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Interleukin-5 in growth and differentiation of blood eosinophil progenitors in asthma: effect of glucocorticoids

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1 There are increased numbers of circulating $CD34^+$ progenitor cells for eosinophils in patients with atopic asthma, with a further increase following allergen exposure or spontaneous worsening of asthma. We investigated the expression of IL-5 and IL-5Rα receptor in circulating $CD34^+$ progenitor cells in allergic asthmatics and the effects of corticosteroids.

2 Using double-staining techniques, up to 50% of $CD34^+$ cells expressed intracellular IL-5, and by RT–PCR, there was significant expression of IL-5 mRNA. When cultured in a semi-liquid methylcellulose medium, there were more eosinophil colony-forming units grown from asthmatic non-adherent mononuclear cell depleted of T cells in the presence of the growth factors GM-CSF, SCF and IL-3, but not of IL-5.

3 An anti-IL-5Rα receptor antibody and an anti-sense IL-5 oligonucleotide reduced the number of eosinophil colony forming units. No IL-5 mRNA or protein expression on T cells was observed in asthmatics or normal subjects. In the presence of growth factors including IL-5, there were significantly greater colony numbers with eosinophilic lineage grown from either asthmatics or normal subjects.

4 Dexamethasone (10^{-6} M) suppressed IL-5 mRNA and protein expression in $CD34^+$ cells, and reduced eosinophil colony-forming units in asthmatics, but not in normal subjects. Dexamethasone did not change the expression of IL-5Rα on $CD34^+$ cells.

5 We conclude that there is increased expression of IL-5 on blood $CD34^+$ cells of patients with asthma and that this expression may auto-regulate eosinophilic colony formation from these progenitor cells. Corticosteroids inhibit the expression of IL-5 in circulating $CD34^+$ progenitor cells. *British Journal of Pharmacology* (2001) **134,** 1539–1547

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Abbreviations: CFU, colony-forming units; CFU-GM, granulocyte-macrophage CFU; Dex, dexamethasone; EPO, eosinophil peroxidase; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony-stimulating factor; IgG, immunoglobulin G; IL-5, interleukin-5; IL-5Rα, IL-5 receptor subunit-α; NAMNC, non-adherent mononuclear cells; NANT, non-adherent non-T cells; PE, phycoerythrin; RT–PCR, reverse-transcription polymerase chain reaction

Introduction

A characteristic feature of asthma is the presence of chronic airway inflammation with infiltration of eosinophils and T cells, and the degree of eosinophilic inflammation in the airways mucosa correlates with the clinical severity of asthma (Bousquet *et al*., 1990; Azzawi *et al*., 1990). Eosinophils are derived from progenitor cells in the bone marrow under the influence of growth factors such as IL-5, and there is increased production and trafficking of inflammatory progenitor cells committed to the eosinophil lineage in asthma (Denburg *et al*., 1985a; Gibson *et al*., 1990; Wang *et al*., 1999). Using semi-solid liquid cultures to induce maturation of progenitor cells, an increase in circulating progenitors for eosinophils and basophils has been demonstrated in patients with allergic asthma during exacerbations of asthma induced by withdrawal of inhaled steroid therapy, and following experimental exposure to allergen (Denburg *et al*., 1985a; Otsuka *et al*., 1986; Gibson *et al*., 1990; 1991). Following allergen challenge of allergic asthmatic subjects, an increase in the number of bone marrow progenitor cells favouring the eosinophilic lineage has been observed (Sehmi *et al*., 1997), and an increase in IL-5 expression has been reported in a similar situation in mice (Minshall *et al*., 1998). Increased progenitor cells have been localized to the airways mucosa of patients with asthma (Robinson *et al*., 1999), indicating that these cells originate from the bone marrow, are released into the circulation, and may be attracted to the airways mucosa.

Primitive haematopoietic progenitor cells of all lineages express the CD34 O-sialylated glycoprotein and this can be used as a marker of these cells by flow cytometry (Sutherland *et al*., 1994). An increased number of $CD34^+$ cells in the blood and in the bone marrow of atopics compared to non-atopic control subjects has been demonstrated (Sehmi *et al*., 1996). We have previously demonstrated that patients with more severe asthma have more $CD34^+$ cells in blood than in

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patients with mild asthma or in non-asthmatics (Wang *et al*., 1999). The number of $CD34^+$ cells in blood correlated significantly with serum levels of IL-5 or GM-CSF, which can prime progenitor cells to proliferate and differentiate into eosinophils (Wang *et al*., 1999). $CD34^+$ blood progenitors obtained from atopic individuals or from atopic asthmatics mature into increased numbers of eosinophil-basophil colony forming units in the presence of IL-5 in methylcellulose cultures (Wang *et al*., 1999; Sehmi *et al*., 1996). An increase in the surface expression of IL-5Rα in bone marrow $CD34^+$ cells after allergen challenge has been reported (Sehmi *et al*., 1997) and this up-regulation of IL-5Rα may result from stimulation by circulating or bone marrow IL-5 to favour eosinophilopoiesis. In addition, enhanced expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine that prolongs eosinophil survival, has been observed in circulating eosinophil progenitors (Gauvreau *et al*., 1998).

Because IL-5 expression has been shown to regulate the expression of its own receptor α-subunit (Tavernier *et al*., 2000), we hypothesized that progenitor cells from asthmatic patients synthesize and release IL-5 in an autocrine fashion to act on IL-5Rα on $CD34^+$ cells, leading to a preferential development into the eosinophilic lineage. We examined the role of IL-5 and IL-5Rα in the regulation of progenitor cell growth and colony development in asthmatic patients and normal subjects. Previous studies have demonstrated that inhaled corticosteroid therapy can reduce the increased numbers of progenitor cells in blood and the enhanced expression of GM-CSF observed after allergen challenge (Gauvreau *et al*., 2000), and in bone marrow inflammatory cell progenitors in asthmatic subjects (Wood *et al*., 1999). We therefore examined the effects of corticosteroids on the ability of progenitor cells to proliferate and differentiate into eosinophils, and on the expression of IL-5 and IL-5Rα in these cells.

