Allergen-induced Increases in IL-5 Receptor α-subunit Expression on Bone Marrow–derived CD34$^+$ Cells from Asthmatic Subjects

A Novel Marker of Progenitor Cell Commitment towards Eosinophilic Differentiation

Roma Sehmi,* Lorna J. Wood,* Rick Watson,* Ronan Foley,* Qutayba Hamid,‡ Paul M. O'Byrne,* and Judah A. Denburg*

**Asthma Research Group, Division of Clinical Immunology and Allergy, Department of Medicine, McMaster University, Hamilton, Ontario L8N 3Z5, Canada; and ‡Department of Medicine & Pathology, Meakins-Christie Laboratories, McGill University, Montreal, Canada 3HX 2P2*

Abstract

We have proposed previously that hemopoietic myeloid progenitors contribute to the ongoing recruitment of proinflammatory cells, namely eosinophils, to sites of allergen challenge in allergic diseases such as asthma. In this study, we investigated the involvement of bone marrow–derived progenitors in the development of allergen-induced pulmonary inflammation in mild asthmatic subjects. By flow cytometry, we enumerated the level of expression of CD34, a hemopoietic progenitor cell marker, on bone marrow aspirates taken before and 24 h after allergen challenge. In addition, the coexpression of the α-subunits of IL-3 receptor (IL-3R) and IL-5 receptor (IL-5R) on CD34$^+$ cells was investigated. After allergen-challenge, although no significant change in total BM CD34$^+$ cell numbers was observed, a significant increase in the proportion of CD34$^+$ cells expressing IL-5Rα, but not IL-3Rα, was detected in the 24-h post-allergen, compared with the pre-allergen bone marrow. This was associated with a significant blood and sputum eosinophilia and increased methacholine airway responsiveness, 24 h post-allergen. Using simultaneous in situ hybridization and immunocytochemistry, we colocalized the expression of messenger RNA for membrane-bound IL-5Rα to CD34$^+$ cells. In summary, our data suggest that increased expression of IL-5Rα on CD34$^+$ cells favors eosinophilopoiesis and may thus contribute to the subsequent development of blood and tissue eosinophilia, a hallmark of allergic inflammation. (*J. Clin. Invest.* 1997. 100:2466–2475.) Key words: CD34 • IL-5 receptor • hemopoiesis • asthma

Introduction

Asthma is a complex disorder characterized by reversible airflow obstruction, airway hyperresponsiveness, and infiltration of the airways by activated inflammatory cells. Increasing evidence suggests that the clinical severity of asthma parallels the degree of eosinophilic inflammation and, as such, highlights a role for eosinophils in the pathology of asthma (1–4). However, the mechanism(s) underlying the development and persistence of tissue eosinophilia in allergic inflammation remains unresolved.

We have investigated the hypothesis that activation of specific hemopoietic pathways in the bone marrow may contribute to the allergic diathesis through increased production and traffic of lineage-committed inflammatory progenitor cells such as those for eosinophils. This is supported by findings (in semisolid liquid cultures) that circulating progenitors (colony-forming units, CFU)[1] for eosinophils and basophils (Eo/Baso–CFU) are constitutively increased in atopic individuals (5) and that these cell numbers change in parallel with acute exacerbations and steroid-controlled resolution of clinical asthma (6). Selective and relevant fluctuations in the numbers of blood Eo/Baso–CFU in allergic rhinitics during seasonal exposure to allergen (7, 8) and in atopic asthmatics after allergen inhalation (9) highlight a link between disease severity and progenitor cell numbers. More direct evidence for the involvement of the bone marrow in allergen-driven airway responses is demonstrated in a canine model of airway hyperresponsiveness, where numbers of bone marrow–derived granulocyte–macrophage CFU are increased significantly 24 h after allergen inhalation challenge and are abolished by pretreatment with inhaled corticosteroids (10). These studies suggest that a feedback mechanism exists between the lungs and bone marrow that triggers increased production of bone marrow–derived inflammatory cell progenitors during allergic inflammatory reactions. However, the potential involvement of the bone marrow in the genesis of allergic asthma in humans has not been directly investigated to date.

CD34 is an *O*-sialylated glycoprotein (105–120 kD), whose expression within the hemopoietic system is restricted to primitive progenitor cells of all lineages (11, 12). By flow cytometry, we have shown recently that increased numbers of CD34$^+$ cells are present in the blood and bone marrow of atopics compared with nonatopic control subjects (13). In addition we showed that in atopics, blood progenitors are skewed towards an increased responsiveness to IL-5 as demonstrated by the increased numbers of Eo/Baso–CFU detected in methylcellulose cultures with IL-5 (13). Therefore, we hypothesized that IL-5R$^+$ progenitors would be detectable in the blood and bone marrow of allergic asthmatic subjects and that the level of ex-

Address correspondence to Dr. Judah A. Denburg, Department of Medicine, Room 3V46, Health Sciences Centre, McMaster University, 1200 Main St. West, Hamilton, Ontario, L8N 3Z5, Canada. Phone: 905-521-2100 ext. 6714; FAX: 905-521-4971.

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1. *Abbreviations used in this paper:* CFU, colony forming units; DR, dual responders; Eo/Baso–CFU, eosinophil and basophil CFU; FSC, forward light scatter; IER, isolated early responders; IL-3Rα, interleukin-3 receptor alpha-subunit; IL-5Rα, interleukin-5 receptor alpha-subunit; NAMNC, nonadherent mononuclear cells; PE, phycoerythrin; PerCp, peridinin chlorophyll protein; SSC, orthogonal or side light scatter; WBC, white blood cells.

pression of IL-5R would be increased preferentially in situations where eosinophilic inflammation is induced. In order to investigate this hypothesis, we enumerated the level of coexpression of CD34 and receptors for eosinophilopoietic cytokines such as IL-5 and IL-3, on bone marrow–derived progenitors from a group of stable atopic asthmatics, taken both before and 24 h after allergen inhalation challenge (a time point associated with increases in circulating and airway eosinophils).

Methods

Materials. Materials were obtained as follows: Percoll from Pharmacia Biotech AB (Uppsala, Sweden); McCoys 5A, Iscove's modified Dulbecco's medium, and FCS from GIBCO BRL (Gaithersburg, MD); methylcellulose, BSA grade V, heparin, sodium azide, and paraformaldehyde from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada); May Grunwald Giemsa (MGG) stain from BDH (Mississauga, Canada).

Antibodies. Phycoerythrin (PE)-conjugated IgG_1 CD34 antibody (HPCA-2), FITC-conjugated IgG_1 CD45 antibody (anti-HLE1), PE-conjugated isotype control antibody (i.e., anti–IgG_1-PE specific for keyhole limpet hemocyanin), and streptavidin-conjugated peridinin chlorophyll protein (PerCp) were purchased from Becton Dickinson, Canada (Mississauga, Ontario, Canada). Nonneutralizing monoclonal antibodies directed against the α-subunit of IL-3R (IL3Rα; 7G2) and IL-5R (IL5Rα; A16) were kind gifts from Dr. A. Lopez (Institute of Medical and Veterinary Science, Adelaide, Australia) and Roche Laboratories (Ghent, Belgium), respectively. Cytokine receptor antibodies and the isotype-matched controls were biotinylated using a long-arm biotin procedure (14) in which biotin was coupled to azide free protein via a hydroxysuccinimide ester after incubation with *N*-hydroxysuccinobiotin (Sigma-Aldrich Canada Ltd.); excess biotin was then removed by dialyses against borate buffered solution, pH 8.6.

