

Kinetics of Bone Marrow Eosinophilopoiesis and Associated Cytokines after Allergen Inhalation

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Allergen inhalation is associated with increased eosinophil/basophil progenitors in bone marrow 24 hours after allergen inhalation. This study examined the kinetics of eosinophilopoiesis in dual ($n = 14$), compared with isolated early, responders ($n = 12$). Dual responders, in contrast to isolated early responders, develop significant sputum and blood eosinophilia and prolonged airway hyperresponsiveness. Bone marrow aspirates were taken before and 5, 12, 24, and 48 hours after allergen inhalation. In dual responders, increases in interleukin (IL)-3-responsive progenitors were detected as early as 5 hours after allergen inhalation, and IL-5-responsive progenitors were detected at 12 and 24 hours. No changes were detected in isolated early responders. Bone marrow IL-5 protein levels increased at 12 and 24 hours in dual responders only and these increases correlated with increases in IL-5-responsive progenitors. In addition, bone marrow IFN- γ levels increased in dual responders at 48 hours. These data demonstrate that, in dual responders, there is allergen-induced activation of an eosinophilopoietic process that is rapid and sustained, and a relationship between increased bone marrow IL-5 levels and increased eosinophil production. We propose that after allergen inhalation, time-dependent changes in cytokine levels in the bone marrow control differentiation of eosinophil/basophil progenitors.

Keywords: allergy; cellular differentiation; eosinophils; human; lung

Asthma is recognized by the presence of reversible bronchoconstriction, airway hyperresponsiveness (AHR), and airway inflammation. Environmental allergens are an important cause of asthma and can be studied in the laboratory by allergen inhalation challenge. Allergen inhalation challenge in sensitized subjects typically induces an immediate bronchoconstriction (the early asthmatic response), which is maximized within 30 minutes and resolves between 1 and 3 hours. A proportion of subjects will proceed to develop a second, delayed bronchoconstrictor response (the late asthmatic response), which is associated with prolonged AHR and pronounced airway eosinophilia (1–4). Although in a given individual the development of either a single or a dual response is generally consistent, it is believed that the development of the late response occurs as a continuum within and between individuals, largely on the basis of the dose of allergen inhaled (5). The development of the late response and associated increase in airway responsiveness results in a lowering of the threshold for subsequent allergen exposure, setting up a vicious circle for continuing allergic inflammation in the airways (6, 7).

The size of the late response and the increase in AHR have been shown to correlate with the degree of airway eosinophilia (8). Likewise, studies comparing numbers of circulating eosinophils have shown significant increases 24 hours after allergen inhalation in dual responders only (9–11). Eosinophils, through their ability to release granule-associated proteins, including proinflammatory lipid mediators and cytokines, are considered to play a central role in the development of airway inflammation leading to the production of the late asthmatic response. This role, however, is based on circumstantial evidence and identifying mechanism(s) contributing to the development of tissue eosinophilia will provide a better understanding of the importance of this cell in asthma (12–14).

There is now substantial evidence supporting the view that activation of specific hematopoietic pathways within the bone marrow is associated with allergen-induced eosinophilic airway inflammation. Early studies demonstrated higher numbers of circulating eosinophil/basophil colony-forming units (Eo/B-CFU) and CD34⁺IL-5R α ⁺ (interleukin [IL]-5 receptor-positive) cells 24 hours after allergen inhalation in dual responders (15, 16). In addition, bone marrow IL-5-responsive Eo/B-CFU were shown to increase 24 hours after allergen inhalation only in dual responders (17). The coinciding increases in both eosinophils and eosinophil progenitors, in dual responders only, has led to the hypothesis that the bone marrow hematopoietic processes are an essential component to the persistence of airway eosinophilia. As such, helper T cell Type 2 cytokines, with the ability to promote the terminal differentiation and maturation of eosinophil-committed lineages, are likely important in promoting eosinophilopoiesis within the bone marrow. In agreement with this, we have shown significant increases in the number CD3⁺ cells expressing IL-5 mRNA in the bone marrow of dual responders compared with isolated early responders 24 hours after allergen inhalation (18). In addition, Sehmi and coworkers have demonstrated a significant increase in the number of bone marrow-derived CD34⁺ cells expressing IL-5R α 24 hours after allergen inhalation challenge in dual, but not isolated early, responders (16). Together these results suggest that an IL-5-driven phenotypic switch in the bone marrow environment occurs in dual responders only, favoring eosinophil production and contributing to the development of blood and airway eosinophilia. On the basis of this view, it is plausible that, in isolated early responders—who do not develop a significant or sustained eosinophilic response—cytokines such as IFN- γ and IL-10 may be produced that downmodulate the helper T cell Type 2 environment and therefore prevent or ameliorate the development of airway eosinophilia.

The aims of the current study were twofold. The first was to investigate whether the short-lived airway eosinophilic response mounted by early responders is due to a limited activation of bone marrow eosinophilopoietic processes. For this reason, we examined the changes in bone marrow eosinophilopoiesis, before and 5, 12, 24, and 48 hours after allergen inhalation, both in isolated early and dual responders. The second was to investigate whether any observed differences in bone marrow responsiveness

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between the two groups is due to the differences in bone marrow cytokines, including the cytokines necessary for eosinophilopoiesis (IL-5 and IL-3) and the downregulatory cytokines (IFN- γ and IL-10). Overall, understanding how these changes relate to AHR and airway inflammation will help to understand the importance of bone marrow eosinophilopoiesis in the persistence of allergen-induced airway responses.