Methods

Subjects

Twenty-seven non-smoking adults with mild-to-moderate asthma were recruited from outpatient clinics of Chang Gung Memorial Hospital, Taiwan (Table 1). Asthma severity was assessed by the degree of asthma symptoms, and by increased requirements for bronchodilator therapy. Airflow limitation was present at the time of the laboratory visit in all patients and patients had a $>15\%$ improvement in FEV_1 with inhaled β-agonist, fenoterol (200 μg). None of the patients had received inhaled or oral corticosteroids (including nasal corticosteroids), cromoglycate, theophylline or other medications 6 weeks before presentation except for inhaled β_2-agonists which were used as a rescue medication. Inhaled β-agonists were withheld for 12 h before venepuncture and methacholine testing, and subjects rested for 30 min prior to testing. Eighteen normal non-smoking subjects (seven males/11 females) without evidence of allergic rhinitis or asthma as identified by history were recruited from the staff of Chang Gung Memorial Hospital. They also had negative tests for serum specific IgE (Phadiatop) and total IgE levels within the normal range as well as eosinophil counts (Table 1). Spirometry and metacholine airway responsiveness were within the normal range.

Table 1 Demographic features of patients

	Normal subjects	*Asthmatic patients*
Age (years)	32.5±4.6	33.4±2.4
Sex (male/female)	7/11	12/15
FEV1% predicted	80.9±4.6	73.2±4.0
FEV1/FVC (%)	82.3±2.4	75.8±2.9
PC_{20} (mg ml^{-1})	>25	3.4±1.5

PC_{20}, concentration of methacholine to induce 20% decrease in FEV_1. Data are mean±s.e.mean.

Preparation of progenitor cells

Peripheral blood mononuclear cells (PBMC) were separated from whole blood using Ficoll-Hypaque density gradient centrifugation at $400 \times g$ for 30 min. The mononuclear cells (MNC) at the interface were harvested, washed twice, and resuspended in Iscove modified Dulbecco medium (GIBCO-BRL, Gaithersburg, MD, U.S.A.). The non-adherent mononuclear cell (NAMNC) fraction was separated by adherence technique, and were depleted of E-rosette-forming cells by a second Ficoll-Hypaque centrifugation of a mixture of NAMNC cells and sheep red blood cells. T cell depleted (NANT) cells obtained at the interface were harvested and resuspended in Iscove modified Dulbecco medium. There were less than 5% of $CD34^+$ cells remaining in NANT cell preparations.

Measurement of $CD34^+$ and co-localization of IL-5 and IL-5Rα in NANT cells

NANT cells (1×10^5 cells ml^{-1}) were centrifuged and the cell pellet was incubated with 5 μl anti-CD34-FITC monoclonal antibody (Dakopatts, Glostrup, Denmark) in the dark for 30 min at 4°C. For control studies, cells were incubated with 5 μl of IgG1-FITC conjugated (Dakopatts). After washing with resuspension in PBS, some cell suspensions were incubated with mouse monoclonal anti-human IL-5Rα-PE antibody (R&D Systems, Minneapolis, MN, U.S.A.) for 30 min at 4°C in the dark. Cells were then washed in RPMI once, and the cells were fixed and permeabilized with 500 μl of permeabilization solution for 10 min in the dark at room temperature. In separate cells already incubated with anti-CD34-FITC conjugated monoclonal antibody, these were washed with penetration buffer (Becton Dickinson, Mountain View, CA, U.S.A.) and then incubated with mouse monoclonal anti-human IL-5-PE antibody (Pharmingen, Los Angeles, CA, U.S.A.).

These cell suspensions were then analysed separately with a FACScan flow cytometer equipped with an argon ion laser (Becton Dickinson, Mountain View, CA, U.S.A). Mouse IgG conjugated with PE was used as control (Dakopatts, Glostrup, Denmark). Compensation settings were established using CalBrite beads (Becton Dickinson Instrument Systems). A multi-parameter sequential gating strategy that excluded non-specific staining of CD34 as previously described (Sehmi *et al*., 1996; Sutherland *et al*., 1994) was used to enumerate $CD34^+$ progenitor cell numbers. This is described in detail in our previous study (Wang *et al*., 1999). Briefly, a region

representing $CD45^+$ events was set up to quantitate total nucleated white blood cells and to exclude contaminating cells such as red blood cells, platelet aggregates and other debris. Simultaneous staining by $CD34^+$ antibody then defined $CD34^+$ stem/progenitoir subfraction. $CD45^+$ events were then analysed for CD34 staining.

Assessment of apoptosis

To measure apoptosis of progenitor cells, the cells were first fixed and permeabilized in iced 70% alcohol at room temperature for 10 min. Then, the cells were treated with 10 μl RNase A (100 μg ml^{-1}; Sigma, St. Louis, MO, U.S.A.), 10 μl propidium iodide (40 μg ml^{-1}; Sigma, St. Louis, MO, U.S.A.) and incubated at 37°C for 30 min. The proportion of cells within the hypodiploid DNA region was assessed by flow cytometry.

Granulocyte-macrophage progenitor (CFU-GM) assay

NANT cells were cultured at a concentration of 2×10^5 cells in 1 ml of 0.9% methylcellulose supplemented with Iscove modified Dulbecco medium, 5×10^{-5} mol l^{-1} 2-mercaptoethanol, 1% bovine serum albumin, 30% foetal calf serum, with growth factors, GM-CSF (500 u ml^{-1}), stem cell factor (50 ng ml^{-1}) and IL-3 (100 u ml^{-1}) in 35-mm dishes (Wang *et al*., 1999). Colonies were scored 14 days after plating using an inverted light microscope. Aggregates of $\geqslant$ 40 cells were defined as 'colonies'. Colonies were classified as two main types (1) eosinophil-type (Eo), which typically were tight accumulations of round, refractile cells and (2) neutrophil-macrophage type (GM), which typically were loose accumulations of cells varying in size and shape. To identify whether the colonies were Eo- or GM-type for each culture of cells from a given subject, eight Eo-type and GM-type colonies were picked at random from dishes, placed on slides and stained with Liu's stain for morphological identification and differential cell count determination. Differential staining of Eo-type colony cells showed two distinct patterns of colonies with either $\geqslant$95% metachromasia or with mixtures of metachromatic and eosinophilic staining cells.

In the CFU-GM assay, NANT cells were cultured on single layer agar culture system. On day 14 of culture, the agar disks containing colonies from each well were fixed with citrate buffer acetone/methanol fixative (Sigma, St. Louis, MO, U.S.A.), dried overnight, and stained for eosinophil peroxidase (EPO). This was used to count the percentage of eosinophils in the colonies. All experimental procedures were performed with endotoxin-free plastic wares; according to the manufacturers' information, the levels of endotoxin contamination in the cytokine preparations were three endotoxin units mg^{-1} measured by the limulus assay, and the antibodies used in all experiments contained $<$1 ng ml^{-1} of endotoxin.

