL-5 also correlated inversely with the percentage of macrophages (not shown), reflecting the decreased macrophage percentage as eosinophil percentage increases. Further, BAL fluid IL-5 after segmental challenge correlated strongly with the magnitude of the late-phase fall in FEV_1 after whole-lung challenge ($r_s = 0.79$, $p < 0.0001$). IL-3 also correlated with late-phase FEV_1 fall but less well ($r_s = 0.51$, $p = 0.03$). GM-CSF ($r_s = 0.37$, $p = 0.12$) and IFN-γ ($r_s = 0.36$, $p = 0.13$) did not correlate with late-phase FEV_1 fall.

Levels of β_2 and reactivity with mAb24 of BAL eosinophils correlated significantly with the concentration of IL-5 in BAL fluid 48 h after segmental Ag challenge (Fig. 10), as did level of α_M ($r_s = 0.70$, $p = 0.04$)(not shown). These integrins did not correlate with concentrations of the other cytokines (not shown). There was no correlation with BAL fluid IL-5 before segmental challenge (not shown). Integrins of blood eosinophils did not correlate with cytokine concentrations in BAL fluid 48 h after segmental Ag challenge (not shown). Thus, a greater IL-5 concentration in BAL fluid in a subject is associated with greater $\alpha_M\beta_2$ upregulation and β_2 activation on BAL eosinophils after segmental challenge.

Discussion

We observed changes in integrins on eosinophils in blood and airway of 19 subjects with mild allergic asthma after segmental bronchial allergen challenge that were related to the reactivities of subjects in the whole Ag challenge protocol. Eosinophils in blood 48 h after challenge had increased expression of the α_D integrin subunit and, in asthmatics with a dual response phenotype, increased reactivity with activation-sensitive anti-β_1 integrin mAb N29 compared to before challenge. Eosinophils in BAL obtained 48 h after challenge had increased expression of β_2 and α_M integrin subunits and increased reactivity of activation-sensitive anti-β_2 integrin mAb24 compared to eosinophils in blood. β_2, α_M, and mAb24 epitope expression on BAL eosinophils correlated with the concentration of IL-5 in BAL fluid obtained 48 h after segmental Ag challenge. In addition, α_D expression and, in dual responders, N29 reactivity were higher on BAL eosinophils than on blood eosinophils before challenge. Integrin expression patterns on BAL eosinophils were more homogeneous and symmetric than on blood eosinophils. The observations of N29 epitope and α_D expression are compatible with a scenario whereby extravasated eosinophils appearing in the airway are derived from a subpopulation of the total, more heterogeneous population of circulating eosinophils. However, the observations of $\alpha_M\beta_2$ expression, β_2 activation state, and IL-5 levels in BAL fluid indicate that after leaving the circulation eosinophils undergo IL-5-triggered activation of β_2 and upregulation of $\alpha_M\beta_2$.

Analysis of neutrophils and monocytes in the same blood samples revealed that α_D expression increased on all three leukocyte types upon segmental challenge; thus, α_D upregulation may occur through a common mechanism. At baseline, N29 reactivity was higher on neutrophils than on eosinophils and even higher on monocytes. N29 reactivity increased significantly on neutrophils in dual responders upon challenge, but to a relatively lesser degree than on eosinophils; whereas it did not change significantly on monocytes. The mechanisms responsible for maintaining the baseline β_1 activation levels on the different leukocytes and responsible for the elevation of activation state upon challenge are unknown. Finally, neutrophil and monocyte α_M and monocyte β_2 were upregulated upon challenge, in contrast to eosinophil αM and β2, which were upregulated in BAL but not blood. Thus, circulating eosinophils are less sensitive than neutrophils or monocytes to increased surface expression of $\alpha_M\beta_2$.

β_1 activation on blood eosinophils, assessed by reactivity with N29, has been shown to correlate inversely with FEV_1 in an inhaled corticosteroid (ICS) withdrawal study (35). The present results complement the steroid withdrawal study by demonstrating persistent β_1 activation in dual responders with more eosinophilic inflammation in the airway. Blood eosinophils from dual responders, but not from single responders, have also been shown to have enhanced activation of FcγRII (CD32), as assessed with the phage mAb A17, 6 h after challenge (42). Whether activation of β_1 integrin and CD32 on circulating eosinophils are triggered by the same or different stimuli and signaling pathways is an interesting question that remains to be investigated.

The changes observed here in BAL eosinophils reproduce observations in the literature, mostly on isolated eosinophils, that BAL eosinophils have higher α_M and β_2 than blood eosinophils (43–46); activated $\alpha_M\beta_2$, as monitored by anti-active α_M mAb CBRM1/5 (22,36); and increased $\alpha_M\beta_2$-inhibitable adhesion to diverse ligands (22,36). Our finding that dual responders have higher total numbers of cells and eosinophils and higher proportion of eosinophils in BAL after segmental Ag challenge than single responders also is in accord with prior reports (47–50). We also found that β_1 activation state and β_2 surface expression were significantly higher on BAL eosinophils from dual responders than on those from single responders. Activation of β_1 and β_2 integrins, as assessed with the activation-sensitive mAbs N29 and mAb24, respectively, on BAL eosinophils correlated with eosinophil percentage in BAL, indicating that activation of both these subfamilies of integrins is important for eosinophil recruitment. This idea is consistent with *in vivo* studies supporting the involvement of $\alpha_4\beta_1$ in eosinophil appearance in the airway and *in vitro* studies showing involvement of both β_1 and β_2 integrins in eosinophil transendothelial migration (22,51–56). The evidence for involvement of both $\alpha_4\beta_1$ and β_2 integrins in eosinophil migration *in vivo* is further strengthened by a recent report that eosinophil recruitment to airway after Ag challenge was severely attenuated in both a conditional α_4 integrin knockout mouse and a β_2-deficient mouse (57). Finally, α_D expression and β_1 activation on blood eosinophils as well as β_2 and α_M expression and β_2 activation on BAL eosinophils after segmental Ag challenge all correlated with the magnitude of the late-phase fall in FEV_1 in response to whole-lung Ag challenge.

A number of mediators are increased in airway after challenge (28,29,49,58,59). Of these, the most likely candidates to account for upregulation and activation of $\alpha_M\beta_2$ are the IL-5 family cytokines (36,60,61). Concentrations of IL-5, GM-CSF, IL-3, and IFN-γ in BAL fluid recovered 48 h after segmental Ag challenge were all increased significantly compared to in BAL fluid recovered before challenge. IL-5 correlated with BAL eosinophil α_M and β_2 expression and β_2 activation 48 h after segmental challenge. The median concentration of IL-5 in BAL fluid from dual responders after segmental challenge was about 600 pg/ml, which was more than 20-fold the median concentration of the other cytokines. Further, the IL-5 concentration in the five BAL samples containing eosinophils with the most highly activated β_2 was 100 to > 1000 pg/ml. During recovery of BAL the volume of the fluid lining the airway epithelium *in vivo* is estimated to become diluted 100-fold (62). Thus, the levels of IL-5 in the epithelial lining fluid of these subjects (all of whom were dual responders) is estimated to be about 10–100 ng/ml. Treatment of blood eosinophils *in vitro* with IL-5 at concentrations in this range is known to saturate the IL-5 receptor (63–68) and lead to $\alpha_M\beta_2$ upregulation and activation and induction of adhesion to ICAM-1 and other substrates (36,60,61), priming and enhanced response to chemoattractants (69), enhanced viability (70), degranulation and granule protein release (30), IL-5 receptor α downregulation (71), and enhanced expression of certain genes (72). In contrast, median concentrations of the other IL-5 family cytokines prior to dilution are estimated to be about 1–3 ng/ml. Thus, the concentration of IL-5 in the lining fluid *in vivo*, assuming that a significant portion of the IL-5 measured represents active IL-5, is estimated to be sufficient to cause $\alpha_M\beta_2$ upregulation and activation of β_2 integrins. These results are in accord with the earlier observation that the IL-5 receptor is downregulated specifically compared to the GM-CSF receptor on eosinophils recovered by BAL 48 h after segmental Ag challenge (30). We also found that BAL fluid IL-5 correlated with the percentage of eosinophils in BAL and with the magnitude of the late-phase fall in FEV_1. A correlation between BAL fluid IL-5 and eosinophil recruitment to the airway after segmental Ag challenge has been reported before (38,73–76) and is consistent with the observation that anti-IL-5 therapy causes a significant decrease in sputum eosinophils (1).

