

ORIGINAL PAPER

Effect of inhaled interleukin-5 on eosinophil progenitors in the bronchi and bone marrow of asthmatic and non-asthmatic volunteers

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Summary

Background Asthma is characterized by increases in mature eosinophils and their progenitors within the bronchus and bone marrow. IL-5 plays a key role in eosinophil development in the bone marrow and at the site of allergic inflammation. We therefore studied the effects of nebulized IL-5 on eosinophils, their progenitors and *in situ* haemopoiesis within the airway and bone marrow.

Methods Nine atopic asthmatics and 10 non-atopic non-asthmatic control volunteers inhaled 10 µg of IL-5 or placebo via a nebulizer in a double-blind, randomized, cross-over study. Bronchoscopy, bone marrow aspiration and peripheral blood sampling were performed 24 h after nebulization. Four weeks later, volunteers inhaled the alternative solution and underwent a repeat bronchoscopy and bone marrow aspiration.

Results Inhalation of IL-5 significantly decreased CD34⁺/IL-5Rα mRNA⁺ cells within the bronchial mucosa and the percentage of CD34⁺ cells that were CCR3⁺ within the bone marrow of atopic asthmatic, but not control, volunteers. Inhalation of IL-5 also induced a significant increase in bronchial mucosal eosinophils in the non-atopic non-asthmatic control volunteers, but not in the asthmatics. IL-5 had no effect on spirometry or airways hyper-reactivity in either group.

Conclusions Inhaled IL-5 modulated eosinophil progenitor numbers in both the airways and bone marrow of asthmatics and induced local eosinophilia in non-asthmatics.

Keywords asthma, eosinophil, IL-5

Submitted 7 April 2006; revised 8 February 2007; accepted 29 March 2007

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Introduction

Eosinophilic infiltration of the bronchial mucosa has been demonstrated in patients with asthma [1]. Until recently, the eosinophil was thought of as a key effector cell in asthma pathogenesis – the eosinophil hypothesis of asthma. The ability of eosinophils to generate toxic products to fight parasitic infections was thought to contribute towards the inflammation associated with eosinophil accumulation at sites of allergic inflammation. However, the role of the eosinophil is in the balance following a study examining the lack of effect of targeted anti-eosinophilic therapy on clinical parameters of asthma [2]. Rather than eosinophils being responsible for a single facet or indeed all the features of asthma, it is more likely that they form part of a complex inflammatory process in which they favour some aspects of the pathophysiology, such as cough and genesis of exacerbations [3].

There is evidence that the eosinophilia observed within the airways is not an isolated phenomenon, but part of a systemic response involving the bone marrow [4], which in turn plays a role in producing and sustaining tissue eosinophilia. Increased numbers of mature and immature eosinophils are found in the bone marrow of atopic asthmatics compared with normal control individuals [5]. These findings, in combination with animal models [6], suggest that there is an increased bone marrow pool of eosinophils available for rapid mobilization in asthmatics.

Eosinophil differentiation and maturation from CD34⁺ progenitors occur largely within the bone marrow. An early stage in eosinophil development is up-regulation of surface expression of the IL-5 receptor α chain (IL-5Rα) on CD34⁺ cells [7]. The percentage of bone marrow CD34⁺/IL-5Rα⁺ cells increases 24 h after inhaled allergen challenge in dual responder (DR) asthmatics [8], suggesting a direct signal between the bronchial mucosa and bone

marrow that is able to increase eosinophil production at times of increased allergic inflammation. Up-regulation of CCR3 on CD34⁺ cells also occurs following allergen challenge in DR asthmatics [9], which may favour increased traffic of progenitor cells from the bone marrow to the peripheral circulation in response to eotaxin.

Once these progenitors have migrated to the site of allergic inflammation, they may differentiate into mature eosinophils; this hypothesis has been named *in situ* haemopoiesis. Evidence to support it includes increased numbers of CD34⁺/IL-5R α mRNA⁺ cells in the bronchial mucosa of asthmatics [10] and the presence of CD45⁺/CD34⁺ cells with eosinophil/basophil colony-forming activity in nasal polyp tissue [11].

IL-5 is a key cytokine for eosinophil differentiation and maturation in the bone marrow as well as in recruitment and activation at sites of allergic inflammation [12]. IL-5 stimulates the expansion and differentiation of eosinophil precursors [13] and up-regulates surface expression of its own specific receptor α -chain during early eosinophil development [7]. Clinical studies have shown an increase in IL-5 in bronchoalveolar lavage fluid (BALF) and bronchial biopsies in asthma at baseline [14]. Concentrations of IL-5 correlate with clinical features [15] and IL-5 mRNA is up-regulated in the bronchial mucosa after allergen-provoked asthma [16] and decreased after successful treatment with corticosteroids [17].