Subjects. In an attempt to establish if the differential airway responses to allergen are reflected by different progenitor cell responses in the bone marrow, two groups of asthmatic subjects were examined based on their airway responses to allergen: (*a*) subjects ($n = 6$) who only developed an isolated early asthmatic response with no definite late asthmatic response and no change in airway responsiveness to methacholine after allergen challenge and, (*b*) subjects ($n = 7$) who developed both an early- and a late-asthmatic response after allergen inhalation together with increased airway responsiveness to methacholine (Table I). All subjects were atopic as determined by skin prick test positivity and were studied at a time when their asthma was mild, stable and treated by inhaled β_2-agonist only. All subjects were nonsmokers and had a baseline forced expiratory volume in one second (FEV_1) < 70% of the predicted normal on all study days (15) and none had had a respiratory tract infection for at least 4 wk before entering the study. The study was approved by the Ethics Committee of the McMaster University Health Sciences Centre, and each subject gave written informed consent.

Study design. Subjects attended the laboratory on three separate occasions. Visit 1: 1 wk before allergen challenge when documentation included a full medical history, skin prick test sensitivity to allergen extracts, spirometry, methacholine inhalation test, and induction of sputum to assess baseline airway inflammation. Visit 2: subjects underwent the allergen challenge procedure. Before allergen challenge, a bone marrow aspirate was collected and spirometry measurements were taken for 7 h after allergen inhalation in order to follow the allergen-induced bronchoconstrictor response. Blood samples were taken before and 5 h after allergen challenge to enumerate allergen-induced changes in the white blood cell count. Visit 3: blood, sputum, and bone marrow aspirates were collected 24 h after allergen challenge. Spirometry measurements and methacholine inhalation

Table I. Subject Characteristics and Allergen-induced Airway Responses

						PC_{20} Methacholine	
	Age	Gender	Allergen	EAR	LAR	Pre-allergen	Post-allergen
	yr		*Inhaled dilution*	*Max percent fall in FEV_1*		*mg/ml*	
Isolated Early Responders							
1	23	F	HDM (1:32)	25.0	5.4	2.48	8.00
2	22	M	Ragweed (1:8)	30.5	−8.5	1.00	2.45
3	19	F	HDM (1:64)	31.5	11.1	0.27	0.13
4	23	M	HDM (1:4)	17.9	7.7	2.99	4.00
5	22	F	HDM (1:128)	25.0	11.8	0.13	0.50
6	30	M	HDM (1:8)	20.5	4.5	3.48	1.86
Mean				25.1	8.2	1.73	2.82
SEM				2.2	1.2	0.59	1.18
Dual-Responders and Delayed Hypperresponsiveness							
1	24	M	HDM (1:512)	25.0	40.8	0.17	—
2	22	F	HDM (1:4096)	21.2	21.2	1.80	0.29
3	26	F	HDM (1:64)	23.5	16.9	2.44	1.65
4	22	M	Grass (1:2048)	21.6	22.4	3.38	0.73
5	22	M	Cat (1:128)	21.1	15.5	3.31	1.00
6	31	M	HDM (1:256)	40.7	18.6	0.62	0.31
7	22	M	HDM (1:256)	29.4	23.5	1.30	0.39
Mean				26.1	22.7*	1.86	0.73‡
SEM				2.7	3.2	0.47	0.20

EAR, early asthmatic response; LAR, late asthmatic response; PC_{20}, provocation concentration of methacholine causing a 20% fall in FEV_1; HDM, house dust mite. *$P < 0.001$ for between group comparisons of maximal % fall in FEV_1 during the LAR; ‡$P < 0.001$ for within group comparison of pre-allergen versus post-allergen log PC_{20} values.

challenge were also performed to assess the development of airways hyperresponsiveness.

Methacholine and allergen inhalation challenge. Methacholine inhalation was performed as described by Cockcroft et al. (16). Spirometry was measured with a Collins water sealed spirometer and kymograph. The test was terminated when a fall in FEV_1 of 20% of the baseline value occurred, and the provocative concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}) was calculated. Allergen challenge was performed as described by O'Byrne et al. (17). The early bronchoconstrictor response was taken to be the maximal percent fall in FEV_1 within 2 h after allergen inhalation and the late asthmatic response was taken to be the maximal percent fall in FEV_1 between 3 and 7 h after allergen inhalation.

Sputum and blood differential counts. Sputum was induced by saline inhalation and processed according to the method of Popov et al. (18). Cytospins of cell plugs collected from the sputum sample and processed using 0.1% dithiothreitol (Sputolysin; Calbiochem Corp., San Diego, CA) and Dulbecco's PBS (GIBCO BRL) were stained with Diff-Quik (American Scientific Products, McGaw Park, IL). Differential counts are expressed as the mean of duplicate slides (500 cells counted per slide). Venous blood was collected into ethylenediaminetetracetic acid (EDTA)-treated tubes. Total cell counts were performed using a Neubauer hemocytometer and differential cell counts were made from blood smears stained by Diff-Quik. Differential cell counts were obtained from the mean of two slides (300 cells counted per slide) and cell populations were expressed as the absolute counts (10^9 cells per liter).

Cells and myeloid cell lines: controls for cytokine receptor staining. Both HL60 clone 15 cells and KG1 myeloid leukemic cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were cultured in RPMI 1640 medium (GIBCO BRL). The B9 cell line, an IL-6–dependent mouse B cell hybridoma, was obtained from Dr. Lucien Aarden (Red Cross Transfusion Services, Amsterdam, The Netherlands) and cultured in RPMI 10 medium plus recombinant human IL-6 (19). Peripheral blood-derived neutrophils and eosinophils were isolated by positive and negative selection, respectively, using a CD16 dependent magnetic cell separation technique (20) and monocytes were isolated by collecting the adherent cell population after adherence of low density mononuclear cells to plastic, as described below.

Preparation of bone marrow cells and immunofluorescence staining. Heparinized (1,000 U/ml) samples of bone marrow (2–3 ml) were aspirated from the iliac crest and low density mononuclear cells (MNC) were isolated by sedimentation on Percoll density gradients (specific gravity 1.077) as described previously (9, 21). Monocytes were depleted from the MNC fraction by incubation in plastic flasks for 2 h at 37°C. Samples of 1×10^6 nonadherent mononuclear cells (NAMNC) in a final volume of 100 μl of ice-cold PBS containing 0.1% NaN_3 and 0.5% BSA (PAB) were stained with saturating amounts of biotin-conjugated anti–IL-3Rα, anti–IL-5Rα, or IgG_1 isotype control antibody (determined in preliminary studies) for 30 min at 4°C. The cells were then washed and stained with streptavidin-conjugated PerCp, together with saturating concentrations of anti-CD45 FITC and anti-CD34 PE or IgG_1 isotype control in a final volume of 100 μl of PAB for 30 min at 4°C. The cells were then washed with 3 ml of PBS plus 0.1% azide, fixed in 500 μl of PBS plus 1% paraformaldehyde, and refrigerated until ready for analysis.