Reverse transcriptase-polymerase chain reaction (RT–PCR) for IL-5

Total RNA was extracted from NANT cells, purified T cells, or NANT cells cultured for 6 h in the presence or absence of dexamethasone, using the guanidine thiocyanate/phenol/chloroform method described previously (Chomczynski & Sacchi, 1987). cDNA was reverse-transcribed from isolated RNA by incubating 25 ng of DNAse-treated RNA in a reaction buffer containing 10 mM dithiothreitol, 1 mM deoxynucleotide triphophate mixture (dNTP), 50 mol of oligoDT, 10 units RNAsin, and 50 units MMLV reverse transcriptase in 10 μl volumes. The PCR reaction was run in amplification buffer containing 1.5 mM $MgCl_2$, 10 pmol each of forward (5′) and reverse (3′) primers, 2.5 units of KlenTaq polymerase (Clontech, Palo Alto, CA, U.S.A.), 1 mM dNTP, and 10 μl of the RT reaction products in a 25 μl volume. After 25 cycles, 20 μl of the PCR products were run on a 2% agarose gel. The gel was stained with ethidium bromide, photographed, and the bands quantified by densitometer scan. Each gel was run with GAPDH, a housekeeping gene for comparison of expression. An IL-5 positive control was obtained from purified peripheral blood eosinophils of a patient with asthma. All PCR primers were synthesized by GIBCO-BRL (Gaithersburg, MD, U.S.A.).

Transfection with IL-5 anti-sense and sense oligonucleotides

To determine the autocrine role of IL-5 in the regulation of colony growth and eosinophilic lineage development, 2′-O-Methoxyethylchimeric antisense oligonucleotides were utilized for all experiments. The IL-5 antisense, sense and their mutant oligonucleotides contained phosphodiesterase linkage in the 2′-O-methoxyethyl regions and phosphorothioate linkage in the 2′-deoxynucleotide regions, which support RNase H activity in cells. All oligonucleotides were synthesized by Integrated DNA Technologics, Coralville, IA, U.S.A. NANT cells were washed three times with prewarmed (37°C) Opti-MEM. IL-5 antisense or sense oligonucleotides were premixed with 10 μg/ml Lipofectin reagent in Opti-MEM at the desired concentration and applied to washed NANT cells. To ensure that adequate numbers of NANT cells were available in each subject, CFU-GM assay was done in half the number of NANT cells (1×10^5 cells), as described above. NANT cells were incubated with the oligonucleotides for 6 h at 37°C, after which the medium was removed and replaced with standard growth medium.

Protocol

Since there were not enough progenitor cells from each subject for all experimental groups, each subject was randomly assigned for pre-planned experiments. In separate experimental groups, NANT cells were cultured in the presence or absence of exogenous human recombinant IL-5 (100 ng ml^{-1}; R & D Systems, Minneapolis, MN, U.S.A.), or anti-IL-5Rα monoclonal neutralizing antibody (2.5 μg ml^{-1}; Catalogue number AF-253-NA, R & D Systems, Minneapolis, MN, U.S.A.), or dexamethasone (10^{-6} M). In the studies with dexamethasone, NANT cells were incubated with either dexamethasone or with diluent alone, the latter acting as time control, for 16 h. To determine whether any residual T lymphocytes in NANT cell preparation could act as a source of IL-5, NANT cells cultured from 14 asthmatics and 14 normal subjects were compared with those co-cultured with autologous T lymphocytes (at 5×10^4 cells ml^{-1}) from the same study subjects.

Statistical analysis

Standard formulae were used for the analysis. Data did not approximate a Gaussian distribution, whereby the mean

value did not approximate the median value. Nonparametric statistical analyses were therefore employed, and the probability of differences between groups was initially assessed by Kruskall-Wallis analysis. The number of subjects in each group was too small to allow for a strict median test between groups, and subsequent analysis was performed using the Mann–Whitney *U*-test (2-tailed) to assess the significance of differences between groups. To minimize the possibility of obtaining chance significance as a result of multiple comparisons, comparisons between specific groups were made and significance confirmed using Newman-Keuls analysis. Data are represented as mean ± s.e.mean. The null hypothesis was rejected at $P<0.05$.

Results

Progenitor cells in peripheral blood

There was an increase in the proportion of IL-5 positive cells in the NANT fractions from patients with asthma (7.3 ± 1.0%, $n=8$, $P<0.01$) compared to normal subjects (2.6 ± 0.4%, $n=8$) (Figure 1). Using RT–PCR, we showed increased expression of IL-5 mRNA in NANT cells of asthma patients, but not that of normal subjects (Figure 2A). IL-5 expression could not be accounted for by any potential contamination with blood T cells since RT–PCR of purified blood T cells did not show detectable IL-5 mRNA (Figure 2). There was also no intracellular IL-5 staining in $CD34^+$ cells as measured by flow cytometry (data not shown).

As we have previously shown (Wang *et al*., 1999), there was an increase in the number of bone marrow-derived $CD34^+$ cells in the NANT fraction of the peripheral blood of patients with asthma (3.1 ± 1.8%, $n=8$ vs 0.9 ± 0.4% in normal subjects, $n=8$; $P<0.01$; Figure 1). By flow cytometry, there were increased proportions of $CD34^+$ cells (49.3 ± 10.4%, $n=8$) containing intracellular IL-5 in patients with asthma, as compared to that of normal subjects (20.5 ± 2.5%, $n=8$, $P<0.01$; Figure 3). In addition, there were more IL-5Rα positive cells in the NANT fraction in asthmatics (5.7 ± 0.9%, $n=8$) compared to normal subjects (1.8 ± 0.6%, $n=8$; $P<0.01$, Figure 3). There was a greater proportion of $CD34^+$ cells expressing IL-5Rα in asthmatics (34.6 ± 4.5%, $n=8$, $P<0.01$) than in normal subjects (12.4 ± 2.6%, $n=8$; Figure 3).