These results have been previously reported in abstract form (19).

METHODS

Subjects/Design

Twenty-six patients with mild, stable, atopic asthma with baseline FEV₁ > 70% predicted were studied (Table 1). Patients were screened and, after allergen inhalation, isolated early responders (n = 12) developed an early fall in FEV₁ > 20% predicted from baseline between 0 and 2 hours, whereas dual responders developed an early and late fall (FEV₁ > 15% predicted) from baseline between 3 and 7 hours (n = 14). Subjects were nonsmokers, using inhaled β_2 -agonists intermittently (withheld 8 hours before each visit), and had not experienced respiratory infection or were exposed to altered allergen levels 2 weeks before allergen inhalation. One subject withdrew because of discomfort from aspiration. Six additional subjects were studied to examine the direct effects of IFN- γ on Eo/B-CFU. This study was approved by McMaster University Health Sciences Centre Ethics Committee and subjects gave written, informed consent before enrollment.

Because of difficulty in obtaining five consecutive bone marrow aspirates, the study was randomized into phases. In Phase 1, aspirates and blood were taken at baseline and at 5 and 24 hours, sputum was induced at 7 and 24 hours, and methacholine PC₂₀ (provocative concentration inducing a 20% fall in FEV₁) was measured 24 hours after allergen inhalation. In Phase 2, aspirates and blood were taken at baseline and at 12 and 48 hours, sputum was induced at 7, 24, and 48 hours, and methacholine PC₂₀ was measured 24 and 48 hours after allergen inhalation. Phases were separated by 1 month.

TABLE 1. SUBJECT CHARACTERISTICS

	Age (yr)	Sex	FEV ₁ (% Predicted)	Allergen (Inhaled Dilution)
IER				
2	29	F	101.2	Cat (1:8)
3	24	F	85.3	Cat (1:32)
5	30	F	93.9	HDM (1:16)
6	26	M	117.1	RW (1:16)
8	24	F	92.2	Grass (1:1,024)
10	23	M	98.6	Cat (1:64)
14	51	F	80.2	Cat (1:8)
15	21	F	98.9	Cat (1:64)
18	27	F	98.7	Grass (1:512)
20	19	F	80.7	HDM (1:16)
23	23	M	91.4	HDM (1:256)
25	39	M	78.5	HDM (1:512)
Mean $\pm$ SEM	27.8 $\pm$ 2.37	8:4	93.1 $\pm$ 3.18	
DR				
1	26	F	74.2	RW (1:512)
4	23	F	93.1	HDM (1:64)
7	26	M	85.1	Cat (1:16)
9	24	M	77.8	RW (1:128)
11	23	F	97.3	HDM (1:256)
12	52	M	90	Cat (1:64)
13	33	M	81.9	Grass (1:2,048)
16	40	M	109.4	HDM (1:32)
19	32	M	84.5	RW (1:256)
21	41	M	78.4	CAT (1:64)
22	19	F	84.5	RW (1:2,048)
24	27	M	81.7	HDM (1:512)
26	26	F	90.4	HDM (1:1,024)
Mean $\pm$ SEM	30.5 $\pm$ 2.77	5:8	86.9 $\pm$ 2.83	

Definition of abbreviations: DR = dual responders; HDM = house dust mite (*Dermatophagoides farinae*); IER = isolated early responders; RW = ragweed.

Allergen/Methacholine Inhalation Challenge

Methacholine inhalation was performed as described by Cockcroft (20).

Allergen inhalation challenge was performed as described by O'Byrne and coworkers (21).

Sputum Induction

Sputum was induced and processed according to Popov and coworkers (22). Slides were stained with Congo red and counts from duplicate slides (400 cells per slide) were expressed as a percentage. Immunocytochemical staining was performed with murine monoclonal antibodies to human eosinophil cationic protein (EG2, 1.0 μ g/ml; Pharmacia, Uppsala, Sweden).

Bone Marrow Eosinophil/Basophil Progenitors and Blood

Bone marrow aspiration and Eo/B-CFU culture and enumeration were performed according to Wood and coworkers (17). Nonadherent mononuclear cells were cultured with diluent, recombinant human (rh)IL-5 (1 ng/ml), rhIL-3 (1 ng/ml), and rhGM-CSF (granulocyte-macrophage colony-stimulating factor, 10 ng/ml).

Six additional samples were cultured with diluent, recombinant human rhIL-5 (1 ng/ml), and rhIFN- γ (0.1, 1.0, 10, and 100 ng/ml) (BD Biosciences Pharmingen, San Diego, CA).

Blood (400 cells per slide) and bone marrow (1,000 cells per slide) smears were stained with Diff-Quik (VWR International, West Chester, PA) and counts from duplicate slides were expressed as a percentage.

Serum and bone marrow supernatant samples were stored at -70°C . IL-5, IL-3, IL-10, and IFN- γ were quantified using ELISAs (BD Biosciences Pharmingen). The lower limits of detection were 4, 7.4, 4, and 4 pg/ml, respectively. Values below these were assigned a value of 2 pg/ml for statistical analysis.