Our study has several limitations. The data sets were incomplete in that all integrins were not assayed from all samples. Sampling of blood and BAL were performed only at one time point (48 h) after segmental Ag challenge. Future experiments with blood sampling at various time

points after segmental and/or whole-lung Ag challenge are required to record the time-course of α_D and N29 expression on blood eosinophils in dual and single responders and learn when the values diverge. Further, the importance of the integrin expression heterogeneity observed on blood eosinophils, particularly regarding β_2 and α_M, is not known. Such heterogeneity, to our knowledge, has not been described or discussed before. Of note, we subjected whole blood to primary antibody incubation without previous cell isolation or centrifugation. Earlier studies on blood eosinophil integrin expression have been performed on a buffy coat preparation or purified eosinophils (43–45). Dextran sedimentation during buffy coat preparation has been shown to cause upregulation of α_M on blood eosinophils (and neutrophils) compared to in whole, unfractionated blood (77). Eosinophils are likely similar to neutrophils, which are known to store a large proportion of α_M in granules, wherefrom it is translocated to the plasma membrane after cell stimulation (78). Remarkably, eosinophils in BAL had strong homogeneous labeling for β_2 and α_M. One possibility is that higher reacting subpopulations of circulating leukocytes represent cells that have undergone “retrograde” migration back to the blood from tissues, as has been demonstrated for zebrafish neutrophils *in vivo* (79) and human neutrophils *in vitro* (80). Blood sampling at various time points after Ag challenge, including times beyond 48 h, may shed further light on circulating eosinophil subpopulations.

Our results and the literature are compatible with the schematic of eosinophil recruitment after segmental Ag challenge that is depicted in Fig. 11. At baseline, β_1 and β_2 integrins on a circulating eosinophil are in a low activation state, the level of eosinophil surface α_D is low, and VCAM-1 is absent from endothelial surfaces of the bronchial circulation. Yet-to-be-identified stimuli resulting from Ag challenge cause enhanced activation of β_1 integrins and increased surface expression of α_D on circulating eosinophils. Because increased expression of the β_1 activation epitope occurs on most eosinophils, the activation likely takes place in the pulmonary circulation through which the eosinophils constantly pass or in response to release of an activating substance into the circulation (Fig. 11). Such activation contrasts with the model for recruitment of leukocytes (16), including eosinophils (20), in which integrin activation is assumed to occur locally and concurrently with rolling and tethering on endothelium. Interestingly, bone marrow-derived progenitor cells or circulating eosinophils of asthmatics have been shown to be activated upon Ag challenge also as measured by upregulation of IL-5 receptor α and CCR3, activation of CD32, and greater responsiveness to chemoattractants (42,81–83). Further, eosinophils from allergic asthmatics have been shown to have a greater capacity to adhere to and transmigrate through endothelium than eosinophils from normal donors (51,84).

The question then arises as to how activated circulating eosinophils localize to the parts of the lung subjected to Ag challenge in preference to neutrophils and monocytes. As a parallel response to Ag challenge, mediators, including IL-4 and IL-13, are elaborated and activate bronchial endothelium to specifically synthesize and express VCAM-1. VCAM-1 has been shown to be preferentially expressed in the asthmatic lung or after Ag challenge (85,86). Further, VCAM-1 can support adhesion of eosinophils but not neutrophils (9,18,22,87–91). Circulating eosinophils with activated β_1 and upregulated α_D are presumed to have a higher probability of arresting on VCAM-1 on activated endothelium of the bronchial circulation (Fig. 11), since adhesion of purified blood eosinophils to VCAM-1 is mediated by $\alpha_4\beta_1$ (22,92), with a possible contribution by $\alpha_D\beta_2$ (22,31).

α_M and β_2 are shown as being upregulated and β_2 integrins as being activated by IL-5, also elaborated in response to allergen. Eosinophils appearing in the airway lumen have a hyperadhesive phenotype that is marked by activated β_1 integrins, upregulated $\alpha_D\beta_2$, activated β_2 integrins, and upregulated $\alpha_M\beta_2$ (Fig. 11). Eosinophils, particularly after priming by IL-5 (69), responds to chemoattractants, such as eotaxin (9,13)(Fig. 11). $\alpha_M\beta_2$ is believed to be important for eosinophil migration (22,51,53,93–95). Thus, the scenario has migrating

eosinophils using activated $\alpha_M\beta_2$ and possibly other integrins to interact with multiple substrates (22,36,92), including VCAM-1, ICAM-1 and ECM proteins, on endothelial cells, in connective tissue, and on epithelium of the bronchial wall.

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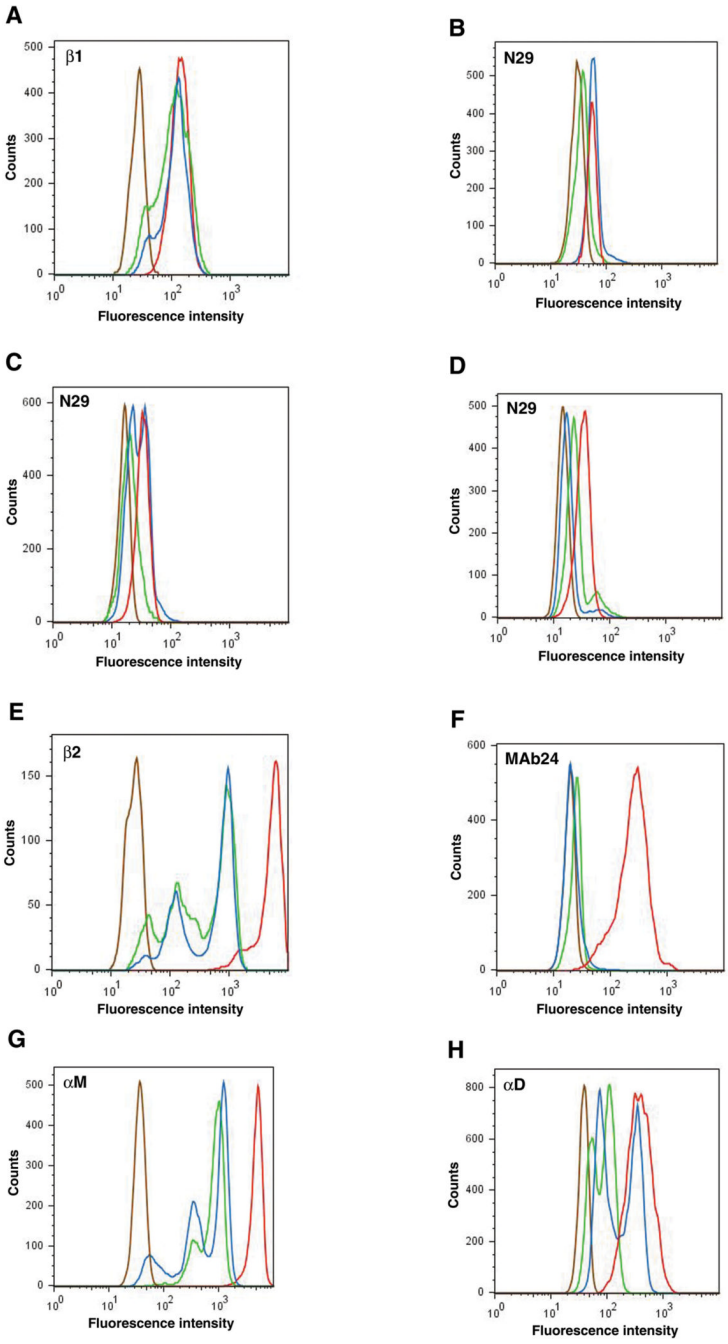