Eosinophil colony formation of peripheral blood NANT cells

There was a significantly greater number of colonies grown from NANT cells cultured in methylcellulose in the presence of growth factors (GM-CSF, SCF and IL-3) in asthmatic patients (252.7 ± 33.8 colonies, $n=10$) than in healthy subjects (86.3 ± 23.0 colonies, $n=10$, $P<0.001$) (Figure 4A). The colonies grown from asthmatics contained significantly more eosinophils (23.2 ± 2.1%, $n=10$) than those from normal subjects (3.9 ± 0.8%, $n=10$, $P<0.01$). To determine whether T cells in the NANT preparations contributed to the enhanced eosinophilic colony growth, a set of progenitor cell colony development from T cell depleted NANT cells was compared with cell preparations without T cell depletion (NA). There was no significant difference in the colony numbers or differential cell counts between NANT (255.4 ± 35.8 colonies, $n=14$) and NA cell culture (277.8 ± 30.9 colonies, $n=14$) (Figure 4B). The number of colonies in the presence of IL-5 (100 ng ml^{-1}) significantly increased in patients with asthma (427.3 ± 85.6, $n=6$) and in normal subjects (208.2 ± 24.5, $n=7$) compared to those cultured without IL-5 in patients with asthma (286.0 ± 48.6, $n=6$, $P<0.03$) and in normal subjects (144.5 ± 27.0, $n=7$, $P<0.05$), respectively.

Role of endogenous IL-5 in regulation of colony growth

To determine the role of endogenous IL-5, we also studied the effect of an anti-IL-5Rα blocking antibody which

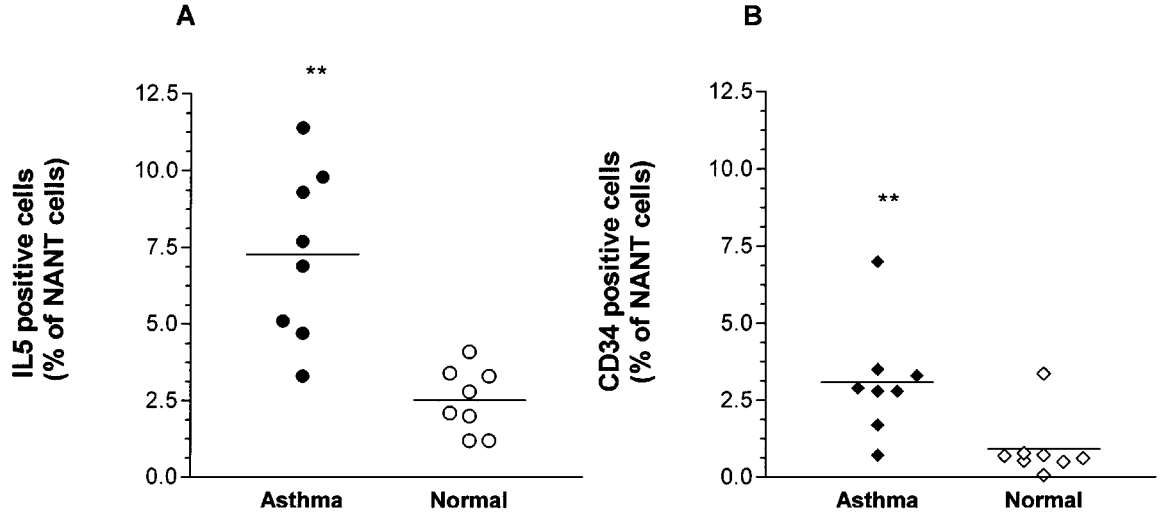

Figure 1 Increased proportion of $IL\text{-}5^+$ (A) and $CD34^+$ (B) cells in non-adherent non-T cell (NANT) preparation from asthmatic patients. Each individual point shows data from normal subjects (Normal, $n=7$) or patients with asthma (Asthma, $n=8$). There are significantly increased numbers of $IL5^+$ and $CD34^+$ cells in asthmatics. Horizontal bars show mean values. $^{**}P<0.01$ compared with corresponding normal subjects.

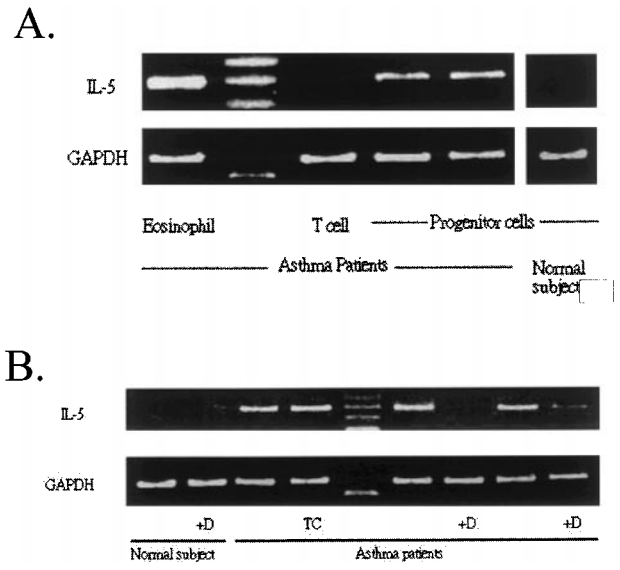

Figure 2 Expression of IL-5 mRNA in progenitor cells and effect of dexamethasone. (A) Agarose gels of PCR-amplified mRNA of IL-5 expression from peripheral blood eosinophils, T cells and progenitor cells from two patients with asthma and one normal subject. Lanes 1, 3, and 4 comes from the same asthmatic patient, while lane 5 is from a separate asthmatic. GAPDH was used as a housekeeping gene for comparison. The second lane is a DNA ladder. (B) IL-5 mRNA expression from progenitor cells from two patients with asthma (lanes 4, 5, 7 and 8 are from one asthmatic, and lanes 8 and 9 from a second) and a normal subject (lanes 1 and 2) in the presence or absence of dexamethasone (+D) or diluent time-control (TC) pretreatment for 16 h. Dexamethasone suppressed IL-5 expression. Lane 5 is a DNA ladder. GAPDH was used as housekeeping gene for comparison.