Statistical Analysis

Results were expressed as means $\pm$ SEM, except for methacholine PC₂₀, which was expressed as geometric mean $\pm$ % SEM. Methacholine PC₂₀, IL-5, IL-3, IL-10, and IFN- γ were log₁₀ transformed before analysis. Because baseline data from all outcomes showed no differences between phases, analyses were performed and data were presented using the average. The effect of allergen on methacholine PC₂₀, cytokine protein levels; sputum, blood, and bone marrow eosinophils; and Eo/B-CFU was analyzed with a mixed model analysis of variance (between group, isolated early versus dual responders; within group, before versus after allergen inhalation). Correlations were analyzed using Spearman's rho. Significance was accepted as $p < 0.05$.

RESULTS

Allergen-induced Bronchoconstriction and Airway Hyperresponsiveness

The mean maximal fall in FEV₁ during the early asthmatic response was $28.3 \pm 1.65\%$ in dual responders and $21.9 \pm 1.54\%$ in isolated early responders. The mean maximal fall in FEV₁ during the late asthmatic response was $20.5 \pm 2.04\%$ in dual responders compared with $5.4 \pm 1.03\%$ in isolated early responders (Table 2).

Dual responders developed methacholine airway hyperresponsiveness 24 and 48 hours after allergen inhalation. The mean methacholine PC₂₀ fell from 2.4 mg/ml (% SEM, 1.3) at baseline to 0.7 mg/ml (% SEM, 1.6) at 24 hours ($p < 0.01$) and 1.1 mg/ml (% SEM, 1.3) at 48 hours ($p < 0.05$) (Figure 1). In contrast, no significant changes in mean methacholine airway responsiveness occurred in isolated early responders after allergen inhalation, being 3.3 mg/ml (% SEM, 1.5) at baseline, 2.2 mg/ml (% SEM, 1.5) at 24 hours, and 2.8 mg/ml (% SEM, 1.5) at 48 hours after allergen inhalation.

Eosinophil Counts in Sputum, Blood, and Bone Marrow

Sputum. After allergen inhalation, there was a significant increase in sputum eosinophils in dual responders at 7, 24, and 48 hours. The numbers increased from $2.4 \pm 0.6\%$ at baseline to

TABLE 2. AIRWAY RESPONSES

	Arm 1				Arm 2				
	PC ₂₀				PC ₂₀				
	BL	24 h	EAR	LAR	BL	24 h	48 h	EAR	LAR
IER									
2	2.4	0.7	-15	-9	1.4	1.0	2	-19	-8
3	5.9	13.6	-23	-4	4.6	2.9	10.2	-20	0
5	11.4	4.8	-32	-4	5.7	3.4	3.2	-14	-12
6	30.3	12.2	-24	2	16	7.8	36.8	-20	-4
8	0.7	0.3	-19	-8	0.5	0.9	1.7	-10	-8
10	7.1	6.7	-25	0	5.3	3.2	2.9	-39	-10
14	0.1	0.2	-29	-6	0.3	0.1	0.1	-19	-8
15	4.6	5.3	-24	-3	3.2	4	6.2	-12	-7
18	14.0	7.9	-23	-8	14.4	6.2	11.3	-27	-11
20	0.6	0.8	-20	+7	0.9	0.7	0.5	-5	0
23	9.3	5.6	-33	-9	5.1	4.4	4	-28	-13
25	2.4	2.6	-26	+2	3.7	1.6	1.1	-21	-10
Mean ± SEM	7.4 ± 2.45	5.1 ± 1.30	-24 ± 1.5	-4 ± 1.4	5.1 ± 1.47	3.0 ± 0.68	6.7 ± 2.93	-20 ± 2.6	-8 ± 1.2
DR									
1	1.2	0.003	-24	-38	4	0.04	0.4	-32	-59
4	1.2	0.9	-22	-18	1.6	1.0	1.3	-19	-17
7	3.0	0.7	-44	-6	7.4	0.7	1.1	-41	-20
9	2.9	1.5	-29	-28	2.6	1.0	1.3	-31	-31
11	7.5	1.2	-23	-18	4.0	1.8	1.4	-18	-10
12	0.9	0.3	-42	-16	0.5	0.1	0.5	-35	-29
13	1.4	0.7	-35	-24	2.3	2.1		-31	-22
16	28.0	11.2	-25	-15	7.7	5.5	3.7	-29	-18
19	7.3	6.1	-19	-17	3.7	1.8	5.6	-19	-7
21	2.6	1.2	-29	-15	2.4	1.5	2.7	-30	-16
22	0.5	0.2	-19	-21	1.0	0.1	0.1	-15	-15
24	3.1	0.3	-24	-28	0.8	0.5	0.6	-36	-25
26	1.3	0.7	-23	-15	1.4	0.7	1.4	-42	-26
Mean ± SEM	4.7 ± 2.04	1.9 ± 0.89	-28 ± 2.2	-20 ± 2.3	3.0 ± 0.64	1.3 ± 0.40	1.7 ± 0.46	-29 ± 2.4	-23 ± 3.6

Definition of abbreviations: BL = baseline; DR = dual responder; EAR = early asthmatic response; IER = isolated early responder; LAR = late asthmatic response; PC₂₀ = provocative concentration of methacholine inducing a 20% fall in FEV₁.