Two groups have previously examined the *in vivo* effects of local IL-5 delivered directly to the airways by the nebulized route. Shi *et al.* [18] reported that inhalation of 10 μ g of IL-5 induced a significant increase in sputum eosinophils and eosinophilic cationic protein (ECP), which was accompanied by an increase in airways hyper-reactivity (AHR). In a subsequent study, inhaled IL-5 produced a significant blood eosinophilia and increase in serum ECP levels 24 h after inhalation [19]. The results of this group were contradicted by a more recent study [20, 21] in which 15 μ g of IL-5 did not induce a change in blood or sputum eosinophils or AHR.

Given the presumed importance of IL-5 in eosinophil development, we have examined the effect of topical IL-5 delivered via a nebulizer on eosinophil progenitors and mature eosinophils both within the airway and bone marrow. In this study, we have compared the effects of topical IL-5 in atopic asthmatics and non-atopic non-asthmatic controls to determine any intrinsic difference in response to the cytokine between different patient groups. We hypothesized that inhalation of IL-5 would increase eosinophil numbers within the bronchial mucosa of asthmatics with no effect on the normal controls. We hypothesized that this tissue eosinophilia would be generated by a combination of *in situ* haemopoiesis and egress of both progenitors and mature eosinophils from the bone marrow.

Additional data from a study that we have already reported [22, 23] examining the effects of repeated dosing

with a monoclonal anti-IL-5 antibody (mepolizumab) in mild atopic asthmatics are also included as they provide extra evidence for the role of IL-5 within the bone marrow.

Methods

Volunteers

Nine volunteers with mild asthma, with a forced expiratory volume in 1 s (FEV₁) of 70% or more, predicted within an 18–55-year-old age range, were recruited. All were atopic as defined by one or more positive skin prick tests (SPTs) to the common aeroallergens. All were well controlled with short-acting β_2 -agonists, with no use of corticosteroids or other anti-inflammatory drugs in the preceding 8 weeks. All volunteers gave a clear history of asthma, demonstrated airway hyperresponsiveness with a histamine PC₂₀ of 4.0 mg/mL or less and were non-smokers.

Ten control volunteers were also recruited. The control volunteers were aged between 18 and 55 and were non-atopic, non-smokers with no evidence of AHR with a histamine PC₂₀ >16.0 mg/mL, and no history suggestive of asthma.

The study was approved by the ethics committee of the Royal Brompton and Harefield NHS trust and all volunteers gave informed consent before participation in the study.

Study design

This was a double-blind, randomized, placebo-controlled study. At an initial screening visit, baseline FEV₁ and histamine PC₂₀ were measured. At visit 2, subjects were randomly allocated to receive either 10 μ g of recombinant human IL-5 (Genzyme, Cambridge, MA, USA) in vehicle [0.1% human serum albumin (BPL, London, UK) in 0.9% saline] or vehicle only, which was inhaled as a 0.5 mL nebulized solution using a Pari LC star nebulizer (Pari Medical Ltd., West Byfleet, UK). The chamber was refilled once to remove any remaining IL-5. The IL-5 was shown to be > 98% pure by SDS-PAGE (Genzyme) and independent testing demonstrated < 0.01 EU lipopolysaccharide/10 μ g of IL-5 before use.

Spirometry was measured at baseline and 6 h after nebulization of IL-5 or placebo. Histamine PC₂₀ was measured 6 h after inhalation. Peripheral blood samples were taken at baseline, 6 and 24 h after nebulization. The following day, bone marrow aspiration and fiberoptic bronchoscopy with endobronchial biopsy and bronchoalveolar lavage (BAL) was performed. Four weeks after visit 2, the volunteers returned and received placebo if IL-5 was administered at visit 2 or vice versa. The pharmacodynamic measurements were repeated as per visits 2 and 3.

Clinical parameters

FEV₁ was measured according to a standard operating procedure on a bellows spirometer (Vitalograph, Bucks, UK). The histamine PC₂₀ was measured as described previously [24].

Processing of bronchial biopsy specimens

Biopsy specimens were immediately fixed in 4% paraformaldehyde (BDH, Leicester, UK) before being mounted in OCT medium (BDH) and snap-frozen in isopentane (BDH) pre-cooled in liquid nitrogen.

Immunohistochemistry

Six-micrometre sections were captured onto 0.1% poly-L-lysine-coated slides (BDH), and immunohistochemistry was performed using a modification of the alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique, as described previously [20]. In brief, mouse monoclonal antibodies against major basic protein (MBP; BMK-13, in house), CD68, neutrophil elastase, CD3 and CD34 (all Dako, Carpinteria, CA, USA) were used as the primary antibody under appropriate conditions. After incubation with a rabbit anti-mouse secondary antibody (Dako), APAAP (Dako) was added before development with fast red solution (Sigma-Aldrich, Gillingham, UK). Appropriate isotype controls were included in staining runs as a negative control.