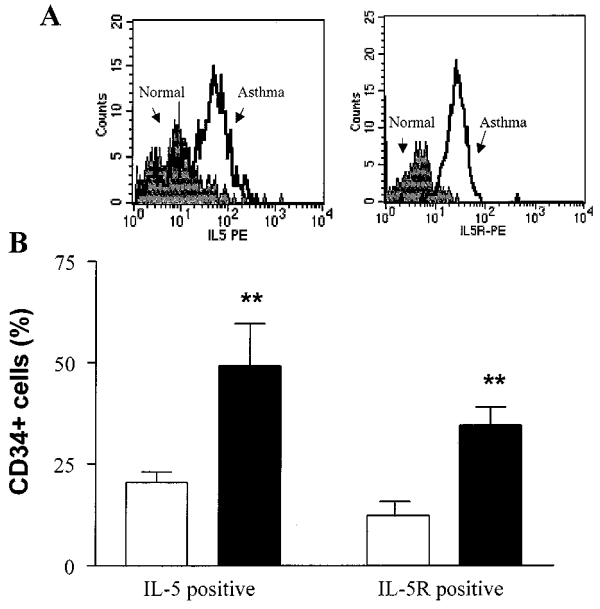

Figure 3 Proportion of $CD34^+$ cells expressing intracellular IL-5 or surface IL-5Rα. (A) Representative flow cytometry plots of $CD34^+$ cells expressing IL-5 or IL-5Rα from an asthma patient compared to a normal subject. (B) Both IL-5 and IL-5Rα expressions were significantly increased in patients with asthma (closed bars, $n=8$) compared to normal subjects (open bars, $n=8$). $^{**}P<0.01$ compared with normal subjects. Data are mean ± s.e.mean.

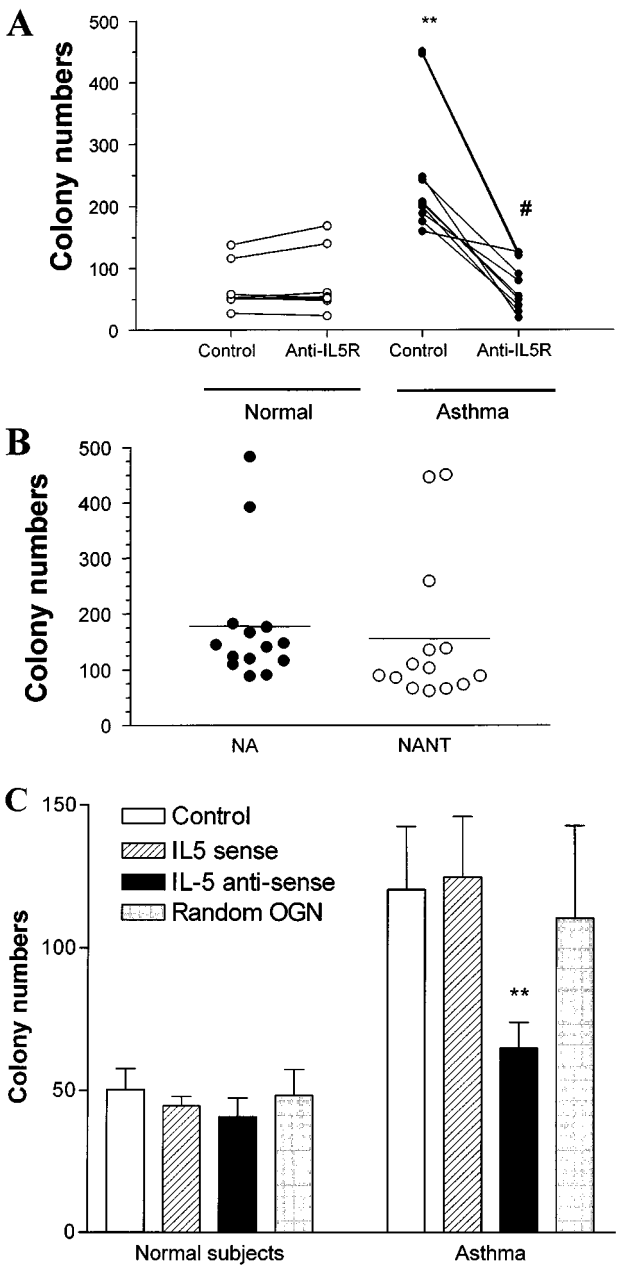

Figure 4 Effect of IL-5Rα antibody, contaminating T cells and anti-sense IL-5 oligonucleotides on colony numbers after 14 days culture of progenitor cells. (A) Effect of IL-5Rα receptor blocking antibody (anti-IL-5R) in normal subjects ($n=10$) or patients with asthma ($n=10$). Anti-IL5R antibody inhibited colony numbers in asthmatics. $^{**}P<0.01$ compared with normal subjects; $\#P<0.01$ compared with corresponding control. (B) Colony numbers from non-adherent non-T cells from asthmatics subjects ($n=14$) compared to those obtained from non-adherent cells containing T cells from the same donors. (C) There were no significant changes in progenitor cells from normal subjects ($n=8$); in patients with asthma ($n=6$), transfection with IL-5 anti-sense oligonucleotides (OGN) 6 h before culture inhibited colony formation. $^{**}P<0.01$ compared to those without transfection. Data are mean ± s.e.mean.

inhibited eosinophil colony-forming units in patients with asthma (from 252.7 ± 33.8 to 73.9 ± 12.9, $n=10$, $P<0.001$), but not in normal subjects (from 86.3 ± 23.0 to 87.5 ± 20.9, $n=10$, NS) (Figure 4A). There was no significant change in

the percentage of IL-5$^+$ or IL-5Rα^+ cells after 14 days of culture either in the presence or absence of IL-5, or of anti-IL-5Rα blocking antibody (Table 2).

Transfection of NANT cells (1×10^5 cells) with an anti-sense IL-5 oligonucleotide in patients with asthma for 6 h before culture caused an inhibition of eosinophilic colony formation by 66% (65.1 ± 8.9, $n=12$) compared to control (120.3 ± 22.3, $n=12$, $P<0.01$). Anti-sense IL-5 oligonucleotide decreased the proportion of eosinophils in eosinophil colony-forming units from 23.2 ± 2.1 to $11.9\pm1.7\%$). By contrast, the sense IL-5 oligonucleotide or random oligonucleotide had no effect on colony formation (124.7 ± 21.3, $n=12$, and 110.5 ± 32.3, $n=6$, respectively) or differential cell counts (Table 3). Neither anti-sense IL-5 oligonucleotide (40.8 ± 6.6, $n=6$) nor sense IL-5 oligonucleotide (44.6 ± 3.3, $n=6$) had any effect on colony formation in normal subjects when compared with their controls (50.3 ± 7.4, $n=6$; Figure 4C). The viability of NANT cells after transfection was greater than 95% in each treatment group.