12.2 ± 2.2% at 7 hours ($p < 0.001$), 11.5 ± 1.6% at 24 hours ($p < 0.001$), and 10.8 ± 2.6% at 48 hours ($p < 0.001$) (Figure 2). Isolated early responders also had an allergen-induced increase in sputum eosinophils from 2.6 ± 0.4% at baseline to 6.9 ± 2.0% at 7 hours, 7.2 ± 1.8% at 24 hours ($p < 0.05$), and 7.6 ± 1.8% at 48 hours ($p < 0.05$) (Figure 2). The magnitude of the allergen-induced change in sputum eosinophils was significantly greater in dual responders than in isolated early re-

sponders at all time points ($p < 0.05$). Baseline values were not significantly different between the two groups of subjects. After allergen inhalation, there was a significant increase in the percentage of activated sputum eosinophils (EG2-positive cells) in dual responders, from 1.65 ± 0.6% at baseline to 7.39 ± 1.7% at 7 hours ($p = 0.05$), 8.68 ± 2.1% at 24 hours ($p < 0.005$), and 8.7 ± 2.4% at 48 hours ($p < 0.005$). In contrast, no significant change in percentage of activated eosinophils was detected in isolated early responders at any time point. In addition, the magnitude of the allergen-induced change in sputum eosinophils was significantly greater in dual responders than in isolated early responders at all time points ($p < 0.05$).

Blood. Allergen inhalation significantly increased the percentage of blood eosinophils in dual responders from 3.1 ± 0.6% at 5 hours and 2.7 ± 0.5% at 12 hours to 5.2 ± 0.8% at 24 hours ($p < 0.05$) and 5.7 ± 0.8% at 48 hours ($p < 0.005$) and had a nearly significant increase from baseline, being 4.0 ± 0.5% ($p = 0.055$) (Figure 2). There was no allergen-induced change in the percentage of blood eosinophils detected in isolated early responders.

Bone marrow. The baseline numbers of eosinophils were not significantly different between the two arms of the study; the mean difference being 1.4 ± 0.3 (Table 3). Allergen inhalation significantly increased bone marrow eosinophils in dual responders at 48 hours when compared with 5 hours ($p < 0.005$) but not when compared with baseline (Figure 2). In contrast, there was no significant allergen-induced change in the percentage of bone marrow eosinophils in isolated early responders at any time point (Figure 2). The percent change in eosinophils was significantly lower at 5 hours ($p < 0.05$) in dual responders when compared with isolated early responders.

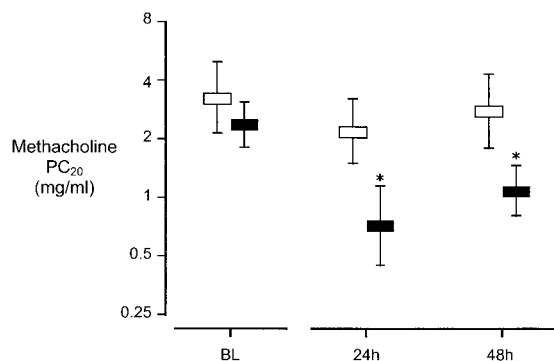


Figure 1. Airway responsiveness to methacholine PC₂₀ at baseline (BL) and after allergen inhalation in isolated early responders (open rectangles) and dual responders (solid rectangles). In dual responders only, airway hyperresponsiveness (AHR) increased significantly at 24 and 48 hours compared with baseline. No significant changes occurred in the isolated early responders over time. Difference compared with baseline, * $p < 0.05$.

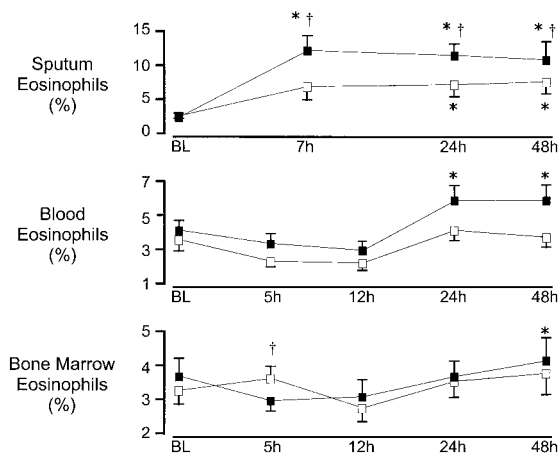


Figure 2. Changes in sputum, blood, and bone marrow eosinophils measured at baseline (BL) and after allergen inhalation in isolated early responders (open squares) and dual responders (solid squares). Sputum eosinophils significantly increased compared with baseline; at 24 and 48 hours in isolated early responders; and at 7, 24, and 48 hours in dual responders after allergen inhalation. The magnitude of the allergen-induced increase in sputum eosinophils was significantly greater in dual responders at 7, 24, and 48 hours when compared with isolated early responders. Blood eosinophils increased significantly in dual responders only at 24 and 48 hours when compared with 5 and 12 hours. Bone marrow eosinophils increased significantly in dual responders only, at 48 hours when compared with 5 hours. This decrease at 5 hours was significantly lower in dual responders compared with bone marrow eosinophils in the isolated early responders at the same time point. Difference over time, * $p < 0.05$; difference between groups, † $p < 0.05$.