Flow cytometry and gating strategy. Cells were analyzed using a FACScan flow cytometer equipped with an argon ion laser (Becton Dickinson Instrument Systems, BDIS, Mississauga, Canada). Five data parameters were acquired and stored in listmode files: linear forward light scatter (FSC), linear side-angle light scatter (SSC), log FITC, log PE, and log PerCp fluorescence; each measurement contained 50,000 events. Compensation settings were established using CalBrite beads (BDIS) and confirmed using NAMNC stained with anti–CD34-PE, anti–CD45-FITC, or anti–IL-5Rα–PerCp. Off-line analysis was performed using the PC lysis software as supplied by BDIS.

We used a multi-parameter sequential gating strategy that, we have previously shown, accurately enumerates $CD34^+$ progenitor cell numbers in various biological samples (22, 23). The rationale for sequential gating was to gradually eliminate contaminating cells that nonspecifically take up anti-CD34 (24). Briefly, a primary gate using CD45 staining versus SSC (region R1) was set up to quantitate total leukocytes and distinguish contaminating events such as platelet aggregates and other debris which can nonspecifically take up anti-CD34 (Fig. 1 *A*, *PLOT 1*). Primitive cells characteristically express CD45 at low to intermediate levels (25) and therefore $CD45^+$ events generate a stable denominator in the calculation of the absolute $CD34^+$ value. Sequential gates were then set up: CD34 staining in region R1 versus SSC (region R2) (Fig. 1 *A*, *PLOT 2*), CD45 versus SSC of the $CD34^+$-gated events in R2 (region R3: to identify blast cells) (Fig. 1 *A*, *PLOT 3*), and FSC versus SSC to confirm the lymphoblastoid characteristics of the gated $CD34^+$ cells in region R3 (i.e., low to medium SSC and FSC; region R4) (Fig. 1 *A*, *PLOT 4*). Without changing any of the gates, analyses of the same cell sample stained with CD45-FITC and PE linked isotype control antibody were performed (Fig. 1 *B*, *PLOT 3* and *PLOT 4*). Enumeration data were derived from the gate statistics: events in gate G4 (= events in R1 to R4) after staining with CD45-FITC/CD34-PE minus events in G4 stained with CD45-FITC/PE–linked control antibody were used to calculate the absolute number of true $CD34^+$ blast cells in the test sample.

In three-color analysis, events in region R4 were back scattered onto a dot plot of CD34-PE versus staining by PerCp linked cytokine receptor mAbs or control antibody (Fig. 2), and data were collected as percent positive cells at the 99% confidence limit (i.e., relative to a marker set to include only 1% of cells stained with control antibody). The data presented are the mean of duplicate assessments. The intra-assay variability was always less than 5%.

Simultaneous in situ hybridization and immunohistochemistry. To confirm the association of the membrane bound form of IL-5Rα messenger RNA to $CD34^+$ progenitor cells, simultaneous in situ hybridization and immunocytochemistry was performed (26). Messenger RNA for membrane bound IL-5Rα was detected by autoradiography and CD34 immunoreactivity was detected by an alkaline phosphatase antialkaline phosphatase technique (APAAP). A population of $CD34^+$ cells was enriched from cord blood by positive selection using a magnetic cell separation technique, as described previously (13). These cells were cytospun on poly-L-lysine–coated slides, fixed in 4% paraformaldehyde in PBS for 30 min, and washed in 15% sucrose in PBS. Preparations were hybridized with ^{35}S- labeled membrane-bound IL-5Rα antisense riboprobe and simultaneously immunostained with a mouse anti–human mAb against CD34 (QBEND 10; Becton Dickinson, San Jose, CA) (26).

Statistical analysis. The data are presented as absolute numbers of $CD34^+$ progenitor cells (Fig. 3) and as arithmetic mean±SEM (Fig. 4, Tables I and II), except PC_{20} values (Table II) that were logarithmically transformed and expressed as geometric means and standard error of geometric means (percent SEM). For statistical analyses of within group comparisons between pre- and post-allergen challenge time points, a paired Student's *t* test (two-tailed) was performed (Table I, Figs. 3 and 4). Student's non-paired *t* tests (two-tailed) were performed for all between group comparisons (Table I) and changes in blood differentials after allergen were assessed for each group using repeated measures analysis of variance (rmANOVA) (Table II). Significance was accepted at the 95% confidence level.

Results

Allergen-induced bronchoconstrictor responses and airway hyperresponsiveness. Subjects ($n = 6$) in whom the maximal percent fall in FEV_1 during the late asthmatic response was $< 15\%$ were labeled isolated early responders (IER; mean percent fall in FEV_1, 8.2±1.2%). Subjects in whom that late maximal percent fall in FEV1 was $> 15\%$ were labeled as dual responders