Effect of corticosteroids

In the presence of dexamethasone (10^{-6} M), there was suppression of IL-5 mRNA expression as measured by RT–PCR, within 16 h of incubation (Figure 2B). By flow cytometric analysis, the number of NANT cells expressing intracellular IL-5 was significantly reduced to $4.2\pm1.4\%$ ($n=7$) in asthmatics, but not in normal subjects ($2.1\pm0.6\%$, $n=4$) compared to the corresponding time-controls ($6.9\pm2.9\%$, $n=7$, $P<0.03$; and $2.5\pm1.1\%$, $n=4$, respectively; Figure 5A). There was no significant effect of dexamethasone on the number of NANT cells expressing IL-5Rα either in asthmatics ($9.6\pm1.1\%$, $n=7$) or in normal subjects ($2.8\pm0.3\%$) when compared with their time controls ($10.2\pm1.1\%$, $n=7$ and $2.7\pm0.4\%$, $n=4$, respectively; Figure 5B). In order to determine whether the effect of corticosteroids may result from increased apoptosis of progenitor cells, NANT cells were incubated with dexamethasone (10^{-6} M), and apoptotic cells were measured by propidium iodide staining with flow cytometric analysis following 3 days of culture in suspension. Dexamethasone increased the number of apoptotic cells in NANT fraction from 7.0 ± 2.1 to $11.7\pm3.1\%$ ($n=7$, $P<0.02$) in asthmatics. By contrast, dexamethasone had no significant effect on cell apoptosis in normal subjects, as measured by propidium iodide staining ($2.7\pm0.8\%$ vs $2.4\pm0.7\%$, $n=5$). In the presence of dexamethasone (10^{-6} M), there were significantly increased total colony numbers grown from asthmatic (328.9 ± 82.3 colonies, $n=7$) and normal subjects (200.4 ± 19.2 colonies, $n=5$) compared to those without dexamethasone (260.5 ± 38.2 colonies in asthmatics, $n=7$, $P<0.03$; and 97.3 ± 14.0 colonies in normal subjects, $n=5$, $P<0.01$). Dexamethasone decreased the percentage of eosinophils from 23.2 ± 2.1 to $5.0\pm0.7\%$ in asthmatics ($n=7$, $P<0.01$), but not in normal subjects (from 3.9 ± 0.8 to $3.5\pm2.7\%$, $n=5$, NS). By contrast, dexamethasone significantly increased the proportion of neutrophils in patients with asthma and in normal subjects (Table 3).

To further examine the suppressive effect of dexamethasone on eosinophilic lineage, NANT cells were cultured on agarose medium for 14 days. Dexamethasone significantly inhibited the eosinophilic colony numbers from 89.7 ± 19.9 to 5.5 ± 3.1 ($n=5$, $P<0.01$) in asthmatics, but caused a non-significant increase in colony-forming numbers from 8.3 ± 2.6 to 10.4 ± 3.5 in normal subjects ($n=5$; Figure 6). Exogenous IL-5 (100 ng ml^{-1}) partially reversed the inhibitory effect of dexamethasone on eosinophilic lineage development by increasing the proportion of eosinophils from 5.0 ± 0.7 to $8.8\pm1.0\%$ ($n=7$, $P<0.02$). Eosinophil colonies were also increased from 4.8 ± 2.0 to 23.1 ± 3.9 ($n=7$, $P<0.01$; Figure 6). There was no significant effect of IL-5 on eosinophil colony development in normal subjects.

Table 2 IL-5 positive (IL-5$^+$) and IL-5 receptor positive (IL-5Rα^+) cells (%) in non-adherent and non-T cell preparation after culture for 14 days

	Control (%)	*IL-5*$^+$ (%)	*IL-5Rα* (%)
Normal ($n=6$)			
IL-5$^+$	2.4 ± 0.6	2.7 ± 0.5	2.5 ± 0.6
IL-5Rα^+	2.1 ± 0.7	2.2 ± 0.7	1.9 ± 0.9
Asthma ($n=6$)			
IL-5$^+$	7.4 ± 2.8*	7.7 ± 3.1*	7.2 ± 3.0*
IL-5Ra$^+$	6.0 ± 1.1*	5.8 ± 1.2*	6.1 ± 0.9*

*$P<0.01$ compared with corresponding group of normal subjects. Data are mean$\pm$s.e.mean.

Discussion

Our results demonstrate that circulating CD34$^+$ progenitor cells express both intracellular IL-5 and its receptor, IL-5Rα with a greater expression in cells from patients with asthma. IL-5 is uniquely able to promote the terminal differentiation and maturation of progenitors committed to the eosinophil/basophil lineage (Denburg *et al*., 1985b; Clutterbuck *et al*., 1989), and acts through the IL-5 receptor which is made of heterodimeric structures consisting of a low-affinity α–subunit, and a common shared β-subunit which forms high-affinity cytokine binding sites in association with the α-subunit (Tavernier *et al*., 1991). The cytoplasmic domain of the α-subunit is essential for signal transduction, mediating growth signals stimulated by (Takaki *et al*., 1994). Therefore, the concomitant expression of IL-5 and of IL-5Rα on CD34$^+$ cells indicates that there are specific binding sites for IL-5 on the same progenitor cell. Progenitor cells obtained from patients with asthma and cultured in semi-solid methylcellulose medium without IL-5 developed more eosinophilic colonies. Anti-IL-5Rα monoclonal antibody inhibited colony growth and differentiation into an eosinophilic lineage, suggesting that IL-5 endogenously synthesized in circulating progenitor cells is crucial for their proliferation, maturation and terminal differentiation into eosinophils. Thus, the increase in IL-5Rα receptor expression on CD34$^+$ cells in the blood of asthmatics may lead to a greater response to IL-5, leading to a greater number of progenitor cells maturing into eosinophils and basophils. Indeed, progenitor cells obtained from circulating blood of patients with asthma and cultured in the presence of IL-5 led to significantly greater colony numbers with an eosinophilic lineage. Interestingly, Tavernier *et al*. (2000) have recently shown that IL-5 expression in CD34$^+$ cells can drive IL-5Rα expression during *in-vitro* eosinophil development (Tavernier *et al*., 2000). This is in line with our finding that both IL-5 and IL-5Rα expression are increased in circulating CD34$^+$ cells obtained from asthmatic patients.