FIGURE 1. Integrin expression on blood and BAL eosinophils before and after segmental Ag challenge

Representative flow cytometry histograms of expression of total β_1 integrin **(*A*)**, activation-sensitive β_1 integrin mAb N29 epitope **(*B*–*D*)**, total β_2 integrin **(*E*)**, activation-sensitive β_2 integrin mAb24 epitope **(*F*)**, α_M integrin **(*G*)**, or α_D integrin **(*H*)** on blood eosinophils before segmental Ag challenge **(green)**, blood eosinophils 48 h after segmental Ag challenge **(blue)**, or BAL eosinophils 48 h after segmental Ag challenge **(red)**; or isotype control **(brown)**. For N29 epitope expression three examples are shown: *B*; one homogeneous, symmetric peak on blood eosinophils before and after challenge, and higher mean expression level after challenge than before. *C*; one peak on blood eosinophils before challenge and two

peaks after challenge, and higher mean expression level after challenge. *D*; an asymmetric peak with a “shoulder” to the right on blood eosinophils before and after challenge, and decrease in the size of the “shoulder” and lower mean expression level after challenge. Numbers of individuals with the various patterns are summarized in Table II.

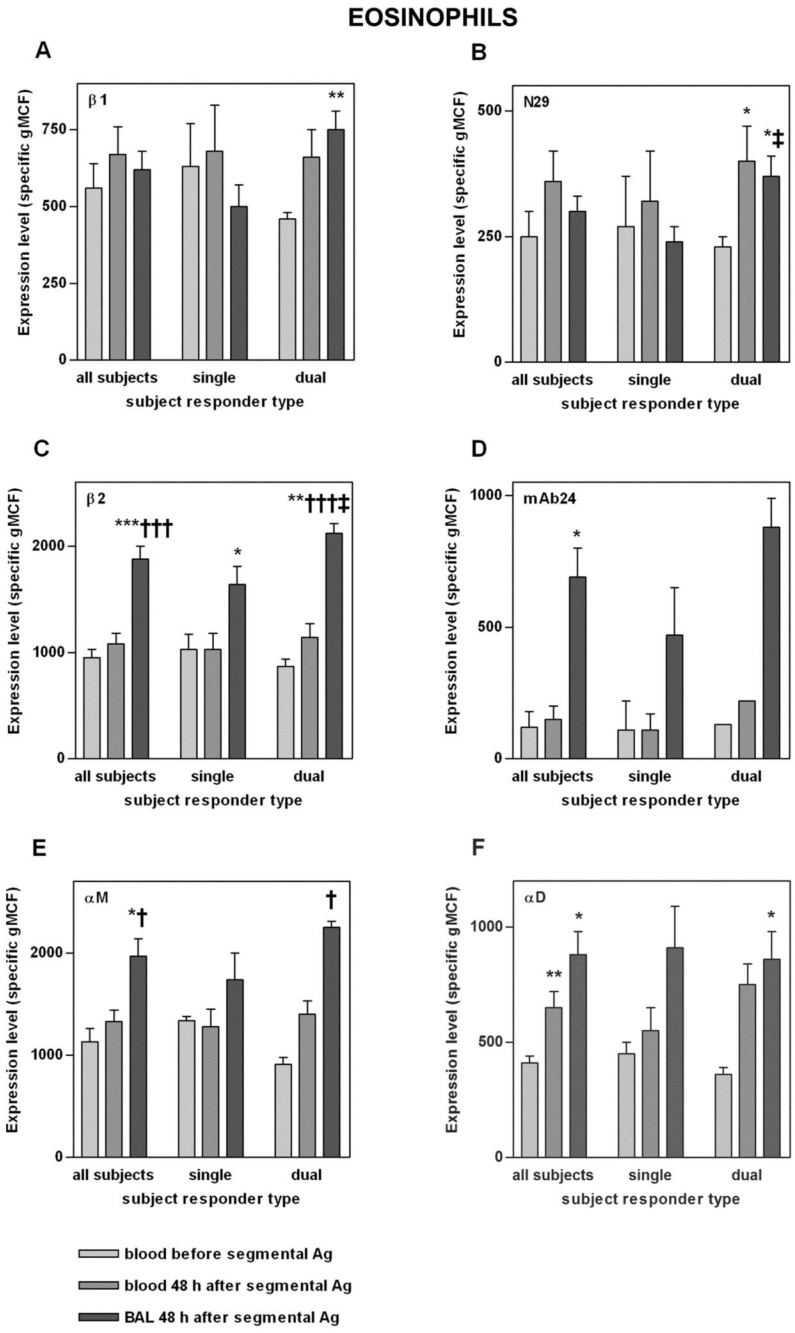

FIGURE 2. Integrin expression on blood and BAL eosinophils before and 48 h after segmental Ag challenge

Expression of total β_1 integrin **(*A*)**, activation-sensitive β_1 integrin mAb N29 epitope **(*B*)**, total β_2 integrin **(*C*)**, activation-sensitive β_2 integrin mAb24 epitope **(*D*)**, α_M integrin **(*E*)**, and α_D integrin **(*F*)** on blood eosinophils before segmental Ag challenge (light gray), blood eosinophils 48 h after segmental Ag challenge (medium gray), and BAL eosinophils 48 h after segmental Ag challenge (dark gray) in all tested subjects, single responders, and dual responders. Values shown are specific mean channel fluorescence (gMCF)(mean ± SEM). For *n* values and expression given as percentage of positive cells, see Table II). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ versus blood eosinophils before segmental challenge; †$p \leq 0.05$, ††$p \leq 0.001$ versus

blood eosinophils after segmental challenge; ‡$p \leq 0.05$ versus single responders. BAL, bronchoalveolar lavage.