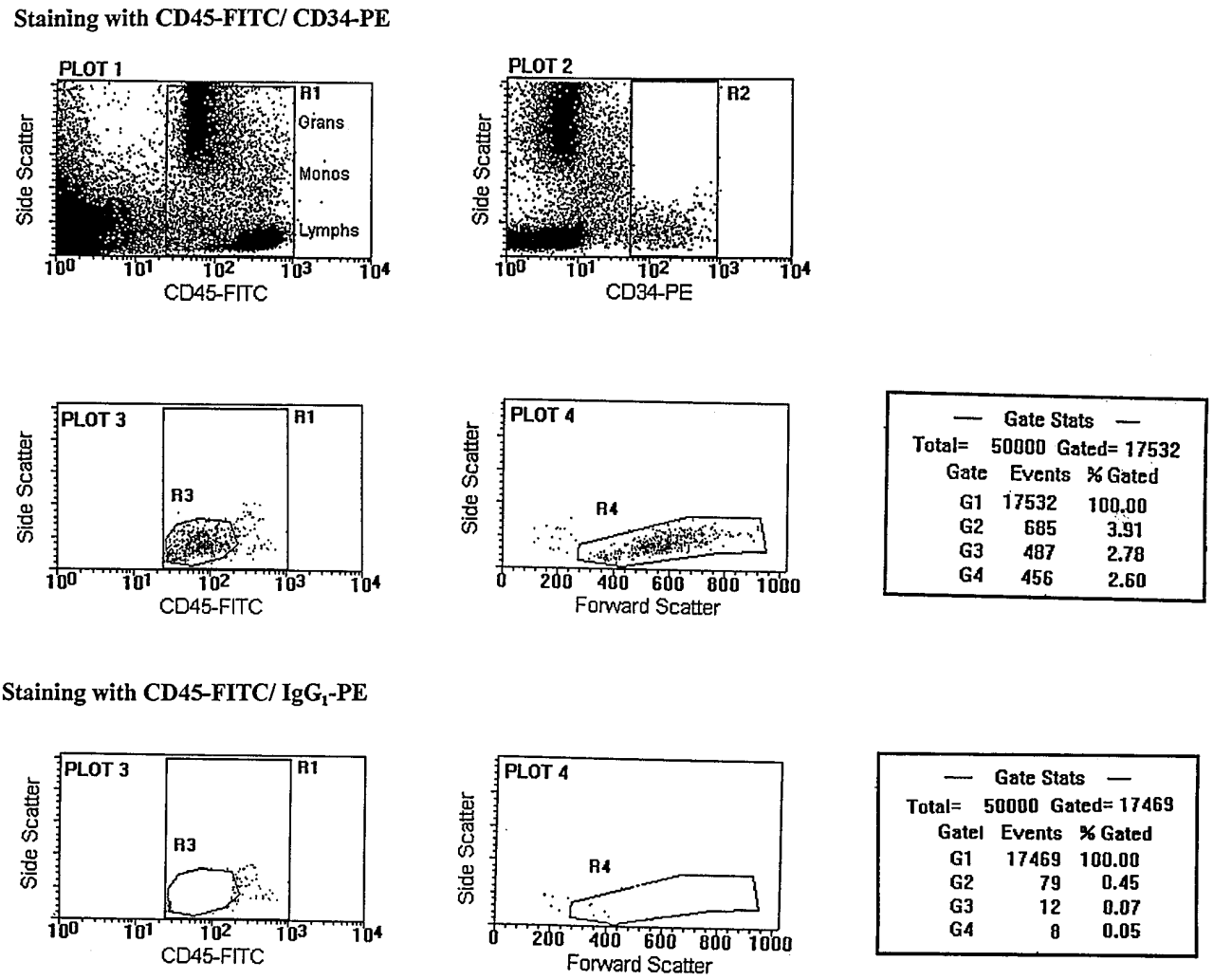

Figure 1. Enumeration of $CD34^+$ progenitor cells from bone marrow samples taken from an atopic asthmatic subject. (*A*) Plots 1–4 represent staining of bone marrow–derived low density nonadherent mononuclear cells with CD45 FITC/CD34 PE. (*B*) Represents staining of low density NAMNC with CD45 FITC/isotype IgG_1 PE. Precise details of the gating strategy are described in Methods. Identical gating regions to those shown in (*A*) were used to analyze the staining of the same bone marrow sample with the control reagents (*B*).

(DR; mean percent fall in FEV_1, 22.7±3.2%). The dual responders, but not the isolated early responders, developed a significant increase in methacholine airway responsiveness 24 h after allergen challenge ($P < 0.001$, Table I).

Airway and blood eosinophilia. A significant increase in sputum eosinophilia was detected in DR asthmatics but not in the IER group, when pre-allergen levels were compared with 24 h post-allergen values (Table II). Similarly, a significant increase in blood eosinophil levels was observed between 5 and 24 h post-allergen in DR asthmatics ($P < 0.05$), although this was preceded by a significant reduction in eosinophil numbers 5 h post-allergen compared with baseline values ($P < 0.05$, Table II). In contrast, in IER, no significant change in the numbers of blood eosinophils was observed at either 5 or 24 h post-allergen compared with pre-allergen values.

IL-3Ra– and IL-5Ra–subunit expression in various cell types. To verify the specific binding capacity of the cytokine receptor antibodies used in this study, we tested the staining of anti–IL-3Rα (7G2) and anti–IL-5Rα (A16) on various leukemic cell lines and mature peripheral blood leukocytes. Compared with the isotype matched control antibody, the significant expression of the IL-3Rα–subunit was detected on KG1 cells [Fig. 3 *A (i)*] and peripheral blood monocytes [Fig. 3 *A (iii)*] (27). As expected, no expression of IL-3Rα was detected on B9 cells, an IL-6–dependent mouse B cell hybridoma cell line [Fig. 3 *A (ii)*] or on mature neutrophils [Fig. 3 *A (iv)*] (27). The expression of IL-5Rα was detected on HL60 clone 15 cells, known to overexpress receptors for IL-5 (8), and on peripheral blood eosinophils (Fig. 3 *B*). No specific expression of IL-5Rα was detected on either neutrophils [Fig. 3 *B (iv)*] or monocytes (data not shown) (28).

Allergen-induced changes in the phenotype of bone marrow progenitors. By multiparameter flow cytometric analyses, allergen-induced changes in the absolute numbers of bone marrow–derived $CD34^+$ progenitor cells and the absolute numbers of $CD34^+$ cells expressing the α-subunit of IL-3R and IL-5R were investigated. In DR asthmatics, the total number of bone marrow–derived $CD34^+$ cells increased from 3,715 cells/0.25×10^6 white blood cells (WBC) (percent SEM 830) before allergen to 5,623 cells/0.25×10^6 WBC (percent SEM 2,627) 24 h