Table 3 Effects of dexamethasone and of anti-sense IL-5 oligonucleotides on colony-forming units from peripheral blood non-adherant non-T cells

	Blast	*Pro*	*Myelo*	*Meta*	*Band*	*Seg*	*Baso*	*Eos*	*Mono*
Normal									
Control ($n=8$)	65.7±3.1	1.9±1.1	6.1±0.6	4.2±0.2	0.1±0.1	0.3±0.1	1.0±0.3	3.9±0.8	20.7±2.8
Dex ($n=5$)	39.0±8.2**	4.8±1.8*	19.0±8.3**	2.9±0.8	9.9±1.8**	19.8±5.0**	2.3±1.2	3.5±2.7	2.2±0.6**
Sense ($n=6$)	64.5±4.1	2.4±1.4	6.7±1.1	0.4±0.3	0.2±0.1	0.4±0.1	0.9±0.4	3.5±0.6	21.1±3.3
Anti-sense ($n=6$)	64.7±2.7	2.8±1.0	6.8±1.1	0.2±0.1	0.3±0.1	0.4±0.1	0.7±0.4	4.5±1.0	19.7±3.5
Asthma									
Control ($n=12$)	44.4±1.9	5.9±1.7	6.8±2.5	1.8±0.5	2.0±0.5	2.2±0.6	1.1±0.3	23.2±2.1	13.0±1.3
Dex ($n=7$)	60.2±1.9**	3.9±0.8	8.6±0.7	5.4±0.8**	4.7±0.6**	2.9±0.8	0.3±0.2	5.0±0.7**	9.0±1.6*
Sense ($n=12$)	42.1±2.2	4.0±1.3	6.9±1.7	1.0±0.2	0.7±0.2	0.5±0.2	1.3±0.2	24.3±2.2	19.4±2.0
Anti-sense ($n=12$)	58.3±2.1**	2.6±0.9	7.7±1.9	1.2±0.5	0.7±0.2**	0.2±0.1**	1.2±0.2	11.9±1.7**	15.4±2.1

Pro, promyelocytes; Myelo, myelocytes; Meta, metamyelocytes; Seg, segmented granulocytes; Baso, basophil; Eos, eosinophils; Mono, monocytes; Dex, dexamethasone (10^{-6} M). Data presented as per cent and mean±s.e.mean. *$P<0.05$; **$P<0.01$ compared with corresponding control group.

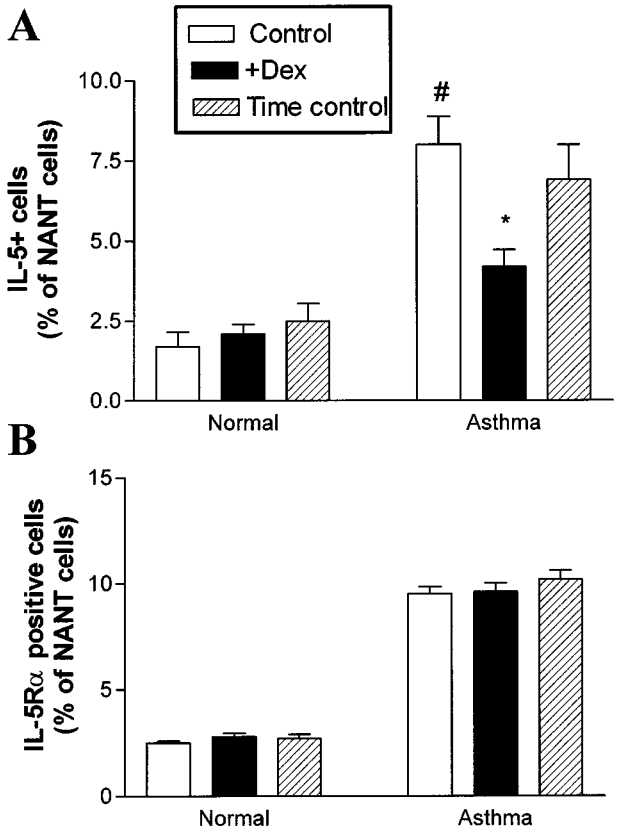

Figure 5 Effect of dexamethasone on expression of IL-5 and IL-5Rα in progenitor cells. Proportion of cells with intracellular IL-5 positive (A) and with surface expression of IL-5Rα (B) as detected by flow cytometry in non-adherent non-T cell preparation after 16 h pre-treatment with or without dexamethasone (10^{-6} M, Dex; +Dex or Time control) from patients with asthma (Asthma, $n=7$) and normal subjects (Normal, $n=4$). Dexamethasone significantly reduced IL-5 progenitor cells without affecting IL-5Rα$^+$ progenitor cells in asthmatic patients. #$P<0.01$ compared with normal subjects; *$P<0.05$ compared with corresponding time control ($n=4$). Data are mean±s.e.mean.

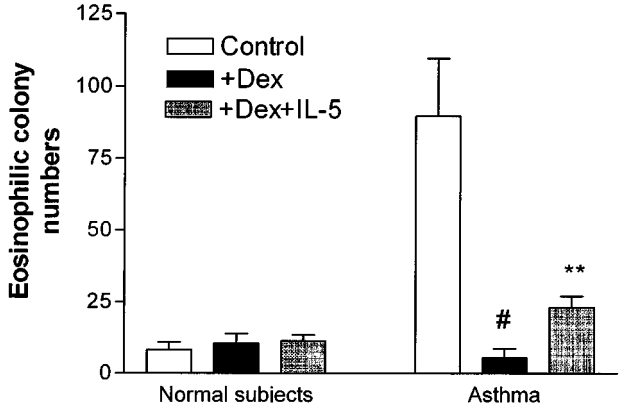

Figure 6 Effect of adding IL-5 on the inhibitory effect of dexamethasone on eosinophil colony forming numbers. Eosinophil colonies grown on agarose medium from progenitor cells of normal subjects ($n=5$) or patients with asthma (Asthma, $n=5$) with or without treatment of dexamethasone (10^{-6} M, Dex) as well as exogenous IL-5 (100 ng ml^{-1}). Dexamethasone inhibited eosinophil colony numbers, an effect partially reversed by exogenous IL-5 in cells from asthmatic patients. #$P<0.01$ compared with control; **$P<0.02$ compared with dexamethasone treatment group. Data are mean±s.e.mean.