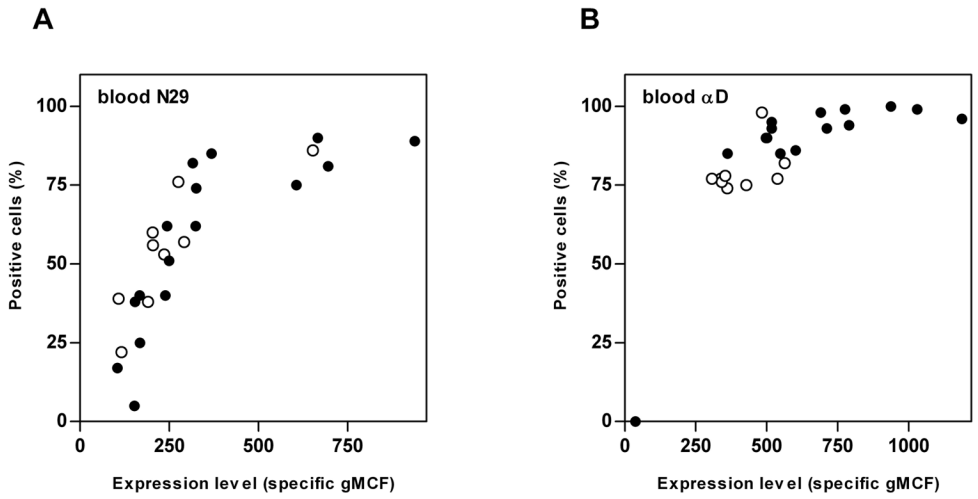

FIGURE 3. Integrin expression on blood eosinophils as percentage of positive cells and expression level

Blood eosinophil expression of activation-sensitive β_1 integrin mAb N29 epitope ***(A)*** or α_D integrin ***(B)*** before (empty symbols) and 48 h after (filled symbols) segmental Ag challenge, expressed as percentage of positive cells (y axes) or as expression level (specific geometric mean channel fluorescence [gMCF])(x axes).

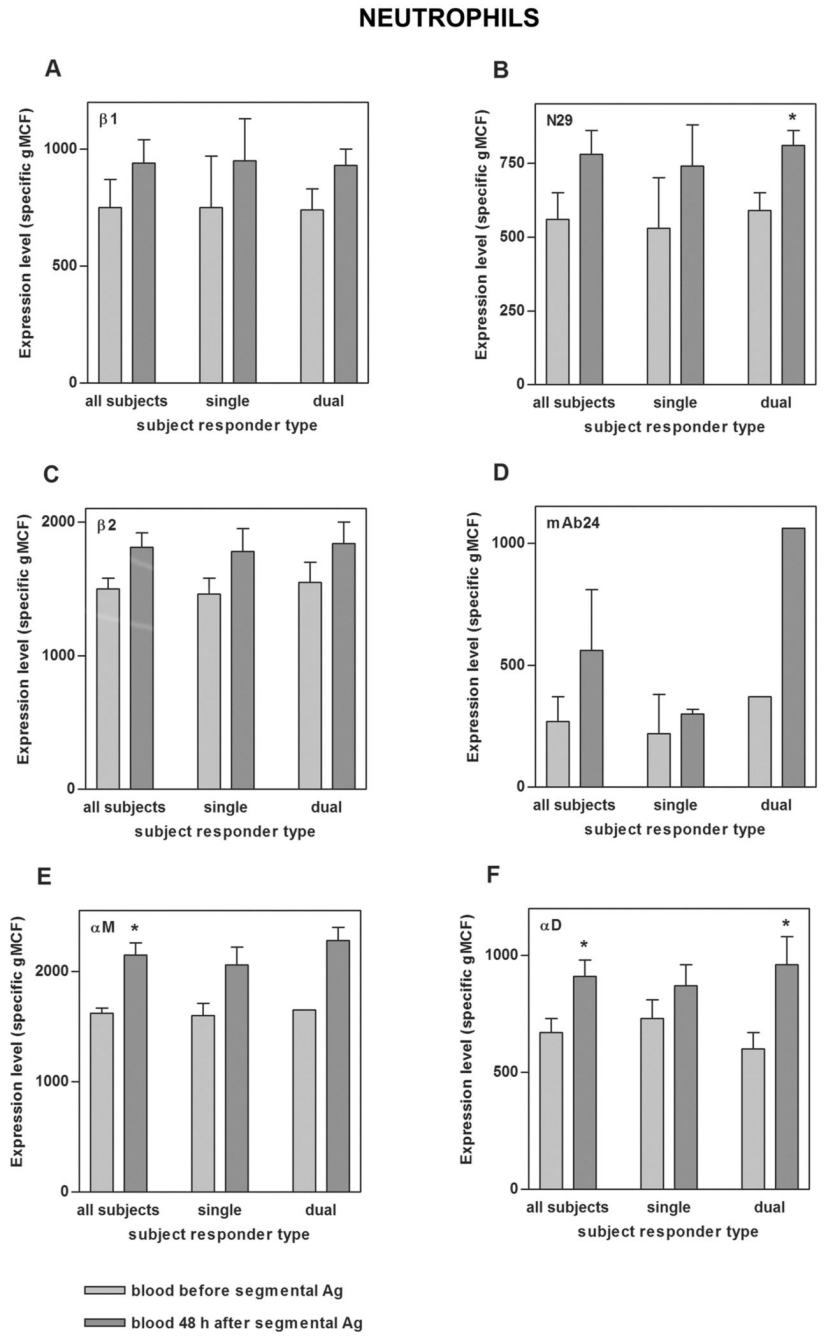

FIGURE 4. Integrin expression on blood neutrophils before and 48 h after segmental Ag challenge

Expression of total β_1 integrin **(*A*)**, activation-sensitive β_1 integrin mAb N29 epitope **(*B*)**, total β_2 integrin **(*C*)**, activation-sensitive β_2 integrin mAb24 epitope **(*D*)**, α_M integrin **(*E*)**, and α_D integrin **(*F*)** on blood neutrophils before segmental Ag challenge (light gray) and 48 h after segmental Ag challenge (medium gray) in all tested subjects, single responders, and dual responders. Values shown are specific mean channel fluorescence (gMCF)(mean ± SEM). $^*p \leq 0.05$ versus before segmental challenge.