Bone Marrow Progenitors

Interleukin-3. The baseline numbers of IL-3-responsive Eo/B-CFU were not significantly different between the two arms of the study; the mean difference being 4 ± 4.5 (Table 3). After allergen inhalation, there was a significantly increased number of IL-3-responsive bone marrow Eo/B-CFU in dual responders at 5 hours, but not at 12, 24, or 48 hours. The numbers increased from 5.1 ± 1.6 at baseline to 12.2 ± 2.7 at 5 hours ($p = 0.05$) (Figure 3). There was no allergen-induced change in the number of IL-3-stimulated Eo/B-CFU from isolated early responders. The number of Eo/B-CFU was significantly greater at 5 hours ($p < 0.05$) in dual responders when compared with isolated early responders (Figure 3).

Interleukin-5. The baseline numbers of IL-5-responsive Eo/B-CFU were not significantly different between the two arms of the study; the mean difference being 8 ± 7.0 (Table 3). After allergen inhalation, there was a significantly increased number of IL-5-responsive Eo/B-CFU in dual responders at 12 and 24 hours after allergen inhalation, increasing from 33.2 ± 4.2 at baseline, to 43.2 ± 5.3 at 5 hours, 51.0 ± 8.1 at 12 hours ($p < 0.01$), 52.7 ± 5.7 at 24 hours ($p < 0.005$), and 46.8 ± 7.3 at 48 hours (Figure 3). In contrast, there was no change in the number of IL-5-responsive Eo/B-CFU in the isolated early responders at any of the time points tested after allergen inhalation, being 29.7 ± 4.0 at baseline, 38.0 ± 4.6 at 5 hours, 36.0 ± 5.5 at 12 hours, 34.9 ± 4.3 at 24 hours, and 30.9 ± 5.6 at 48 hours after allergen inhalation. The magnitude of the change in IL-5-responsive Eo/B-CFU at 24 hours was significantly greater in dual responders when compared with isolated early responders ($p < 0.05$) (Figure 3).

GM-CSF. There was no significant allergen-induced difference over time in the number of GM-CSF-responsive Eo/B-CFU in either isolated early or dual responders (data not shown).

IL-5 Protein

Serum. After allergen inhalation, there was a significant increase in serum IL-5 protein in dual responders at 12 and 24 hours, increasing from 20.1 ± 8.7 pg/ml at baseline to 21.8 ± 8.3 pg/ml at 5 hours, 32.9 ± 10.2 pg/ml at 12 hours ($p < 0.05$), 33.5 ± 10.5 pg/ml at 24 hours ($p < 0.01$) and 28.6 ± 8.9 pg/ml at 48 hours (Figure 4). There was no changes in the level of serum IL-5 protein in isolated early responders at any time points (Figure 4). The level of serum IL-5 protein was significantly greater in dual responders when compared with isolated early responders at all time points ($p < 0.01$).

Bone marrow. The baseline levels of IL-5 were not significantly different between the two arms of the study; the mean difference being 4 ± 6.3 (Table 3). After allergen inhalation, there was a significant increase in bone marrow IL-5 protein in dual responders at 12 and 24 hours increasing from 19.2 ± 8.4 pg/ml at baseline to 25.5 ± 10.3 pg/ml at 5 h, 32.6 ± 10.8 pg/ml at 12 hours ($p < 0.01$), 29.6 ± 9.8 pg/ml at 24 hours ($p < 0.01$) and 22.9 ± 7.2 pg/ml at 48 hours (Figure 4). In contrast, there was no change in the level of bone marrow IL-5 protein in isolated early responders at any time point (Figure 4).

There was a significant positive relationship between levels of IL-5 protein detected in the bone marrow and in the serum as measured by the area under the curve from baseline to 48 hours ($r = 0.973$, $p < 0.0001$). Similarly, there was a significant positive correlation between levels of bone marrow IL-5 protein and the number of IL-5-responsive Eo/B-CFU as measured by the area under the curve from baseline to 48 hours after allergen inhalation ($r = 0.448$, $p < 0.05$).

IFN- γ Protein

Serum. After allergen inhalation there was no change in serum IFN- γ protein levels at any time point in either group of subjects studied.

Bone marrow. The baseline levels of IFN- γ were not significantly different between the two arms of the study; the mean difference being 34 ± 91.7 (Table 3). After allergen inhalation there was a significant increase in the level of bone marrow IFN- γ protein in dual responders at 48 hours compared with baseline increasing from 126.6 ± 27.9 pg/ml at baseline to 146.5 ± 34.4 pg/ml at 5 hours, 148.4 ± 41.5 pg/ml at 12 hours, 165.5 ± 46.5 pg/ml at 24 hours, and 168.9 ± 30.6 pg/ml at 48 hours ($p < 0.01$) (Figure 4). In contrast, there was no change in the level of IFN- γ protein detected in the bone marrow of isolated early responders at any time point tested (Figure 4).

There was a significant positive correlation between level of bone marrow IFN- γ and level of bone marrow IL-5 ($r = 0.54$, $p < 0.05$), but not between bone marrow IFN- γ and bone marrow, blood, or sputum eosinophils or Eo/B-CFU.

IL-3 and IL-10 Protein

Serum and bone marrow. There was no significant allergen-induced difference over time in the amount of IL-3 or IL-10 in either isolated early or dual responders (data not shown).

Cell Culture with IFN- γ

There were no significant changes in the number of IL-5-stimulated eosinophil/basophil colonies grown when incubated with any concentration of IFN- γ . The value with IL-5 alone was 31.8 ± 2.8 ; and values with IFN- γ were as follows: IFN- γ (100 ng/ml), 33.3 ± 8.1 ; IFN- γ (10 ng/ml), 40.8 ± 6.8 ; IFN- γ (1 ng/ml), 40.9 ± 5.5 ; IFN- γ (0.1 ng/ml), 40.4 ± 6.8 .