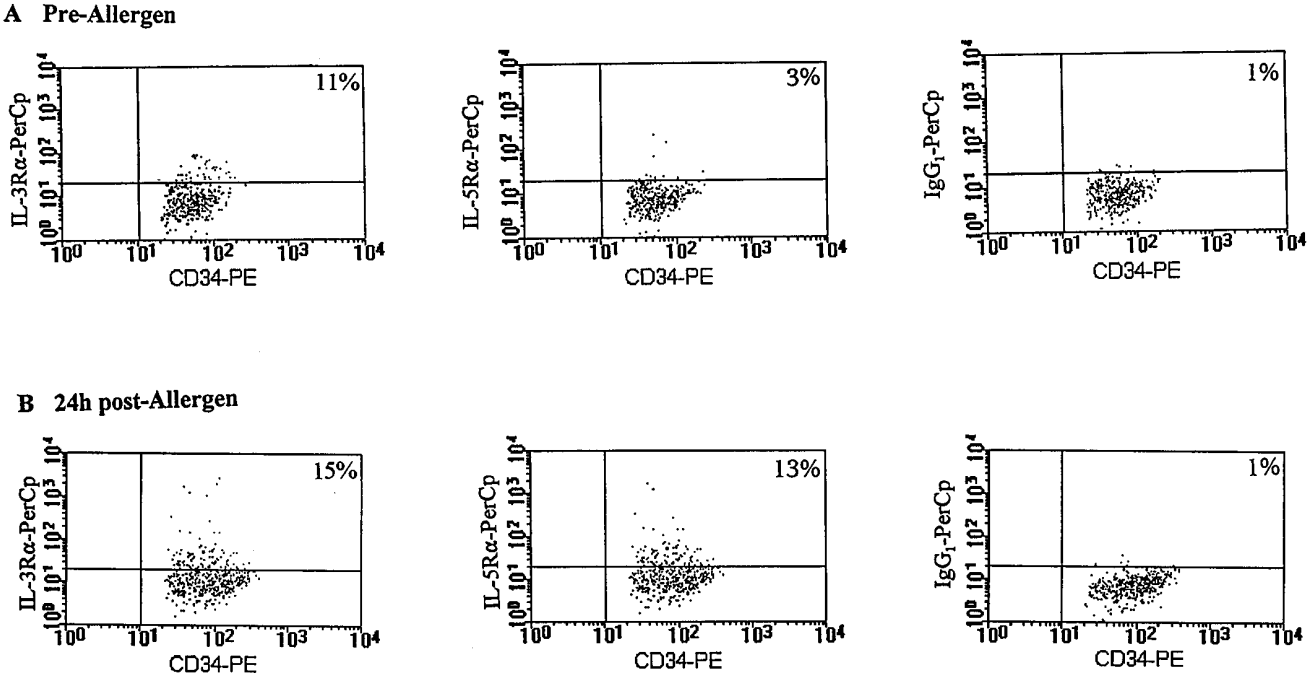

Figure 2. Enumeration of bone marrow–derived $CD34^+$ cells that express the α-subunit of IL-3R and IL-5R. Samples of bone marrow–derived NAMNC collected pre- and 24 h post-allergen from an atopic asthmatic subject were stained with CD45 FITC/CD34 PE and either PerCp linked IL-3Rα, IL-5Rα, or isotype control antibody. Events in R4 (see Fig. 1 *A*, *Plot 4*) were backscattered onto a dot-plot of CD34 PE versus PerCp staining. Quadrant statistics (i.e., data in upper right hand corner) are presented as the percent of total $CD34^+$ cells that demonstrated positive staining with anti–IL-3Rα, anti–IL-5Rα, or mouse IgG_1 isotype matched control antibody. Comparisons between pre- and post-allergen bone marrow samples demonstrate that increased numbers of $CD34^+$ cells express IL-3Rα and IL-5Rα.

after allergen challenge, although these changes were not significant ($P = 0.06$) (Fig. 4). In IER, no differences in $CD34^+$ cell numbers was detected when pre-allergen levels (2,291 cells/ 0.25×10^6 WBC, percent SEM 1,009) were compared with 24 h post-allergen levels (2,570 cells/0.25×10^6 WBC, percent SEM 782, $P = 0.70$) (Fig. 4). No significant differences in either the baseline values of bone marrow $CD34^+$ cells or the magnitude of increase of $CD34^+$ cells after allergen challenge were detected between the two groups of asthmatic subjects.

In DR asthmatics, the absolute number of bone marrow–derived $CD34^+$ cells expressing IL-5Rα increased 30-fold from 26 cells/0.25×10^6 WBC (percent SEM 3.3) before allergen to 724 cells/ 0.25×10^6 WBC (percent SEM 1.6) 24 h after allergen ($P = 0.04$) (Fig. 4). In contrast, there was a negligible increase in the numbers of $CD34^+IL\text{-}5R\alpha^+$ cells in IER when pre-allergen levels (6 cells/0.25×10^6 WBC, percent SEM 2.7) were compared with post-allergen levels (11 cells/0.25×10^6 WBC, percent SEM 2.7, $P = 0.55$) (Fig. 4). Although there was a ninefold increase in the absolute number of bone marrow $CD34^+IL\text{-}3R\alpha^+$ cells in DR asthmatics when pre-allergen levels were compared with 24 h post-allergen levels, these changes were not significant (37 cells/0.25×10^6 WBC versus 324 cells/ 0.25×10^6 WBC, $P = 0.051$) (Fig. 4). In addition, no significant increase in the absolute numbers of bone marrow $CD34^+IL\text{-}3R\alpha^+$ cells was detected in IER 24 h post-allergen (Fig. 4).

It is conceivable that the observed increases in absolute numbers of cytokine receptor positive progenitor cells 24 h post-allergen may be entirely due to increases in total $CD34^+$ cell numbers (Fig. 4). In order to exclude this effect and to determine whether a distinct phenotypic change of a fixed progenitor cell pool had occurred after allergen challenge, we expressed the data as a percent of total $CD34^+$ cell numbers (Fig. 5). In DR, but not IER asthmatics, a significant increase in the percent of $CD34^+$ cells expressing IL-5Rα was detected when pre-allergen bone marrow samples were compared with 24 h post-allergen aspirates (DR: 6.6±3.1 versus 15±2.7% of $CD34^+$ cells, $P = 0.045$, and IER: 1.9±1.08 versus 2.2± 1.2% of $CD34^+$ cells, $P = 0.84$) (Fig. 5). In contrast, in both groups of asthmatic subjects no significant increase in the proportion of $CD34^+$ cells expressing IL-3Rα, after allergen challenge, was observed (DR: 8.5±3.4 versus 15±4.5% of $CD34^+$ cells, $P = 0.22$, and IER: 2.7±1.2 versus 9.2±3.5% of $CD34^+$ cells, $P = 0.19$) (Fig. 5). In comparisons between the two groups of asthmatics, no significant differences were observed in the baseline values of percent $CD34^+$ cells expressing either cytokine receptors (Fig. 5). However, allergen-induced increases in the proportion of $CD34^+$ cells expressing $IL\text{-}5R\alpha^+$ cells were significantly greater in dual responders compared with isolated early responder asthmatics ($P = 0.038$).

In situ hybridization and immunohistochemistry. Colocalization experiments using in situ hybridization and immunocytochemistry were performed in order to confirm that $CD34^+$ progenitor cells can express IL-5Rα mRNA. Because of the small sample sizes of bone marrow aspirates, these experiments were performed on an enriched population of unstimulated $CD34^+$ cells isolated from cord blood (purity determined by flow cytometry, > 65%). Of all the $CD34^+$ cells, 50% were IL-5Rα mRNA positive. A representative example is shown in Fig. 6.