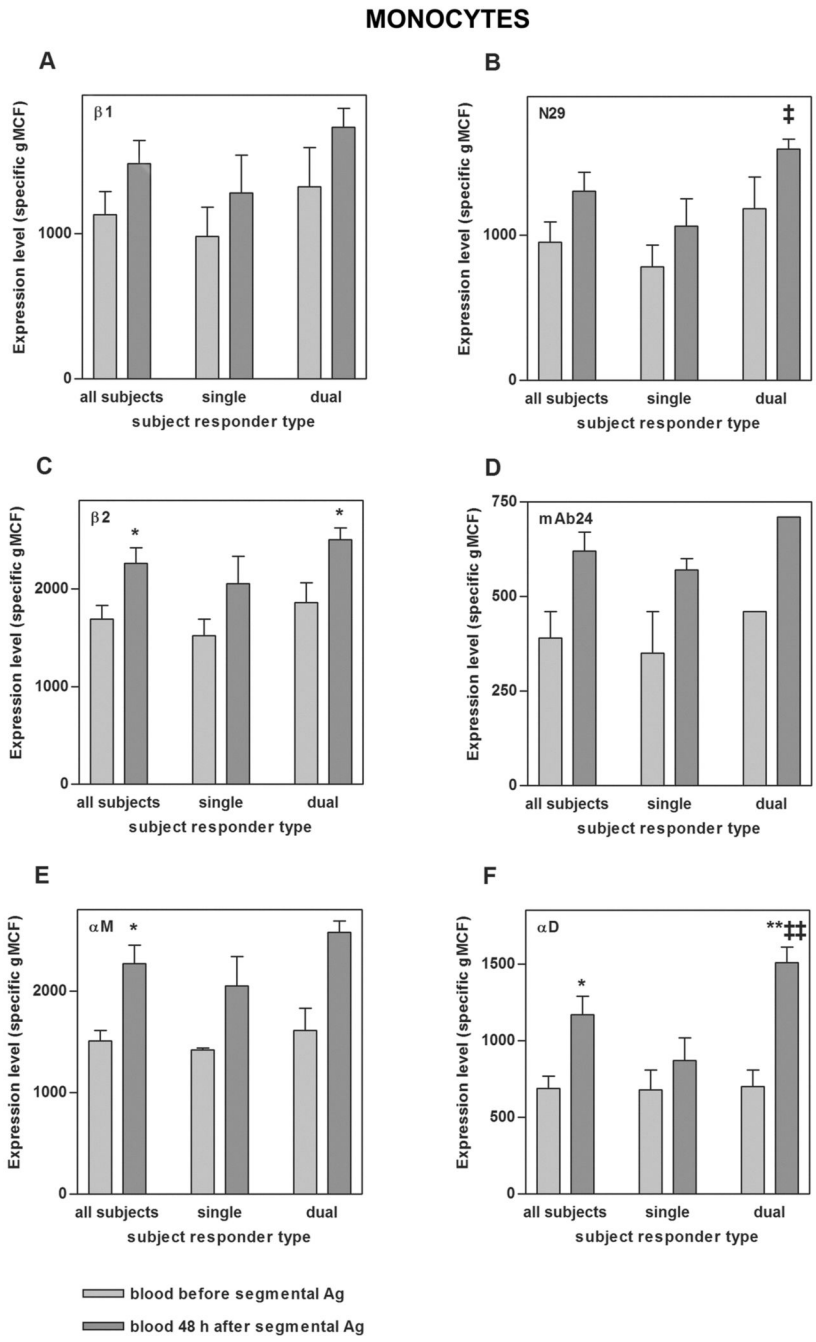

FIGURE 5. Integrin expression on blood monocytes before and 48 h after segmental Ag challenge

Expression of total β_1 integrin **(*A*)**, activation-sensitive β_1 integrin mAb N29 epitope **(*B*)**, total β_2 integrin **(*C*)**, activation-sensitive β_2 integrin mAb24 epitope **(*D*)**, α_M integrin **(*E*)**, and α_D integrin **(*F*)** on blood monocytes before segmental Ag challenge (light gray) and 48 h after segmental Ag challenge (medium gray) in all tested subjects, single responders, and dual responders. Values shown are specific mean channel fluorescence (gMCF)(mean ± SEM). $*p \leq 0.05$, $**p \leq 0.01$ versus before segmental challenge; $‡p \leq 0.05$, $‡‡p \leq 0.01$ versus single responders.

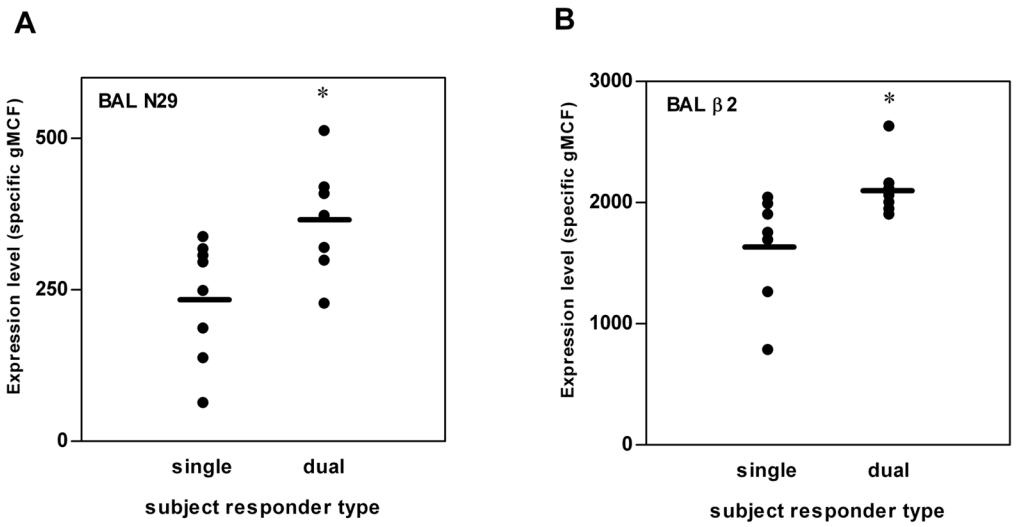

FIGURE 6. Differences in integrin activation and expression on BAL eosinophils between single and dual responders

BAL eosinophil expression 48 h after segmental Ag challenge of activation-sensitive β_1 integrin mAb N29 epitope ***(A)*** or total β_2 integrin ***(B)*** in single and dual responders. Bar = mean, $*p \leq 0.05$ versus single responders. BAL, bronchoalveolar lavage; gMCF, geometric mean channel fluorescence.

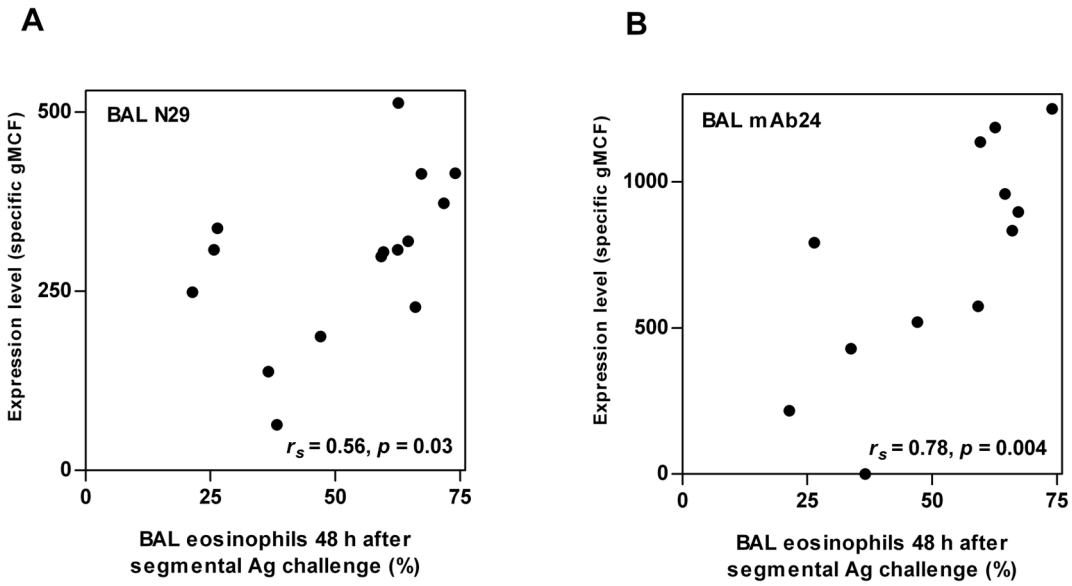

FIGURE 7. Correlations between integrin activation on BAL eosinophils and BAL eosinophil percentage

Correlations between BAL eosinophil expression of activation-sensitive β_1 integrin mAb N29 epitope ***(A)*** or activation-sensitive β_2 integrin mAb24 epitope ***(B)*** and percentage of eosinophils in BAL 48 h after segmental Ag challenge. BAL, bronchoalveolar lavage; gMCF, geometric mean channel fluorescence. r_s, Spearman rank correlation coefficient.