In situ hybridization

The IL-5R α complementary DNA was used as a template for the synthesis of a 92-base pair ³⁵S-labelled riboprobe specific for the membrane-associated isoform, as described previously [8]. Negative controls used hybridization with the sense probe and pre-treatment of slides with Rnase A (Promega, Southampton, UK) before hybridization with the antisense probe.

Simultaneous in situ hybridization and immunohistochemistry

To examine the expression of IL-5R α mRNA by CD34⁺ cells, sections were first stained with an anti-CD34 monoclonal antibody as described above; they were then processed for *in situ* hybridization using the ³⁵S-labelled riboprobe for an IL-5R α membrane-associated isoform.

Quantification of immunohistochemistry and in situ hybridization

Slides were counted in duplicate in a blinded fashion using an eyepiece graticule. Results were expressed as the number of cells/mm² of bronchial biopsy tissue.

Bronchoalveolar lavage

The BAL specimen was obtained and cytopspins were generated as described previously [16]. BALF cytopspins were stained with a modified Wright's stain to enumerate eosinophil numbers. The cytopspins were stained in an automated fashion to ensure consistency using a Hema-Tek (Miles, Elkhart, USA) within the department of haematology at the Royal Brompton Hospital. Cytopspins were counted in a blinded fashion, a minimum of 200 cells were counted per cytopspin and the results were expressed as the percentage of eosinophils present.

Bone marrow aspiration

The bone marrow was aspirated from the posterior iliac crest and transferred to a sterile universal container with 1000 U of heparin. Before preparing cytopspins, red blood cells were removed by osmotic lysis with ice-cold distilled water, before resuspension in McCoy's 5A medium (Sigma-Aldrich) at a concentration of 0.5×10^6 cells/mL. Hundred microlitres of the cell suspension was inserted into each Shandon 2 cytopspin device (Shandon Inc., Pittsburgh, PA, USA) and the cells were captured onto 0.1% poly-L-lysine-coated slides. Cytopspins were stained using a Hema-tek within the department of haematology at the Royal Brompton Hospital and counted in a blinded fashion. Eosinophil numbers and the level of maturity (myelocyte, metamyelocyte and mature eosinophil) were determined as described previously [5] and expressed as a percentage of the total cells present. A minimum of 200 cells were counted per cytopspin.

Preparation of bone marrow and peripheral blood cells for quantification of CD34⁺/CCR3⁺ and CD34⁺/IL-5R α ⁺ cells

Low-density mononuclear cells (MNCs) were isolated by sedimentation on Histopaque 1.077 (Sigma-Aldrich) density gradients as described previously [8]. Monocytes were depleted from the MNC fraction by incubation in plastic flasks for 2 h at 37 °C. Samples of 1×10^6 non-adherent MNCs (NAMNCs) were incubated with saturating amounts of anti-CCR3 (7B11, a kind gift from Dr W. Newman, Leukosite, USA) or biotin-conjugated anti-IL-5R α (a kind gift from Dr Jan Tavernier, Ghent, Belgium) or appropriate isotype control antibodies for 30 min at 4 °C. Next, the cells were washed and stained with saturating concentrations of rat anti-mouse IgG_{2a+b} or streptavidin-conjugated PerCP (both Becton Dickinson, NJ, USA), respectively. Finally, the cells were washed and stained with saturating concentrations of anti-CD45 fluorescein isothiocyanate (FITC) and anti-CD34 PE (both Becton Dickinson). Cells were then washed before fixing in phosphate-buffered solution (PBS) and 1% paraformaldehyde. Samples were

analysed on a BD Facsan (Becton Dickinson) using a sequential gating strategy as described previously [8].

Peripheral blood eosinophils

Blood eosinophils were measured within the department of haematology at the Royal Brompton Hospital using an Advia 120 cell counter (Bayer, Berkshire, UK).

Methods for study 2

We have previously published a study examining the role of repeated high-dose anti-IL-5 (mepolizumab) therapy in mild atopic asthmatics [22, 23]. We include data from that study in this manuscript as it has not been previously published and is complementary to the findings of this study. Below is a brief overview of the relevant parts of the study:

Volunteers

Twenty-four mild asthmatic volunteers, with a FEV₁ of $\geq 70\%$ of the predicted value within an 18–55 age range were recruited. All volunteers were atopic as defined by a positive SPT to one or more aeroallergen. All volunteers were well-controlled on short-acting β_2 -agonist monotherapy, with no use of corticosteroids in the preceding 8 weeks. All volunteers demonstrated airway hyperresponsiveness with a PC₂₀ to histamine of ≤ 4.0 mg/mL and were non-smokers. The study was approved by the ethics committees of the Royal Brompton and Harefield NHS Trust and the London Chest Hospital, and all volunteers gave informed consent prior to participation in the study.