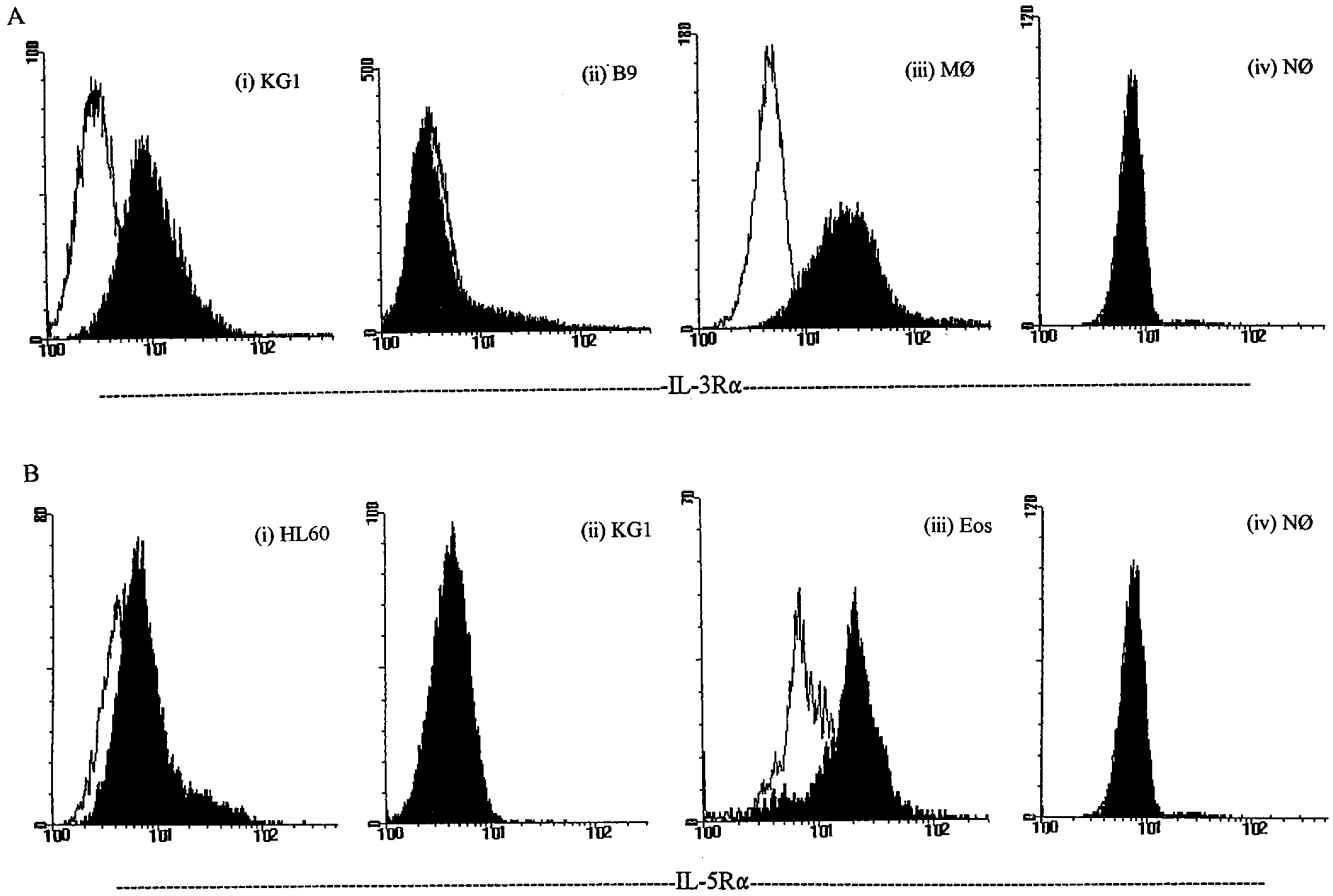

Figure 3. Expression of IL-3Rα and IL-5Rα on various cell types. Cells were incubated with saturating amounts of the biotinylated antireceptor antibodies, washed and incubated with streptavidin-linked PerCp (*shaded area*). For negative control (*blank area*), cells were stained with the equivalent amount of the isotype matched biotinylated antibody (mouse IgG_1) in place of the antireceptor antibody.

Discussion

The novel observation from this study is that the proportion of bone marrow–derived $CD34^+$ cells expressing IL-5Rα is increased preferentially in DR asthmatics who characteristically develop allergen-induced late-bronchoconstrictor responses, methacholine airways hyperresponsiveness, and a significant sputum eosinophilia 24 h after allergen inhalation (Fig. 5, Tables I and 2) (2, 29). In contrast, this distinct change in cytokine receptor expression on bone marrow progenitor cells was not seen in asthmatics who did not develop airway hyperresponsiveness or a marked eosinophil infiltration after allergen inhalation (Fig. 5). Although the data presented herein do not prove a direct association between activation of the bone marrow and development of airway pathology as a result of increased inflammatory cell production, they are consistent with the view that a feedback mechanism exists between tissues involved in allergic inflammation and distal sites such as the

Table II. Inflammatory Cell Count in Sputum and Blood Samples from Asthmatic Subjects

	Early single responders			Dual responders		
	Pre-allergen	5 h Post-allergen	24 h Post-allergen	Pre-allergen	5 h Post-allergen	24 h Post-allergen
Sputum						
Eosinophils (percent)	6.7±2.0	ND	16.4±9.9	3.3±0.9	ND	32.3±11.7*
Neutrophils (percent)	15.7±4.3	ND	28.4±10.2	26.0±10.5	ND	26.9±11.8
Macrophages (percent)	72.2±4.1	ND	48.0±13.5	66.3±10.1	ND	36.1±8.6‡
Blood						
Eosinophils ($\times 10^9$/liter)	0.35±0.04	0.36±0.20	0.46±0.50	0.44±0.12	0.23±0.05*	0.32±0.06§

Data for sputum cell counts are presented as the percentage of 500 white cells counted per slide. Cells were stained with Diff-Quik. Data represent the mean±SEM of $n = 6$ early single responders and $n = 7$ dual responders. *$P < 0.05$; ‡$P < 0.001$ for comparisons of pre-allergen versus post-allergen challenge values; §$P < 0.05$ for 5 h post-allergen versus 24 h post-allergen comparisons.

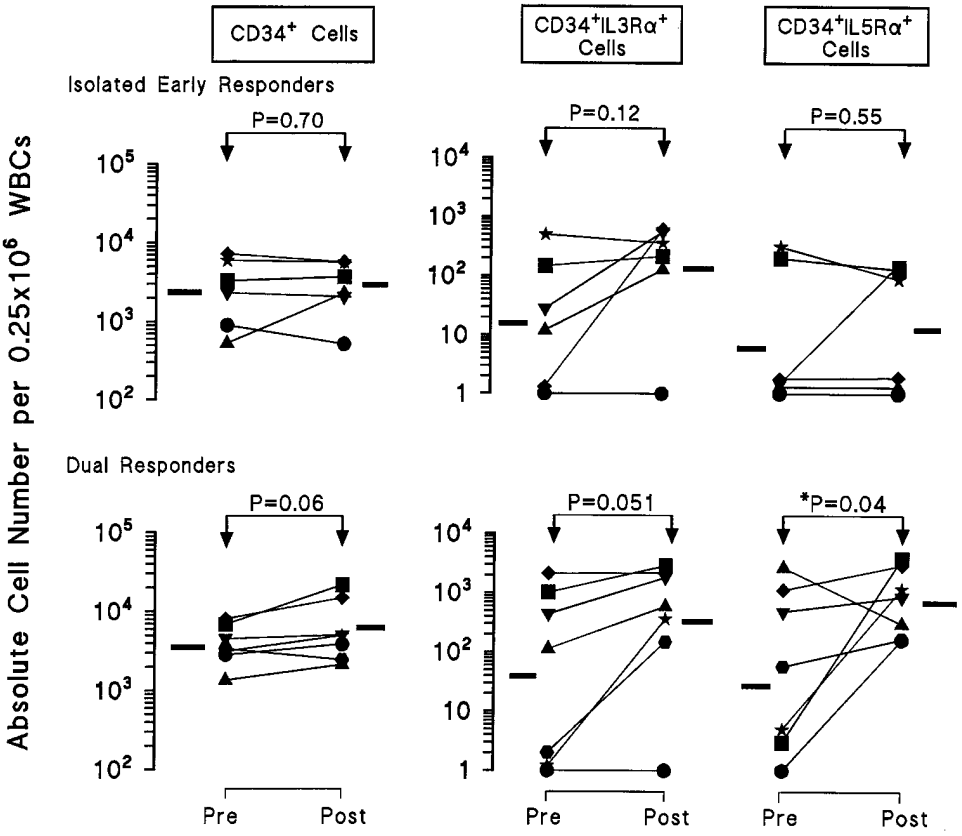

Figure 4. Flow cytometric enumeration of the absolute number of bone marrow derived $CD34^+$ progenitor cells expressing the α-subunit of receptors for IL-3 and IL-5. Samples from ($n = 6$) isolated early- and ($n = 7$) dual-responder asthmatics were taken pre- and 24 h post-allergen challenge. After allergen challenge, a significant increase in the numbers of $CD34^+$ IL-5Rα$^+$ cells were detected in bone marrow samples taken from DR asthmatics. Horizontal bars represent the geometric mean of each data set.