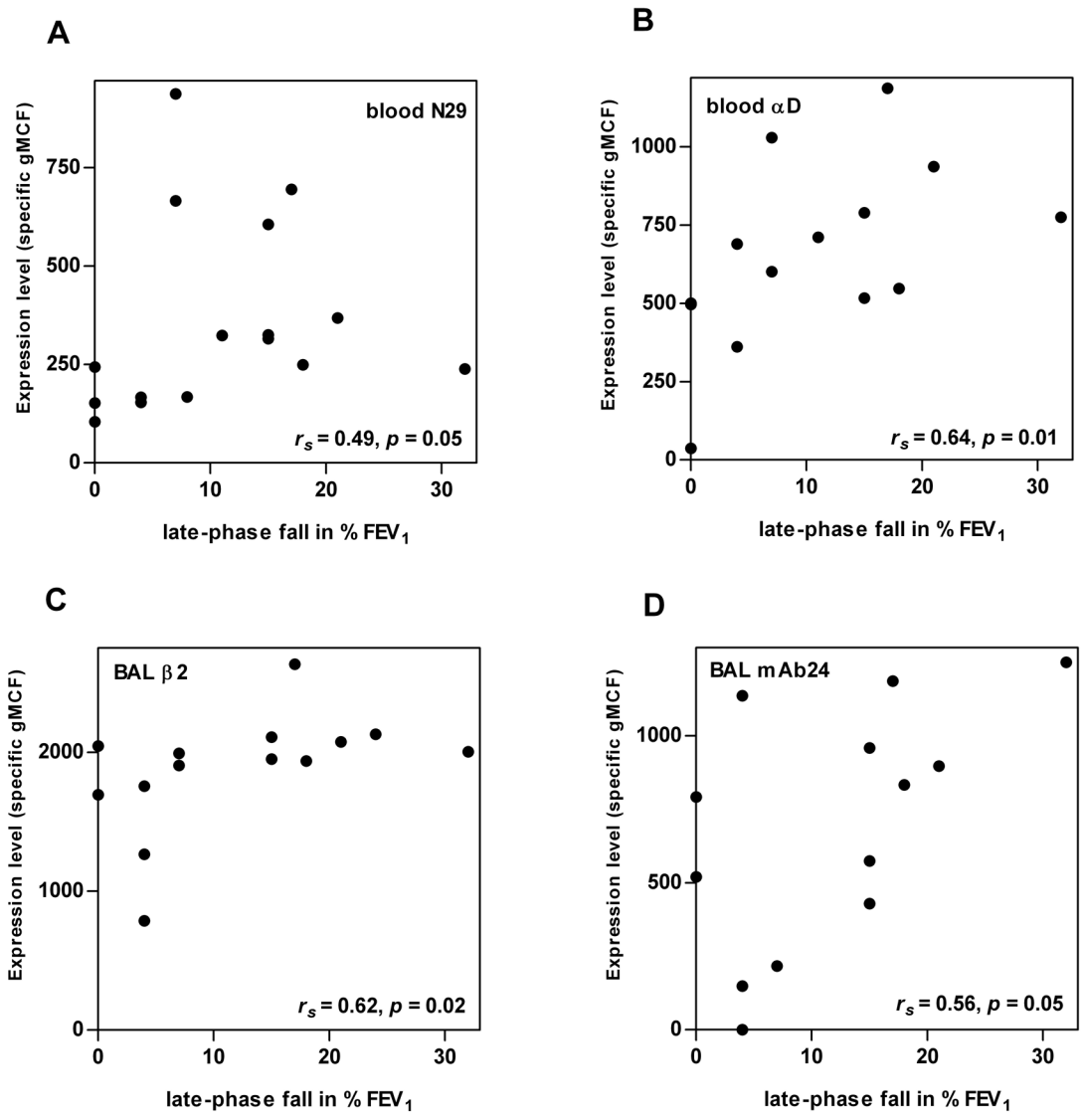

FIGURE 8. Correlations between integrin activation and expression on blood and BAL eosinophils and the magnitude of the fall in FEV_1 3-8 h after whole-lung Ag challenge

Correlations between blood eosinophil expression of activation-sensitive β_1 integrin mAb N29 epitope ***(A)*** or α_D integrin ***(B)*** or BAL eosinophil expression of total β_2 integrin ***(C)*** or activation-sensitive β_2 integrin mAb24 epitope ***(D)*** 48 h after segmental Ag challenge and the magnitude of the late-phase fall in FEV_1 3–8 h after whole-lung Ag challenge. BAL, bronchoalveolar lavage; FEV_1, forced expiratory volume in 1 s; gMCF, geometric mean channel fluorescence. r_s, Spearman rank correlation coefficient.