Study design

This was a two-centre, double-blind, placebo-controlled, parallel group study. At baseline, bone marrow aspiration

was performed. Two days later, the study medication (750 mg of mepolizumab or an equal volume of placebo) was given. This was administered as an intravenous infusion over 30 min in a double-blind fashion. The second and third infusions of the study drug were given 4 and 8 weeks following the first infusion. Bone marrow aspiration was repeated 2 weeks after the final drug infusion. Samples were analysed for CD34⁺/CCR3⁺ cells as described above. Eleven patients received active therapy and 13 placebo.

Statistical analysis

Non-parametric statistical methods were used for within-group paired comparisons (Wilcoxon' signed rank test). For between-group comparisons, the delta values of the effect of IL-5 and placebo or mepolizumab and placebo were compared (Mann–Whitney test). *P*-values of ≤ 0.5 were considered to be significant.

Results

Nine asthmatic and 10 non-asthmatic volunteers completed the study without reporting any adverse events. Inhalation of IL-5 and placebo was well tolerated by both the asthmatic and normal volunteers. There were no adverse events following bone marrow aspirate or bronchoscopy.

Airway eosinophils

At baseline, bronchial mucosal eosinophil numbers were significantly higher in the asthmatics than normal controls (*P*=0.002, Table 1). Inhalation of IL-5 induced a significant increase in eosinophil numbers in the bronchial mucosa of the normal volunteers 24 h after inhalation (*P*=0.049), but had no effect on eosinophils in the

Table 1. Bronchial mucosal inflammatory cell and bronchoalveolar alveage eosinophil numbers sampled 24 h after inhalation of IL-5 or placebo in the asthmatic (*n* = 9) and normal (*n* = 10) volunteers; numbers in bold are statistically significant

	Asthmatics			Normals			A vs. NA
	Placebo median (range)	IL-5 median (range)	<i>P</i> -value	Placebo median (range)	IL-5 median (range)	<i>P</i> -value	
Eosinophils (MBP ⁺ cells/mm ²)	15.7 (4.9–86.24)	35.8 (1.2–68.6)	0.93	4.1 (0.0–29.4)	12.25 (1.6–24.5)	0.049	0.002
Progenitors (CD34/IL-5R α mRNA ⁺ cells/mm ²)	17.09 (6.1–27.2)	9.24 (2.29–15.32)	0.012	5.87 (0.64–13.49)	9.55 (0.97–35.2)	0.13	0.004
Basophils (BB1 ⁺ cells/mm ²)	0.0 (0.0–18.38)	0.0 (0.0–1.23)	0.16	0.0 (0.0–3.5)	0.0 (0.0–0.0)	–	0.9
Neutrophils (elastase ⁺ cells/mm ²)	43.12 (27.44–88.2)	31.4 (18.38–125)	0.82	57.07 (6.53–100.5)	68.25 (36.4–158.4)	0.11	0.21
Macrophages (CD68 ⁺ cells/mm ²)	33.85 (8.58–120.1)	64.52 (23.28–232.8)	0.25	102.5 (46.55–196)	94.33 (66.15–129.9)	0.92	0.9
Lymphocytes (CD3 ⁺ cells/mm ²)	101.7 (49.8–372.4)	107.8 (34.3–263)	0.91	117.8 (0.0–260.5)	73.0 (49.0–362.6)	0.43	0.78
BALF eosinophils (% of total cells)	1.0 (0.0–6.5)	0.5 (0.0–4.5)	0.94	0.0 (0.0–1.5)	0.0 (0.0–0.5)	0.5	0.09