DISCUSSION

This study has demonstrated, for the first time, that allergen inhalation by dual responders is associated with a rapid onset of

TABLE 3. BONE MARROW RESPONSES

	Arm 1					Arm 2				
	% BM Eo	Eo/B-CFU		BM		% BM Eo	Eo/B-CFU		BM	
		IL-3	IL-5	IL-5	IFN- γ		IL-3	IL-5	IL-5	IFN- γ
IER										
2	NA	3.0	29.5	18	285	NA	10.5	36.5	16	246
3	NA	2.5	54.0	5	138	NA	5.5	69.0	6	128
5	3.4	1.0	21.5	NA	NA	2.7	2.0	30.0	3	122
6	3.1	3.0	4.5	3	80	1.4	0.5	30.5	3	59
8	2.9	2.0	34.0	5	80	5.5	1.0	15.0	5	92
10	2.6	6.5	20.0	4	70	7.4	6.5	24.5	4	79
14	4.1	0.5	42.5	13	81	3.4	11.5	32.5	11	66
15	NA	10.5	21.5	4	63	5.8	4.0	23.0	5	71
18	1.9	1.5	29.5	6	70	NA	0.5	24.5	4	93
20	1.7	4.0	16.5	136	NA	2.9	4.0	19.0	119	NA
23	2.9	8.5	44.5	12	59	2.8	2.0	42.0	4	75
25	3.9	2.0	26.0	4	71	2.6	1.0	23.0	4	73
Mean $\pm$ SEM	4.0 $\pm$ 0.6	3.8 $\pm$ 0.9	29 $\pm$ 3.9	19 $\pm$ 2	100 $\pm$ 2	3.3 $\pm$ 0.5	4.1 $\pm$ 1.1	31 $\pm$ 4.1	15 $\pm$ 9	101 $\pm$ 16
DR										
1	NA	5.0	32.5	4	74	NA	1.0	19.0	3	80
4	NA	4.5	27.0	8	77	NA	10.0	22.0	8	93
7	3.0	0.0	29.5	3	60	2.4	0.0	43.0	10	94
9	3.2	1.5	77.5	2	50	4.4	5.0	61.5	2	61
11	5.2	0.0	34.5	4	61	2.6	2.5	36.0	4	71
12	7.2	3.0	47.0	57	282	4.6	19.0	36.0	52	313
13	NA	14.0	23.5	4	109	3.5	1.0	33.0	30	558
16	5.3	8.5	38.0	31	205	6.7	8.0	45.5	28	206
19	2.9	16.5	19.5	4	66	2.2	4.0	25.0	4	70
21	0.9	9.5	28.5	114	NA	1.0	9.0	24.0	99	NA
22	3.1	0.5	26.5	4	82	3.6	0.5	27.0	4	69
24	5.5	0.0	31.0	6	91	2.0	0.0	9.5	9	126
26	4.1	0.0	10.0	3	67	4.5	0.0	13.0	3	74
Mean $\pm$ SEM	2.9 $\pm$ 0.2	5.3 $\pm$ 1.6	35 $\pm$ 4.4	19 $\pm$ 9	102 $\pm$ 20	3.7 $\pm$ 0.6	5.0 $\pm$ 1.6	30 $\pm$ 4.0	20 $\pm$ 8	151 $\pm$ 43

Definition of abbreviations: BM = bone marrow; DR = dual responder; Eo = eosinophils; Eo/B-CFU = eosinophil/basophil colony-forming unit; IER = isolated early responder; IL = interleukin; NA = not available.

IL-3-dependent eosinophilopoiesis in the bone marrow, detectable as early as 5 hours. In addition, there is sustained IL-5-dependent eosinophilopoiesis at 12 and 24 hours after allergen inhalation in dual responders. IL-5 protein levels increased significantly in serum and bone marrow at 12 and 24 hours after allergen inhalation in dual responders only. Furthermore, there was a significant correlation between IL-5-responsive Eo/B-CFU and IL-5 protein levels in the bone marrow, suggesting that both airway and blood eosinophilia in dual responders are sustained by an IL-5-responsive, eosinophil-differentiative process within the bone marrow. Finally, bone marrow IFN- γ protein levels increased 48 hours after allergen inhalation, in association with the reduction of bone marrow IL-5 protein levels and eosinophilopoiesis. Together these results are consistent with the hypothesis that upregulation of bone marrow IL-5 and IL-5-responsive Eo/B-CFU plays an important role in the persistence of allergen-induced airway eosinophilia and airway hyperresponsiveness over 48 hours and that increases in bone marrow IFN- γ downregulate these responses.

The development of both early and late asthmatic responses after allergen inhalation is known to be associated with increases in circulating eosinophils (11), greater increases of activated eosinophils in the airway (23), and the development of airway hyperresponsiveness (24) when compared with isolated early responders. All of these changes were confirmed in this study, although markers for eosinophil activation may not be ideal (25). Contrasting these two groups allows for a useful clinical model of increased eosinophilic airway responses and associated airway functional changes. The groups are, however, not dichotomous and the levels of bronchoconstriction used to discriminate

between them are arbitrary. Also, increases in airway eosinophils were seen in the isolated early responders in the present study and in other studies from our laboratory (17, 23). Despite this, it was only the significantly greater increases in circulating and airway eosinophils in dual responders that were associated with increases in bone marrow eosinophilopoiesis.