We also showed that corticosteroids inhibit eosinophilic colony development by suppressing IL-5 mRNA expression and protein synthesis, but not IL-5Rα expression. Ectopic expression of IL-5R in multipotent progenitor cells induced by transfection with a retroviral vector containing IL-5Rα subunit did not increase eosinophilic development but stimulated survival and proliferation (Pierce *et al*., 1998). Our results are also consistent with the observations of Gauvreau *et al*. (2000) in that the inhaled corticosteroid, budesonide, taken by patients with asthma reduce the numbers of circulating eosinophil progenitors together with their expression of GM-CSF. In addition, inhaled corticosteroids also reduced the number of bone marrow eosinophil progenitors in the bone marrow of patients with asthma (Wood *et al*., 1999). We extend these effects of corticosteroids by demonstrating an inhibitory effect on eosinophil colony forming activity, through the inhibition of autocrine IL-5 expression. Our results also indicate that synthesis of IL-5 rather than IL-5Rα expression in progenitor cells plays a more important role in the development of eosinophilia in patients with asthma.

The cellular source of IL-5 in our NANT cell preparation is not completely elucidated in this study. Up to 50% of

peripheral blood $CD34^+$ cells express intracellular IL-5, accounting for approximately 25% of $IL\text{-}5^+$ cells. Because the NANT cell preparation is a mixture of progenitor cells at different stages of maturation, the other IL-5 positive cells may represent more mature progenitor cells. Although activated T lymphocytes, mast cells and mature eosinophils are potentially major cellular sources of IL-5 (Hamid *et al*., 1991; Bradding *et al*., 1994; Broide *et al*., 1992), we did not detect tryptase-positive cells or mature granulocytes in the NANT cell preparations. We do not believe that any contaminated T cells in the NANT preparations could have been an important source of IL-5 for the following reasons. Firstly, there was no detectable IL-5 protein or mRNA in purified T cell populations from the same patients. Secondly, the presence or absence of T cells did not affect progenitor cell colony formation. It is possible that the culture medium used for progenitor cells is not suitable for lymphocyte growth, since there were less than 1% of lymphocyte that survived after 24 h of culture in methylcellulose medium. Thirdly, T cells do not release cytokines without adequate exogenous stimulation (Bohjanen *et al*., 1990; Enokihara *et al*., 1989). Thus, IL-5 is mostly derived from progenitor cells but may also be expressed in other cells such as immature eosinophils. The autocrine role of IL-5 in progenitor cells was supported by the transfection of progenitor cells with antisense IL-5 oligonucleotide, which led to a significant inhibition of colony formation of cultured $CD34^+$ cells, as well as the development of the eosinophilic lineage. The autocrine role of IL-5 is only seen in progenitor cells obtanined from asthmatics, since there was no significant effect of IL-5Rα blocking antibody or antisense IL-5 oligonucleotide on colony growth of progenitor cells from normal subjects. The IL-5 positive cells are not always confined to IL-5Rα-positive cells, indicating that IL-5 synthesized from progenitor cells may also act in a paracrine fashion to regulate other progenitor cell colony growth.

Corticosteroids have been used for the treatment of asthma for the past 50 years, and inhibit the number of circulating eosinophils (Evans *et al*., 1993), as well as causing a reduction in the number of eosinophils in the airways submucosa (Djukanovic *et al*., 1992; Laitinen *et al*., 1992). Corticosteroids inhibit the number of circulating eosinophil progenitors and their expression of GM-CSF after allergen challenge in subjects with atopic asthma (Gauvreau *et al*., 2000). Corticosteroids also attenuate allergen-induced increases in blood eosinophils, and sputum eosinophils (Gauvreau *et al*., 1996). Inhaled corticosteroids inhibit the number of bone marrow $CD34^+$ cells, $CD34^+$ $IL\text{-}5R\alpha^+$ cells and IL-5 induced eosinophil-basophil colony forming units from cultured bone marrow $CD34^+$ cells (Wood *et al*., 1999). Our study demonstrates similar effects on blood $CD34^+$ cells in that corticosteroids inhibit the eosinophil colony forming units specifically in patients with asthma, but not in normal subjects, despite the increase in total colony-forming units. This inhibitory effect of corticosteroids, perhaps related to an increase in the number of apoptotic cells, is partially attributed to the inhibition of IL-5 expression that occurs at the mRNA level. Exogenous IL-5 partially reversed the inhibitory effect of corticosteroids on eosinophilic lineage development or colony formation in asthmatics but not in normal subjects. On the other hand, corticosteroids did not affect the expression of IL-5Rα. This also indicates that the expression of IL-5 and of IL-5Rα is independent of each other. These data confirm the role of endogenous generation of IL-5 by progenitor cells in prolonging cell survival and in the development of eosinophil lineage. Our results also reveal that the suppression of eosinophilia by corticosteroids may be partly attributed to their inhibition of progenitor cell numbers and of IL-5 expression. It is interesting that dexamethasone had no effect on eosinophil colony numbers or $IL\text{-}5^+$ number of cells cultured from NANT cells from normal subjects. This lack of effect is unlikely to be due to reduced corticosteroid receptor expression or corticosteroid receptor activation on $CD34^+$ progenitor cells. Rather, this is likely to represent low levels of IL-5 expression in these cells from normal subjects, and consequently no inhibition of such IL-5 low levels.

We have shown for the first time that $CD34^+$ cells in the blood of asthmatics express IL-5, using both intracellular staining techniques with an anti-IL-5 antibody, and RT–PCR to demonstrate the gene product. This is in accord with the demonstration of IL-5 mRNA in the bone marrow $CD34^+$ cells of mice following allergen challenge exposure (Minshall *et al*., 1998). We found that twice more $CD34^+$ cells from blood of asthmatics expressed IL-5 when compared to blood from non-asthmatics, and that the percentage of $CD34^+$ cells did not change with culture in agar or in the presence of the anti-IL-5Rα antibody. The essential role of IL-5 in the maturation of circulating $CD34^+$ cells into eosinophils has been demonstrated by the use of anti-sense IL-5 oligonucleotide which caused significant inhibition of colony-forming units. Our data show that the circulating $CD34^+$ cell in patients with asthma express both IL-5 and IL-5Rα, making this cell unique in terms of being capable of controlling its own maturation. These *in vitro* studies suggest that eosinophil maturation may occur within the circulation outside the bone marrow.

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