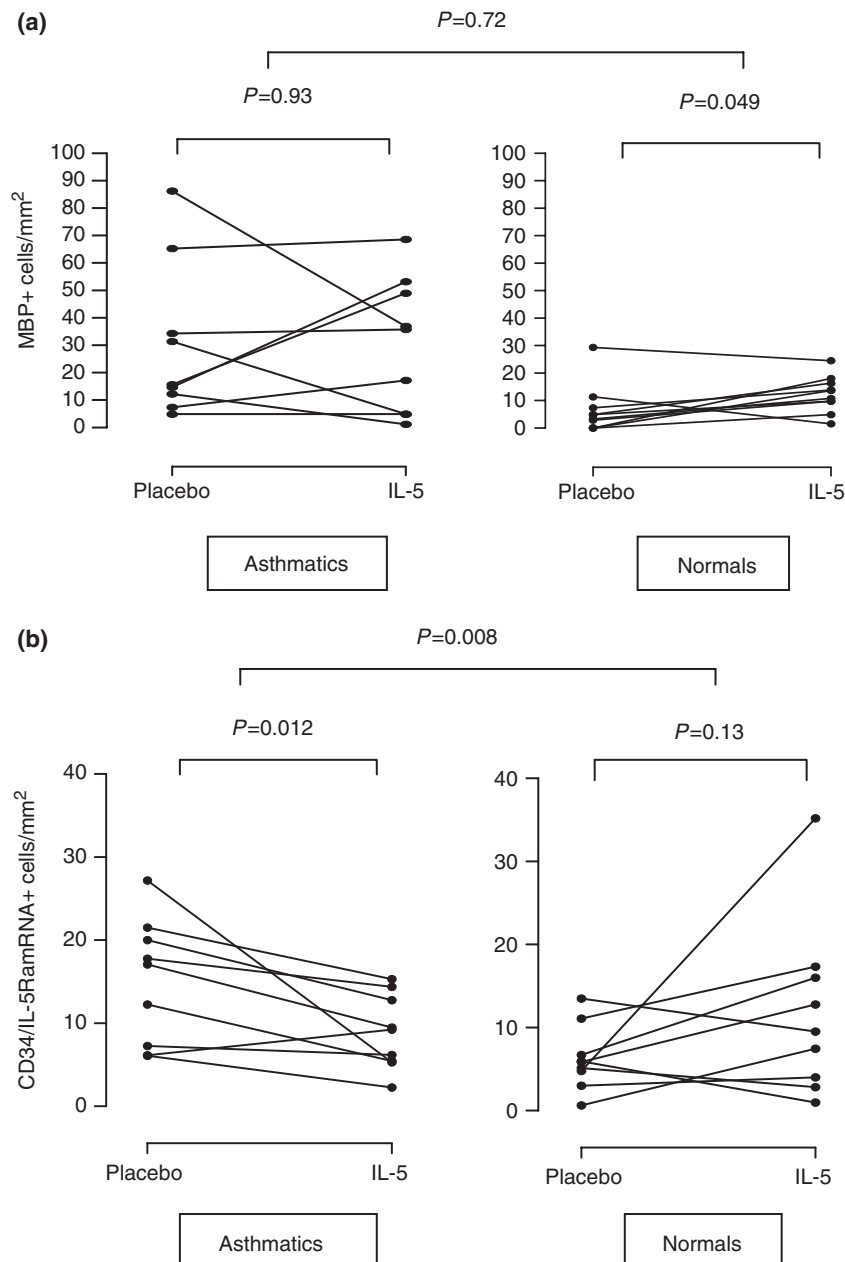


Fig. 1. Effects of IL-5 inhalation on (a) eosinophils, and (b) CD34⁺/IL-5Rα mRNA⁺ cells in the bronchial mucosa of asthmatics and normal controls.

asthmatics ($P=0.93$) (Fig. 1a and Table 1). The number of eosinophils within the BALF of both asthmatics and normals was unaffected by IL-5 inhalation (Table 1). There was, however, a trend towards higher eosinophil numbers in the asthmatics at baseline ($P=0.09$) (Table 1).

Bronchial mucosal CD34/IL-5Rα mRNA⁺ cells

At baseline, bronchial mucosal CD34/IL-5Rα mRNA⁺ cell numbers were significantly higher in the asthmatic volunteers when compared with the normal controls

($P=0.004$, Table 1). Twenty-four hours after inhalation of IL-5, there was a significant decline in the number of CD34/IL-5Rα mRNA⁺ cells within the bronchial mucosa of the asthmatic volunteers ($P=0.012$), with no effect on the numbers of these cells in the normal volunteers (Fig. 1b and Table 1).

Other inflammatory cells within the bronchial mucosa

In both the asthmatic and normal volunteers, IL-5 inhalation had no effect on the numbers of basophils,