An increase in peripheral blood Eo/B-CFU was first shown in atopic, compared with nonatopic, subjects (26), and in dual responders 24 hours after allergen inhalation (15). Subsequently, Wood and coworkers (17) described a significant increase in IL-5-responsive Eo/B-CFU 24 hours after allergen inhalation in dual responders, but not in isolated early responders. An increase in the expression of IL-5R α on bone marrow CD34 $^{+}$ cells in dual responders 24 hours after allergen inhalation helped explain these initial findings (16). The data from the present study extend these observations, demonstrating a rapid activation of eosinophilopoiesis, detectable as early as 5 hours after allergen inhalation, which is at first IL-3 dependent followed by an IL-5-dependent phase for at least 24 hours after allergen inhalation.

The development of tissue eosinophilia in allergic inflammatory responses is known to be orchestrated by a number of mediators, of which IL-5 appears to be central. Studies have shown that IL-5 protein levels are increased in induced sputum after allergen inhalation challenge (4, 27). In addition, we have also shown that IL-5 mRNA-positive cells colocalized with CD3 $^{+}$ cells are increased significantly in the bone marrow of dual responders when compared with isolated early responders (18). This suggests that the local production of IL-5 by T cells in the bone marrow is involved in inducing bone marrow hematopoietic processes. However, it is likely that other mediators are also

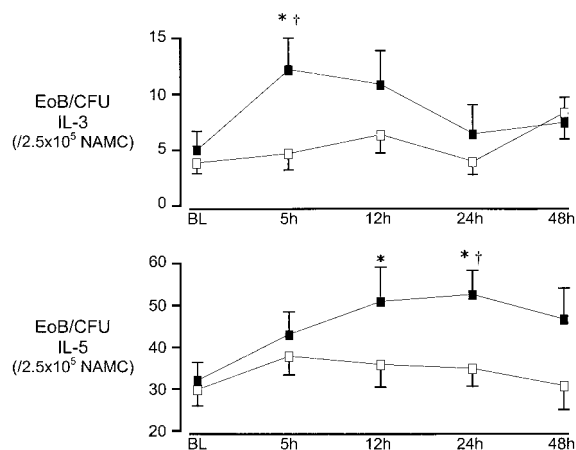


Figure 3. Changes in eosinophil/basophil colony-forming units (Eo/B-CFU) measured at baseline (BL) and after allergen inhalation in isolated early responders (open squares) and dual responders (solid squares). IL-3-responsive Eo/B-CFU significantly increased after allergen inhalation at 5 hours, compared with baseline in dual responders, and the magnitude of this change was significantly greater when compared with isolated early responders. IL-5-responsive Eo/B-CFU significantly increased after allergen inhalation at 12 and 24 hours compared with baseline in dual responders, and the magnitude of this change at 24 hours was significantly greater when compared with isolated early responders. Difference compared with baseline, * $p < 0.05$; difference between groups, † $p < 0.05$. NAMC = nonadherent mononuclear cells.

involved in the generation of mature eosinophils from pluripotent hematopoietic stem cells, including IL-3. Indeed, IL-3, GM-CSF, and IL-5 all regulate the commitment of progenitor cells along a basophil/eosinophil lineage. IL-3 can induce eosinophil/basophil differentiation, but has other hematopoietic activities,

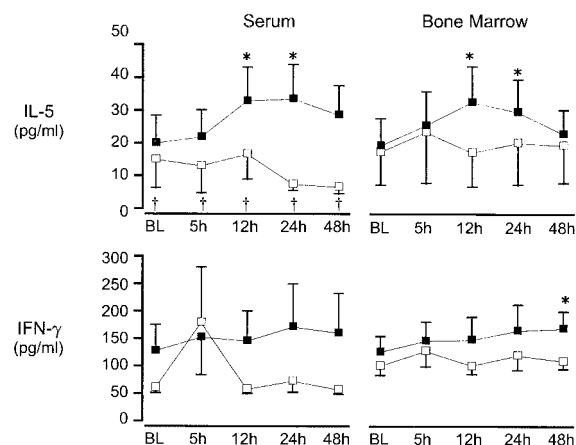


Figure 4. Changes in protein levels of IL-5 and IFN- γ in serum and bone marrow supernatant measured at baseline (BL) and after allergen inhalation in isolated early responders (open squares) and dual responders (solid squares). In dual responders only, IL-5 protein levels significantly increased after allergen inhalation at 12 and 24 hours, compared with baseline, in both serum and bone marrow supernatant. Serum IL-5 protein levels were significantly higher at all time points in dual responders compared with isolated early responders. In dual responders only, IFN- γ levels significantly increased after allergen inhalation at 48 hours compared with baseline, in bone marrow supernatant. Difference over time, * $p < 0.05$; difference between groups, † $p < 0.01$.

whereas IL-5 is a specific eosinophilopoietin. Early stem cells express receptors for IL-3 and Clutterbuck and coworkers have shown that IL-3 has the ability to enhance the number of eosinophil clusters (28). Tavernier and coworkers, on the other hand, have shown that IL-5 is able to upregulate its own receptor on CD34⁺ cells (29). Therefore, with early stimulation via IL-3 the number of eosinophil progenitor clusters increases and in the presence of IL-5 the number of these cells differentiating into mature eosinophils also increases.