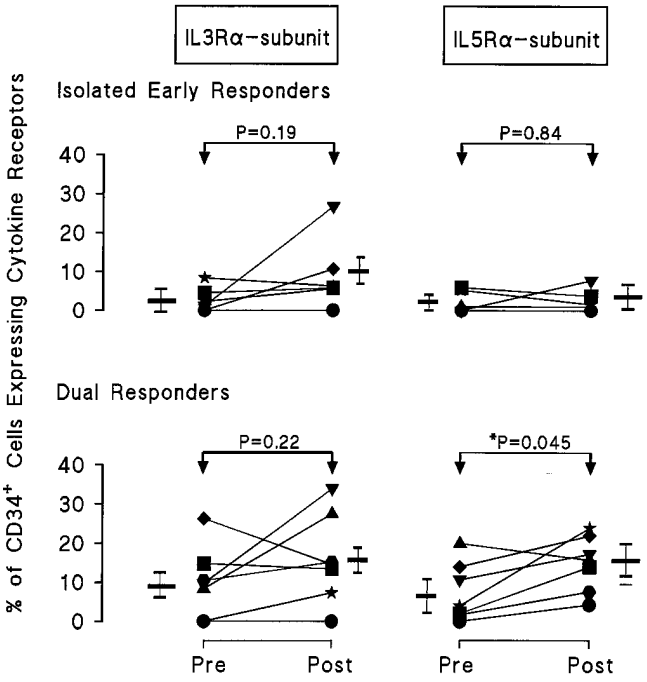

Figure 5. Percent of bone marrow derived $CD34^+$ cells that express the α-subunit of receptors for IL-3 and IL-5. Samples from isolated early- ($n = 6$) and dual-responder ($n = 7$) asthmatics were taken pre- and 24 h post-allergen challenge. After allergen challenge, a significant increase in the percent of $CD34^+$ cells expressing IL-5Rα, but not IL-3Rα, was observed in bone marrow samples taken from dual responder asthmatics but not isolated early responders. Horizontal bars represent the arithmetic mean of each data set.

bone marrow. We have shown that a distinct phenotypic switch occurs within the bone marrow progenitor cell population and we suggest that in the presence of eosinophil growth factors such as IL-5, increased expression of IL-5Rα on $CD34^+$ cells may favor eosinophilopoiesis and thus contribute to the subsequent development of blood and tissue eosinophilia, a hallmark of allergic inflammatory diseases such as asthma.

Of the cytokines which can support eosinophilopoiesis (IL-5, IL-3, and GM-CSF), IL-5 is unique in its ability to specifically promote the terminal differentiation and maturation of eosinophil/basophil lineage–committed progenitors in liquid and semi-solid cultures (30, 31). In mice that overexpress the IL-5 transgene, IL-5 has been shown to be the predominant regulator of eosinophilia (32). Furthermore, a pivotal role for IL-5 in chronic allergic inflammation has been confirmed by the capacity of neutralizing anti–IL-5 mAb to inhibit antigen- or virus-induced airway hyperresponsiveness and eosinophil infiltration in the airways of mice, guinea pigs, and primates (33–39). In contrast, IL-3 is a pluripotential hemopoietic factor; mice that overexpress either IL-3 or GM-CSF, show only modest eosinophilia, but succumb early owing to massive tissue infiltration and destruction by myeloid cells, especially neutrophils and macrophages (32). Our evidence suggests that both GM-CSF and IL-3 commit pluripotential $CD34^+$ $CD33^-$ progenitors to an eosinophil lineage and that IL-5 brings about the terminal differentiation of the less primitive, myeloid lineage-committed progenitor cells (i.e., $CD34^-CD33^+$ cells) derived from $CD34^+$ $CD33^-$ precursors (40). In this study, however, we have demonstrated for the first time the expression of IL-5Rα on $CD34^+$ cells indicating the existence of specific binding sites for IL-5 on more primitive progenitor cells. This