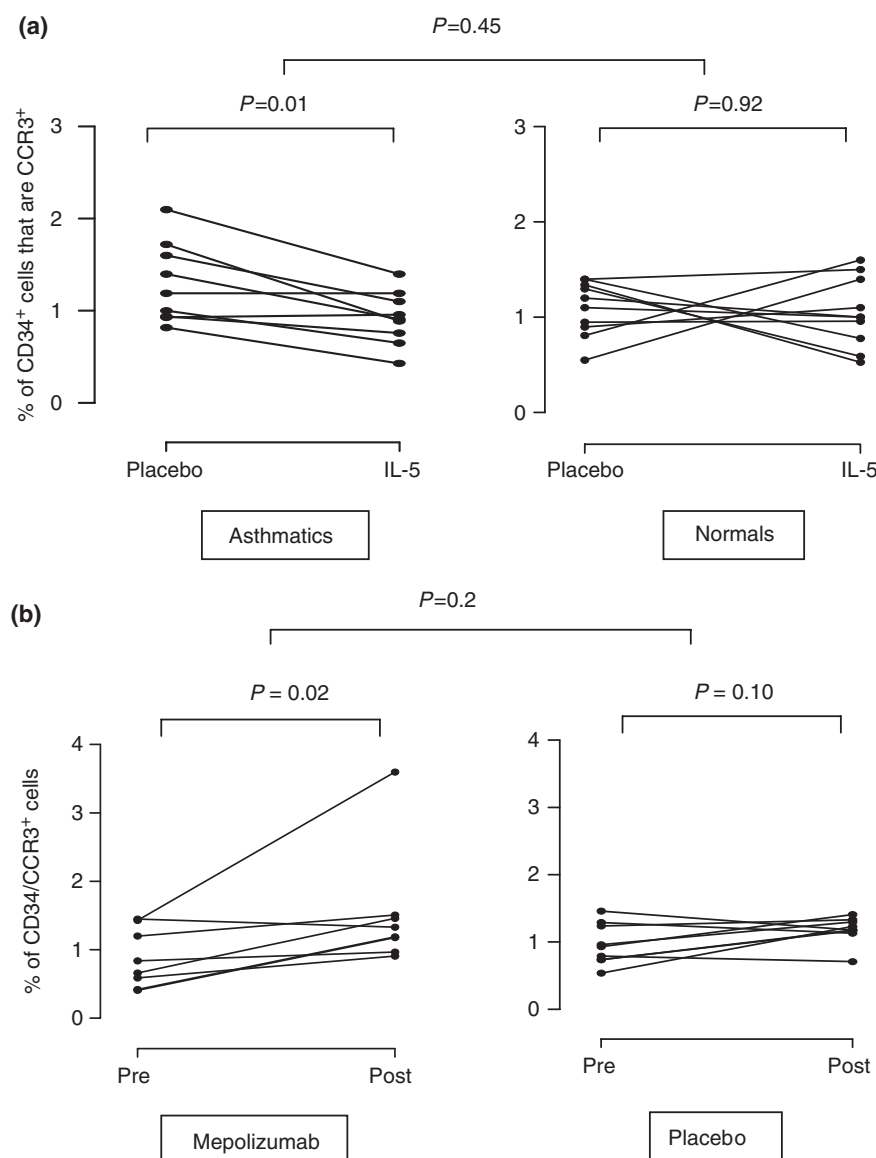


Fig. 2. Percentage of bone marrow CD34⁺ cells that are CCR3⁺ in asthmatics after (a) inhalation of IL-5, and (b) mepolizumab therapy.

neutrophils, macrophages or CD3⁺ cells within the bronchial mucosa (Table 1).

Bone marrow-derived CD34⁺, CD34/IL-5R α ⁺ and CD34/CCR3⁺ cells

Inhalation of IL-5 significantly decreased the percentage of CD34⁺ cells within the bone marrow of atopic asthmatics who were CCR3⁺ 24 h after inhalation (Fig. 2a and Table 2). In parallel with this finding, prolonged therapy with a monoclonal antibody against IL-5 (mepolizumab) in 11 mild asthmatics [20] induced a significant increase in the percentage of CD34⁺ cells that were CCR3 positive (Fig. 2b).

Inhalation of IL-5 had no effect on total CD34 numbers or the percentage of CD34⁺ cells that were IL-5R α ⁺ in both asthmatic and normal volunteers (Table 2).

Bone marrow-derived eosinophil myelocytes, metamyelocytes and mature eosinophils

There were significantly higher numbers of both mature ($P=0.02$) and total ($P=0.01$) eosinophils in the bone marrow of the asthmatic volunteers compared with the normal controls at baseline (Table 2). The numbers of eosinophil myelocytes, metamyelocytes and mature eosinophils were not affected by IL-5 inhalation in either volunteer group (Table 2).

Table 2. Bone marrow eosinophil and progenitor levels twenty four hours after inhalation of IL-5 or placebo in the asthmatic ($n = 9$) and normal ($n = 10$) volunteers

	Asthmatics			Normals			A vs. NA
	Placebo median (range)	IL-5 median (range)	<i>P</i> -value	Placebo median (range)	IL-5 median (range)	<i>P</i> -value	<i>P</i> -value
Bone marrow CD34 ⁺ cells/10 ⁶ NAMNC	28550 (10440–61530)	34120 (11860–42940)	0.65	32100 (18200–41370)	30370 (18200–41370)	0.625	0.72
% of bone marrow CD34 ⁺ cells that are IL-5Rα ⁺	1.2 (0.78–1.95)	0.95 (0.66–3.1)	0.74	1.15 (0.53–2.8)	1.05 (0.43–1.6)	0.85	0.78
% of bone marrow CD34 ⁺ cells that are CCR3 ⁺	1.19 (0.82–2.1)	0.91 (0.43–1.4)	0.01	1.15 (0.55–1.4)	1.0 (0.53–1.6)	0.92	0.45
Bone marrow eosinophil myelocytes (% total cells)	1.5 (0.0–2.5)	1.0 (0.0–2.5)	0.69	1.0 (0.0–2.5)	1.0 (0.0–2.5)	0.94	0.55
Bone marrow eosinophil metamyelocytes (% total cells)	0.5 (0.0–2.5)	0.0 (0.0–1.5)	0.47	0.0 (0.0–1.5)	1.0 (0.0–3.0)	0.11	0.97
Bone marrow mature eosinophils (% total cells)	4.0 (1.5–13.0)	3.5 (2.5–5.0)	0.55	2.5 (0.0–5.5)	2.0 (1.0–4.0)	0.74	0.02
Bone marrow total eosinophils (% total cells)	6.0 (4.0–13.5)	5.5 (2.5–6.5)	0.14	3.0 (0.5–8.0)	4.0 (2.0–7.5)	0.3	0.01