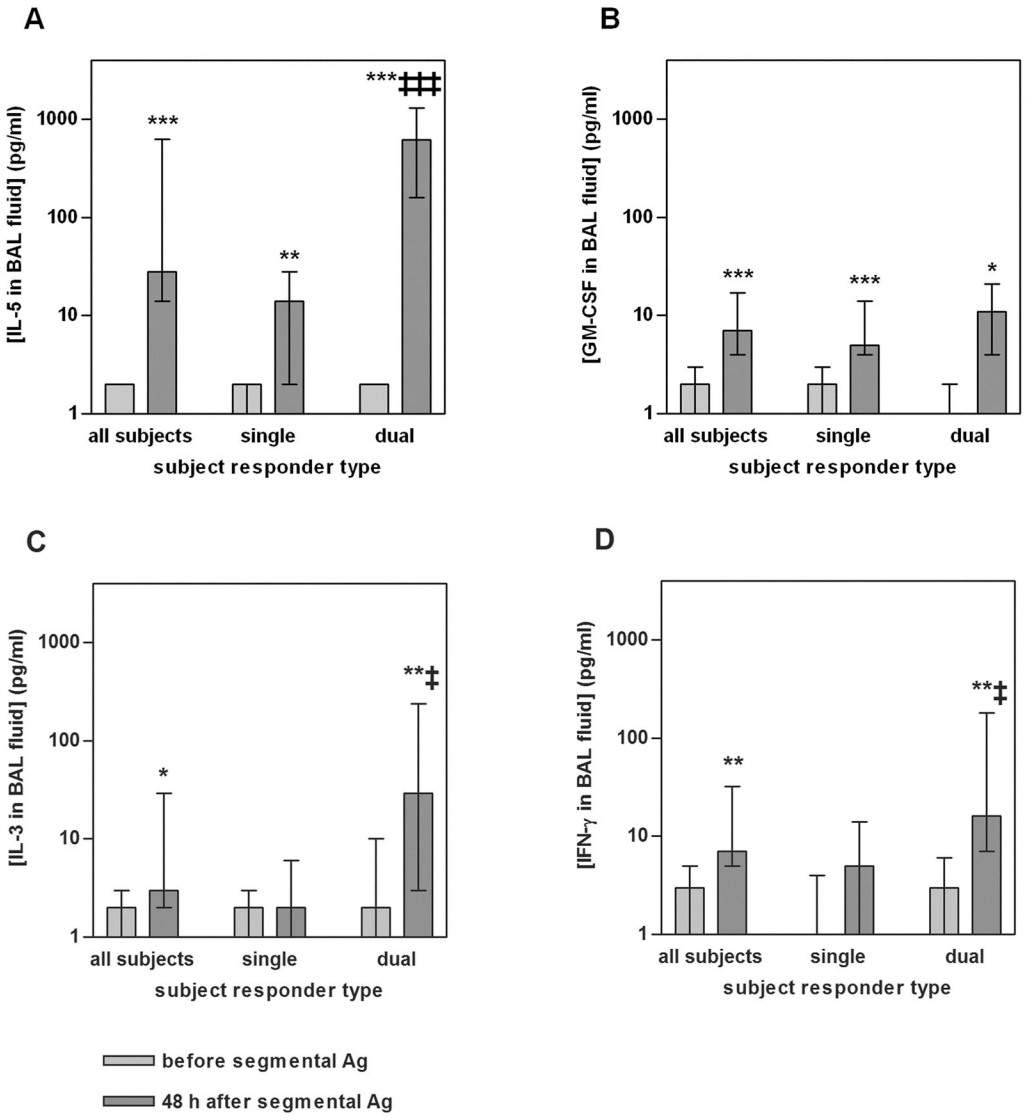

FIGURE 9. Cytokine concentrations in BAL fluid before and 48 h after segmental Ag challenge

Concentrations of IL-5 **(*A*)**, GM-CSF **(*B*)**, IL-3 **(*C*)**, and IFN-γ **(*D*)** in BAL fluid before (light gray bars) and 48 h after (medium gray bars) segmental Ag challenge from all tested subjects ($n = 19$), single responders ($n = 10$), or dual responders ($n = 9$). Values shown are medians with 25th and 75th percentiles of concentrations in recovered, unconcentrated BAL fluid. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ versus before segmental challenge; ‡$p \leq 0.05$, ‡‡‡$p \leq 0.001$ versus single responders. BAL, bronchoalveolar lavage.

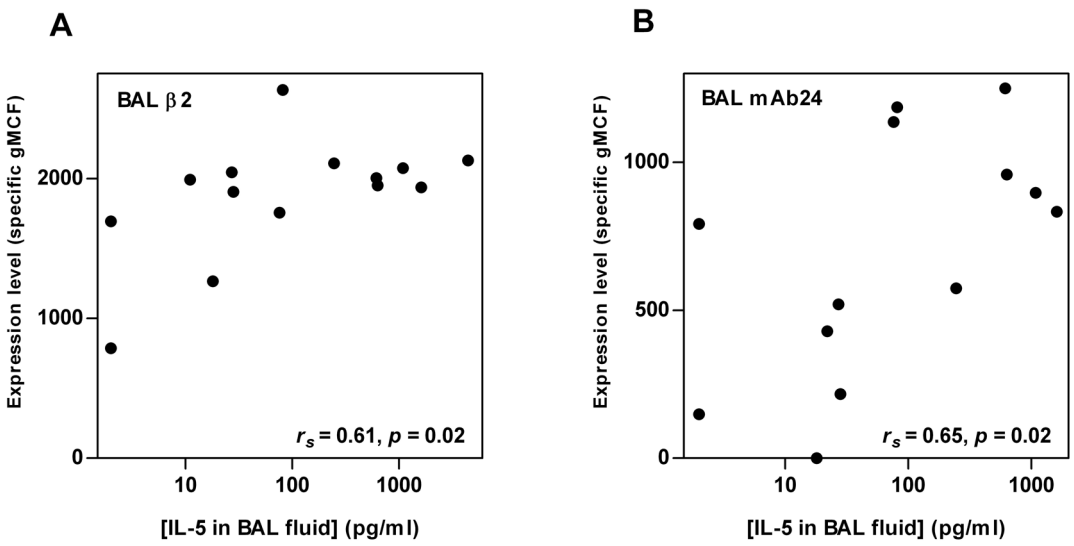

FIGURE 10. Correlations between integrin activation and expression on BAL eosinophils and IL-5 in BAL fluid

Correlations between blood eosinophil expression of total β_2 integrin **(*A*)** or activation-sensitive β_2 integrin mAb24 epitope **(*B*)** and the concentration of IL-5 in BAL fluid 48 h after segmental Ag challenge. BAL, bronchoalveolar lavage; gMCF, geometric mean channel fluorescence. r_s, Spearman rank correlation coefficient.

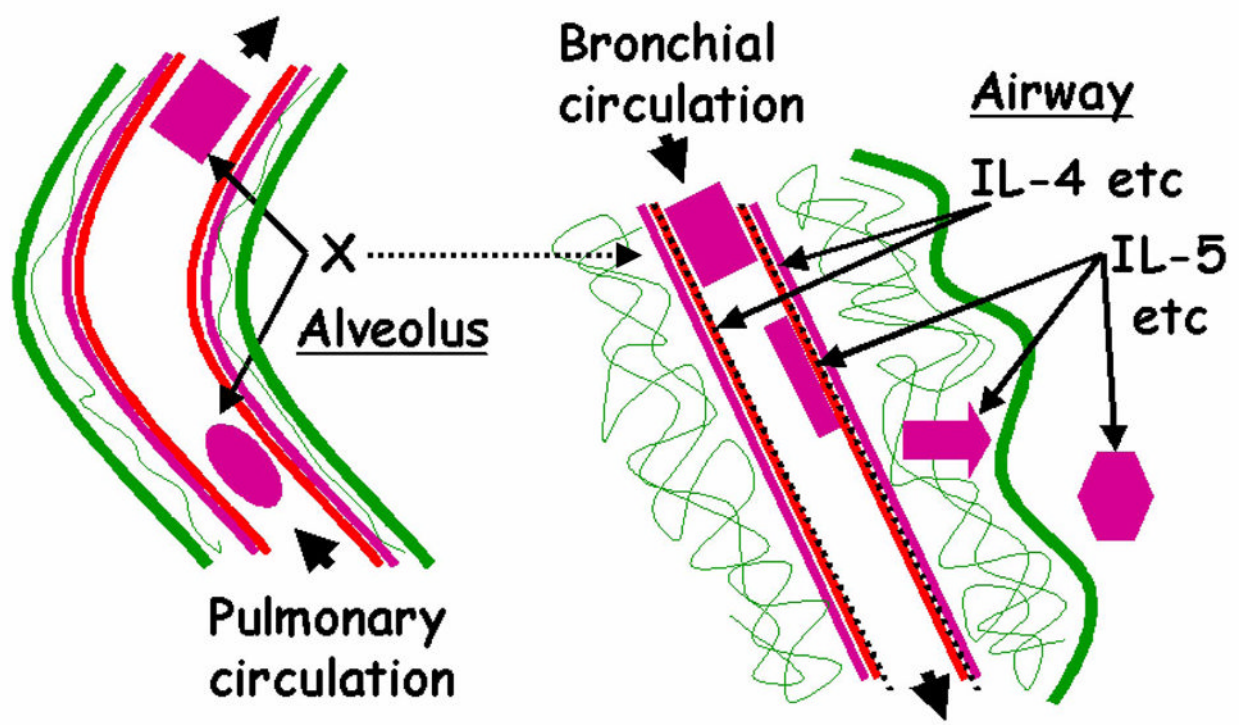

FIGURE 11. Sequential upregulation and activation of eosinophil integrins during recruitment to airway after exposure to allergen