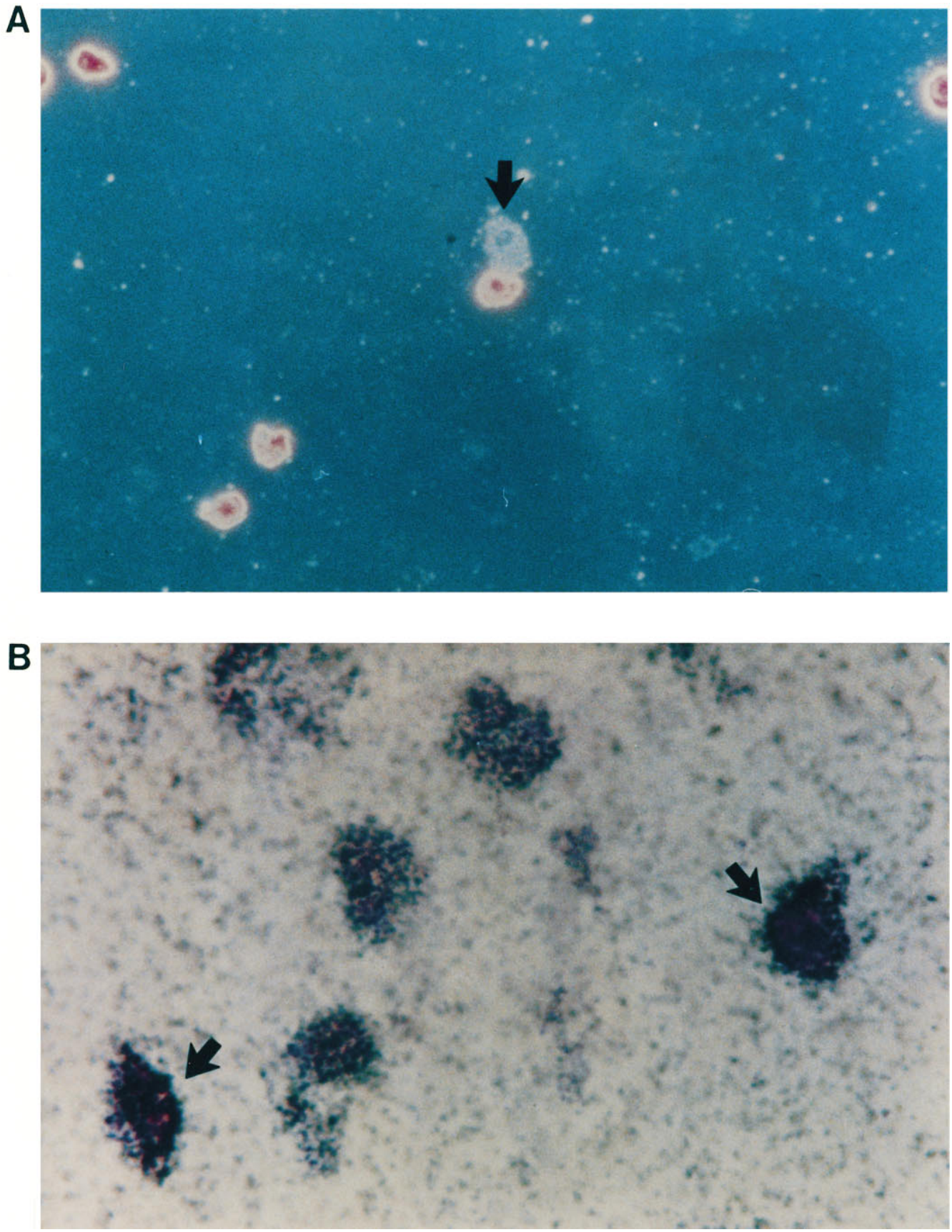

Figure 6. Detection of mRNA for membrane bound form of IL-5Rα in human cord blood–derived CD34$^+$ cells. (*A* and *B*) CD34 enriched cell preparations were hybridized with ^{35}S-labeled membrane bound IL-5Rα antisense riboprobe and simultaneously immunostained with a mouse anti-human CD34 (QBEND10). (*A*) Messenger RNA for membrane bound IL-5Rα was detected by autoradiography and visualized by dark field illumination (*arrow*). (*B*) Subcellular localization of membrane bound IL-5Rα mRNA in CD34$^+$ cells immunostained simultaneously with mouse anti-human CD34 and APAAP and viewed by bright field illumination (*arrows*).

is supported by results from in situ hybridization which demonstrate the colocalization of mRNA for membrane bound IL-5Rα to cells immunostained with anti-CD34. Therefore, we propose that the $CD34^+$ IL-$5R\alpha^+$ phenotype may be representative of the earliest eosinophil/basophil lineage–committed progenitor. However, until additional cloning experiments have been performed to assess the progeny of progenitors of this specific phenotype, this proposal cannot be confirmed.

Molecular cloning of cytokine receptors have revealed that IL-3R, IL-5R, and GM-CSFR are uniquely composed of heterodimeric structures consisting of a distinct α-subunit that binds the cognate cytokine with low affinity and a common, shared, β-subunit which, although failing to bind the ligand itself, forms high affinity cytokine binding sites in association with the α-subunit (41, 42). Deletion mutation experiments of IL-5R have now revealed that, like the β-subunit, the cytoplasmic domain of the α-subunit is also essential for signal transduction, in particular mediating growth signals through IL-5R (43, 44). Since the α-subunit functions as a cytokine-specific binding site, it has been proposed that this subunit may transduce cytokine-specific growth signals while the common β-chain provides the molecular basis for functional redundancy of IL-3, IL-5, and GM-CSF. Therefore, the preferential increase in the proportion of bone marrow–derived $CD34^+$ cells expressing membrane bound IL-5Rα-subunit on bone marrow progenitor cells may increase the ability of the cells to respond more readily to IL-5, and thus differentiate terminally into mature eosinophils and basophils. In support of our findings, Wood et al. have shown that bone marrow aspirates taken from DR 24 h post-allergen are more responsive to IL-5 in vitro, as determined by the significantly greater numbers of Eo/Baso–CFU detected in methylcellulose cultures with suboptimal doses of IL-5 compared with bone marrow cells from IER (45).

Since increases in expression of cytokine receptors on $CD34^+$ cells were detected within 24 h after allergen challenge in DR asthmatics, these changes may have occurred as a consequence of cell division and proliferation (Fig. 4). However, when expressed as a percentage of total $CD34^+$ cells, the increase in IL-5Rα, but not in IL-3Rα, expression on $CD34^+$ cells was independent of changes in the number of progenitors (Fig. 5). This indicates that after allergen challenge in DR asthmatics, a fixed pool of bone marrow $CD34^+$ progenitors undergo a distinct phenotypic change resulting in increases in IL-5Rα surface expression. In contrast, the increased production of small amounts of primitive progenitor cells may account for the near significant increases in absolute numbers of $CD34^+$IL-3Rα cells observed in DR 24 h post-allergen (Fig. 4) (27).

Evidence of the generation of a serum hemopoietic factor during airway allergen challenge that can prime the bone marrow for increased production of granulocyte progenitor cells has been recently demonstrated in a canine model of airways hyperresponsiveness (46). Similarly, increased numbers of Eo/Baso–CFU were grown from the peripheral blood of atopic individuals when antigen-stimulated lymphomononuclear cell conditioned medium was included in colony assays, suggesting the generation of a hemopoietic signal after in vitro allergen challenge (5). Furthermore, in studies of nematode infection in IL-5 transgenic mice, Strath et al. have provided evidence that the level of blood eosinophilia may not only be controlled by the amount of IL-5 produced but, in addition, by the frequency of eosinophil progenitors in the bone marrow during a chronic inflammatory response (47). Thus, investigation of the nature of the signal(s) that modulate the expression of IL-5R on $CD34^+$ progenitor cells may provide insight into the control of eosinophil differentiation from pluripotential stem cells and, potentially provide a novel therapeutic target for controlling the development of the eosinophilic component of the allergic inflammatory response in asthmatic airways. From in vitro studies, little is currently known regarding the modulation of IL-5R expression on normal progenitor cells. Preincubation of peripheral blood $CD34^+$ cells with GM-CSF and IL-3 enhances their subsequent ability to differentiate into eosinophils in response to IL-5 (48). While this finding implies upregulation of IL-5R expression, this has not been formally demonstrated, at least not for progenitor cells. On mature eosinophils, preincubation with GM-CSF, but not IL-3, will enhance expression of IL-5R (49). Downregulation of the IL-5Rα-subunit at the mRNA level in myeloid leukemic cell lines has been shown to be due to factors that either promote eosinophil apoptosis such as $TGF\beta_1$ (50), or by pharmacological agents, such as all-trans retinoic acid (RA), which inhibit eosinophil/basophil differentiation from pluripotential progenitor cells, while favoring neutrophil maturation (51, 52). This is further support for the view that the level of expression of IL-5Rα on $CD34^+$ cells may be directly related to commitment to the eosinophilopoietic pathway.

In conclusion, the results from this study demonstrate that relevant fluctuations occur in the expression of a specific cytokine receptor, IL-5Rα, on bone marrow progenitors in response to allergen challenge in atopic asthmatics. We propose that this selective increase in expression of IL-5Rα on $CD34^+$ progenitors may favor eosinophilopoiesis that may play a role in the generation of increased numbers of eosinophils during an allergic inflammatory response in the airways of asthmatic subjects.

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