Peripheral blood eosinophils and CD34⁺, CD34/IL-5Rα⁺ and CD34/CCR3⁺ cells

IL-5 inhalation had no effect on the numbers of CD34⁺, CD34/IL-5Rα⁺ or CD34/CCR3⁺ cells in the peripheral blood of both normal and asthmatic volunteers (results not shown).

IL-5 inhalation had no effect on eosinophil numbers in the peripheral blood of either the asthmatics or normals at 6 or 24 h after inhalation (results not shown).

Spirometry and airway hyper-reactivity

IL-5 had no effect on airways' calibre at any of the time-points at which it was measured in either volunteer group. In the asthmatic volunteers, the median FEV₁ at baseline was 2.85 and 2.8 L 24 h after IL-5 inhalation ($P = 1.0$). In the non-asthmatic volunteers median FEV₁ at baseline was 3.7 and 3.6 L 24 h after IL-5 inhalation ($P = 1.0$). Likewise, IL-5 had no effect on AHR when histamine PC₂₀ was measured at baseline and 6 h after inhalation, median 2.0 and 2.6 in the asthmatics, respectively, ($P = 1.0$) and > 16 at both time-points in the normal volunteers.

Discussion

We have demonstrated for the first time that inhalation of IL-5 induces a significant increase in eosinophils within the bronchial mucosa of non-atopic, non-asthmatic individuals, as well as modulating eosinophil progenitor numbers in both the airways and bone marrow of atopic asthmatics.

The discrepancy between the local effects of IL-5 on bronchial mucosal eosinophil numbers in normal volunteers

and asthmatics may be explained by baseline IL-5 levels. Asthmatic individuals have elevated IL-5 levels within their airways, which already enhance eosinopoiesis [14]; the increase produced by 10 µg of nebulized IL-5 may be insufficient to further increase eosinophil levels. In contrast, inhalation of 10 µg of IL-5 may significantly increase local levels in non-atopic, non-asthmatics.

The significant decline in eosinophil progenitor numbers (CD34/IL-5Rα mRNA⁺ cells) within the bronchial mucosa of the asthmatic volunteers 24 h after inhalation of IL-5 is consistent with the nasal explant work of Cameron et al. [25]. In this *in vitro* study, incubation of mucosal tissue with IL-5 for 24 h significantly decreased the number of CD34/IL-5Rα mRNA⁺ cells, with a concomitant increase in the number of MBP⁺ cells. There was no significant increase in the number of MBP⁺ eosinophils within the bronchial mucosa in our study; however, we only examined the bronchial mucosa at one time-point and therefore may simply have missed the increase in eosinophil numbers. Alternatively, the progenitors may not have differentiated into mature cells, but migrated into the airway lumen in response to the chemokinetic activity of IL-5. Dorman et al. [26] have demonstrated an increase in CD34/IL-5Rα⁺ cells within the BALF of DR asthmatics 24 h after allergen challenge. They also showed that bone marrow IL-5 protein concentration increased at 12 and 24 h in DRs after allergen challenge [27].

The lack of effect of IL-5 inhalation on the number of neutrophils and macrophages is not unexpected and confirms the selectivity of IL-5. The lack of effect of IL-5 on basophils is more unexpected, given previous reports of IL-5 responsiveness of these cells [28]. However, basophils are less numerous in the airway infiltrate than eosinophils in asthma [29] and they predominantly

express IL-3 receptors [30]. Nebulized IL-5 had no effect on the number of total T cells (CD3⁺ cells) within the asthmatic airway; we did not examine T cell subsets and it is possible that IL-5 inhalation may have an effect on the number or functional activity of T-helper type 2 (Th2) cells within the airway.

The effect of IL-5 on the percentage of bone marrow-derived CD34⁺ cells that are CCR3⁺ is novel and supported by the finding that IL-5 blockade with mepolizumab increases CCR3 positivity within the same subpopulation. IL-5 has been shown to stimulate eosinophil release from the bone marrow of guinea-pigs [6] and it is plausible that IL-5 selectively promotes the release of progenitors that will then home to the site of allergic inflammation via the CCR3/eotaxin axis. The very low numbers at any one time in transit in the peripheral blood, in conjunction with the limited number of sampling time-points, may explain why we were unable to demonstrate any increase in CD34/CCR3⁺ cells within the systemic circulation. The nature of the signal that produces these changes in CD34/CCR3⁺ cell numbers has not yet been defined; it may be a direct signal following systemic absorption of the nebulized IL-5. Alternatively, nebulized IL-5 may provide a signal to cells within the bronchial mucosa that traffic to the bone marrow where they locally induce the efflux of CD34/CCR3⁺ cells.