Schematic relating activation of β_1 and β_2 integrins to eosinophil trafficking during and after segmental Ag challenge. *Left*, An eosinophil **(oval)** with unactivated β_1 integrins and low surface expression of α_D is shown entering the pulmonary circulation of a segment subjected to Ag challenge. A yet-to-be-identified stimulus or stimuli (X) cause(s) activation of β_1 integrins and increased surface expression of α_D during transit of the eosinophil **(rectangle)** through the vessel. *Right*, Concurrently, IL-4 and other mediators are released and specifically induce surface expression of VCAM-1 (discontinuous black line) on the endothelial cells of a bronchial blood vessel in the challenged segment. An eosinophil with activated β_1 and upregulated α_D **(rectangle)** is shown entering the bronchial circulation. The eosinophil arrests and adheres **(elongated rectangle)** to VCAM-1. After exposure to IL-5 the eosinophil becomes responsive to chemotactic factors, migrates **(arrow)** and assumes a hyperadhesive airway phenotype **(hexagon)** in the lumen. The airway eosinophil displays activated β_1, upregulated α_D, activated β_2, and upregulated α_M and β_2.

Table I

Subject characteristics

				Screening		Whole-lung challenge			Segmental challenge	
							FEV_1 fall[a]		Eosinophils in BAL 48 h after challenge[b]	
Subject No.	Age (years)	Sex[c]	Ag[d]	PC_{20}[e] (mg/ml)	FEV1[f] (L;% pred.)	$AgPD_{20}$[g] (CBU)	Early (% fall)	Late	(%)	total (× 10^6)
Single responders[h]										
1	22	M	RW	9.0	3.8 ; 90	81	37	0	26	9
2	21	F	HDM	1.2	3.2 ; 85	6	52	4	37	150
3	26	F	HDM	1.8	4.0 ; 99	1	26	7	21	7
4	19	M	CD	0.7	4.1 ; 85	85	31	4	ND	ND
5	19	F	HDM	3.5	3.7 ; 105	118	22	0	47	150
6	21	M	RW	0.2	4.4 ; 93	115	23	0	26	24
7	20	M	CD	5.5	4.3 ; 98	68	44	4	60	83
8	27	M	HDM	20	3.7 ; 89	52	35	8	38	24
9	23	F	CD	0.4	3.0 ; 91	2	32	11	24	10
10	26	F	CD	2.9	4.0 ; 109	174	40	7	62	88
Median:	22			2.4	3.9 ; 92		34	4	37	24
Dual responders[i,j]										
11	30	F	HDM	4.8	2.9 ; 95	11	23	25	72	220
12	19	M	CD	3.8	4.9 ; 96	37	22	17	63	230
13	20	F	RW	0.3	2.9 ; 87	1	48	15	34	29
14	20	F	HDM	1.3	3.7 ; 99	8	31	15	59	410
15	18	M	HDM	20	5.1 ; 104	21	21	15	65	120
16	22	M	HDM	2.5	3.9 ; 87	31	23	32	74	760
17	22	M	RW	20	4.7 ; 97	48	35	18	66	420
18	20	F	HDM	0.4	3.4 ; 96	27	31	21	67	280
19	23	M	RW	20	4.4 ; 106	52	48	24	55	380
Median:	20			3.8	3.9 ; 96		31	18***	65**	280**

[a] FEV_1, forced expiratory volume in 1 s.

[b] BAL, bronchoalveolar lavage.

[c] F, female; M, male.

[d] CD, cat dander; HDM, house dust mite; RW, ragweed.

[e] PC_{20}, provocative concentration of methacholine producing a 20% fall in FEV_1.

[f] FEV_1 values from the screening visit are expressed in liters and as percentage of predicted value (% pred.).

[g] $AgPD_{20}$, provocative dose of antigen producing a 20% fall in FEV_1, expressed in cumulative breath units (CBU).

[h] Single responder, subject with FEV_1 fall 3–8 h after whole-lung Ag challenge (late-phase fall) < 15% (Liu et al. 2004, reference No. 28).

[i] Dual responder, subject with FEV_1 fall 3–8 h after whole-lung Ag challenge ≥ 15% (Liu et al. 2004, reference No. 28).

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^{j}** $p \leq 0.01$,

*** $p \leq 0.001$ versus single responders.

Table II

Integrin expression on blood and BAL eosinophils as distribution patterns and percentage of positive cells before and 48 h after segmental Ag challenge[a]

Integrin (mAb)	Blood eosinophils before		Blood eosinophils 48 h after segmental Ag challenge[b]		BAL eosinophils 48 h after[b,c]	
	H/T[d]	%pos[e]	H/T	%pos.	H/T	%pos.
All tested subjects						
Total β_1 (MAR4)	2/9	82±4	6/16	80±5	17/17	85±6
Activated β_1 (N29)	8/9	54±7	11/16	57±7	15/15	55±8
Total β_2 (L130)	1/10	91±2	1/17	93±2	12/14	97±1*,†
Activated β_2 (mAb24)	3/3	23±17	3/3	18±11	10/13	65±11
α_M	1/4	92±5	2/12	95±3	8/9	96±3
α_D	2/9	79 ±2	4/15	87±6**	13/16	81±8
Single responders[f]						
Total β_1 (MAR4)	2/5	85±7	5/9	79±8	9/9	80±9
Activated β_1 (N29)	4/5	53±11	5/9	48±10	8/8	49±10
Total β_2 (L130)	1/5	94±2	0/9	92±4	6/7	95±3
Activated β_2(mAb24)[g]	2/2	30±27	2/2	26±14	6/6	42±19
α_M [h]	1/2	100±0	1/7	93±4	4/5	95±5
α_D	2/5	82±4	3/8	80±12	7/8	77±11
Dual responders[h,i]						
Total β_1 (MAR4)	0/4	78±3	1/7	83±5	8/8	90±7*
Activated β_1 (N29)	4/4	56±8	6/7	70±7	7/7	61±14
Total β_2 (L130)	0/5	88±4	1/8	94±2	6/7	99±0*
Activated β_2(mAb24)[g]	1/1	8	1/1	3	4/7	85±4
α_M [g]	0/2	84±4	1/5	97±1	4/4	98±2
α_D	0/4	76±1‡	1/7	95±2**	6/8	85±11*

[a] BAL, bronchoalveolar lavage.

[b] $*p \leq 0.05$,

** $p \leq 0.01$ versus blood eosinophils before segmental challenge.

[c] $\dagger p \leq 0.05$ versus blood eosinophils after segmental challenge.

[d] H, number of subjects with homogeneous expression distribution; T, total number of subjects.

[e] Expression as percentage of positive cells (mean±SEM).

[f] Single responder, subject with FEV_1 (forced expiratory volume in 1 s) fall 3–8 h after whole-lung antigen challenge < 15% (Liu et al. 2004, reference No. 28).

[g] Note regarding data with $n < 3$: These data are within the ranges of our overall data on blood eosinophils from unchallenged donors.

[h] Dual responder, subject with FEV_1 fall 3–8 h after whole-lung antigen challenge ≥ 15% (Liu et al. 2004, reference No. 28).

[i] ‡p ≤ 0.05 versus single responders.