The finding of increased eosinophil numbers in the bone marrow of asthmatic subjects compared with normal controls is consistent with earlier work [5] and supports the concept that the bone marrow plays a key role in perpetuating allergic inflammation. The lack of effect of IL-5 inhalation is perhaps surprising, given the significant increases observed in IL-5 transgenic mice [31]. This negative result is likely to represent the significant difference in the magnitude of the signal between the topical instillation of IL-5 in our volunteers and the systemic overexpression seen in transgenic mice.

Unlike inhaled allergen in DR asthmatics [8], IL-5 inhalation had no effect on CD34/IL-5R α ⁺ cells or more advanced eosinophil progenitors either within the bone marrow or peripheral circulation; this can be explained by the difference in magnitude of the two signals. Allergen inhalation induces mast cell degranulation and the release of a wide range of inflammatory mediators, which will have a more significant effect than inhalation of a single cytokine. We also did not reproduce the observation of a doubling of CD34⁺ cells within the peripheral blood of asthmatics 24 h after IL-5 inhalation [20]. This may be due to the lower dose used of 10 μ g compared with 15 μ g in the earlier study.

We have demonstrated that inhalation of IL-5 had no effect on spirometry in both asthmatics and normal controls. We have also shown that despite an increase in bronchial mucosa eosinophil numbers in the normal volunteers there was no concomitant increase in AHR,

suggesting that eosinophils may be necessary, but not sufficient in themselves to induce AHR. This hypothesis is supported by work using IL-5 gene transfer to the lungs of naïve mice, which induced a pronounced and selective airway eosinophilia, but did not result in eosinophil degranulation or AHR [32].

Our findings were not in agreement with previous studies investigating the *in vivo* properties of IL-5 [18, 19]. In the first study, inhalation of 10 μ g of IL-5 induced a significant increase in sputum eosinophils and ECP, which was accompanied by an increase in AHR [18]. In a subsequent study, inhaled IL-5 produced a significant blood eosinophilia and rise in serum ECP levels 24 h after inhalation [19]. We did not observe a blood, BAL or bronchial mucosal eosinophilia in atopic asthmatics administered the same dose of IL-5. We also did not find any increase in AHR following IL-5 inhalation. This apparent discrepancy is difficult to explain; however, supporting evidence for our mostly negative data comes from the study reported by Stirling *et al.* [20] and van Rensen *et al.* [21] in which there was also no change in blood or sputum eosinophils or AHR following IL-5 inhalation.

One possible explanation for this discrepancy between studies is a difference in either the level of baseline airway eosinophilia or some other genetic difference between the Chinese and British populations. There was a sputum eosinophilia of 6% in the Chinese volunteers compared with 1% in the previous British study. We did not measure sputum eosinophils in our study population. At baseline, the level of AHR was comparable between the three studies: mean PC₂₀ of the Chinese group 0.9, British group 1.15 and our study 2.95 mg/mL. An alternative explanation is that inhalation of 10 μ g of IL-5 is an insufficient dose in the population that we studied to have a systemic effect. This is supported by the finding that 15 μ g of nebulized IL-5 did not produce a significant increase in serum IL-5 levels 30 min after inhalation [20].

The key importance of circulating over local lung IL-5 has been demonstrated in an animal model of ovalbumin-induced airways' eosinophilia [33]. The authors demonstrated that IL-5 knockout mice fail to develop blood and airway eosinophilia after challenge. Intramuscular IL-5 gene transfer led to increased systemic IL-5 levels and reconstitution of the peripheral blood and airways eosinophilia. In contrast, intranasal IL-5 gene transfer was unable to reconstitute the eosinophilia in any compartment. This is supported by the findings of Stirling *et al.* [20], who demonstrated that 2 μ g of intravenous IL-5 produced a prolonged and significant blood eosinophilia and increased the number of CD34⁺ cells within peripheral blood. The critical importance of circulating IL-5 may explain why we did not see any changes in many of the stages of eosinophil development both in the bone marrow and peripheral blood following inhalation of IL-5.

In conclusion, inhalation of IL-5 induces bronchial mucosal eosinophilia in non-asthmatics and modulates eosinophil progenitors both within the airway and bone marrow of asthmatics, without any concomitant effect on airways calibre or AHR. This provides further evidence that IL-5 plays an important role in eosinophilic inflammation *in vivo*, although it is unlikely to be the sole determinant of airways eosinophilia and AHR in asthma.

Acknowledgement

The work was supported by the Wellcome Trust (UK).

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