IL-3 has been shown in murine models to regulate commitment of early myeloid progenitors to the eosinophil lineage (30). Indeed, this role for IL-3 is supported for the first time in humans by our finding of a rapid increase in *ex vivo* IL-3-responsive eosinophil/basophil progenitors 5 hours after allergen inhalation. Although IL-3 was not detectable in bone marrow supernatant in this study, it may be that the sensitivity of the assay was inadequate. It is likely that the increased colony numbers seen 12 and 24 hours after allergen inhalation are due to both the early regulation by IL-3 and the terminal differentiation by IL-5. This is supported by observations in an IL-5-deficient murine model in which symptoms and signs of allergic rhinitis were delayed, but not abolished, after nasal allergen inhalation challenge (31, 32). Therefore, after allergen inhalation, time-dependent changes in bone marrow cytokines control the expansion and differentiation of eosinophil/basophil progenitors.

The turnover of eosinophils is likely slower in normal hematopoiesis compared with that seen during inflammatory situations. Terashima and coworkers have used bromodeoxyuridine (BrdU) in rabbits to examine progenitor cell expansion and transit times. They have shown a transit time for circulating BrdU⁺ cells beginning at 24 hours and peaking between 66 and 78 hours. However, when *Streptococcus pneumoniae* was instilled in the lung the transit time was significantly shortened, with BrdU⁺ cells increasing at 12 hours and peaking between 24 and 48 hours. They also found significantly shorter time periods for bone marrow cells in both the mitotic and postmitotic pools (33). This suggests that either there is a shorter time for each division or that cells skip a number of divisions. Similarly, we have examined BrdU⁺ cells in dogs exposed to allergen or saline inhalation and found significant increases in BrdU⁺ cells in both the blood and airways of allergen-challenged dogs at 24 hours compared with diluent challenge (34). Together these data show that under stressful circumstances such as during an allergic response, the bone marrow is able to rapidly produce inflammatory cells.

IL-5 is also recognized as a potent eosinophil-activating cytokine (35). Studies have demonstrated the importance of IL-5 in facilitating the release of eosinophils from bone marrow (36). The present study found that in dual responders, IL-5 protein levels peak significantly in the serum before increases in blood eosinophils occur. It is therefore probable that IL-5 regulates eosinophilic responses at several levels, including enhanced bone marrow eosinophil differentiative processes, release of eosinophils from the bone marrow to the blood, and trafficking from the blood into the airways.

Durham and Kay demonstrated an initial fall in circulating eosinophil numbers coinciding with the development of the late airway inflammatory response, followed by a gradual increase peaking at 24 hours (11). Our study corroborates these findings and has shown, for the first time, a similar trend in allergen-induced changes in bone marrow eosinophils, with a drop in eosinophils at 5 and 12 hours, followed by an increase at 24 and 48 hours in dual responders only.

At any given time, the number of eosinophils present in the blood after allergen inhalation depends on three factors: the recruitment of cells into the airways or other tissues, the "margin" of eosinophils in the pulmonary vasculature, and the

recruitment of cells from the bone marrow or other tissues. Likewise, the number of eosinophils present in the bone marrow depends on the release of mature cells into the blood and the ongoing maturation of progenitors. Both margination and bone marrow responses likely contribute to the increases in the number of circulating eosinophils seen at 24 hours, whereas decreased bone marrow eosinophil numbers at 5 hours may be due to egress of mature eosinophils from the bone marrow into the blood. We suggest that movement of eosinophils between compartments is induced, in part, by increases in circulating IL-5, whereas maturation of progenitors at 12 and 24 hours is dependent on bone marrow IL-5 protein levels. As circulating IL-5 decreased, so too did the egress of eosinophils into the blood. Likewise, as bone marrow IL-5 levels diminished, so too did the production of eosinophils by maturing Eo/B-CFU. Therefore, by 48 hours, the number of bone marrow eosinophils was not significantly higher than baseline numbers.

Similarly, in the airways the number of eosinophils present is dependent on three factors: the number of eosinophils available to recruit from the circulation, the quantity of chemokines produced, and the ability of the tissue to express adhesion molecules for the uptake of eosinophils into the airways. The number of eosinophils available to recruit is dependent on the baseline level of circulating eosinophils, the number of eosinophils in reserve in the tissues, and the number of eosinophils produced by the bone marrow. Expression of chemokines and adhesion molecules will depend on the degree of activation and on the baseline levels of inflammatory cells in the airways. The *in situ* production of IL-5 in the bone marrow suggests that the bone marrow is not only responding to the airway's inflammation, but is also driving the ongoing inflammatory response.

In contrast to the stimulatory effects of IL-3 and IL-5, IFN- γ , through the attenuation of helper T cell Type 2-driven processes, may play an important role in the resolution of allergen-induced eosinophilic responses (37). One primary antiinflammatory effect of IFN- γ is the reduction of IL-5 production (38). Rais and coworkers have also shown that IL-12 suppressed eosinophilopoiesis via an IFN- γ -dependent mechanism in a murine model (39). On the basis of these results, the present study looked at the production of IFN- γ as a potential downmodulatory cytokine. We found that in the bone marrow supernatant, there is a delayed increase in IFN- γ protein levels in dual responders only. However, when eosinophil/basophil progenitors were cultured in the presence of IFN- γ and IL-5, we were unable to show a direct suppression of Eo/B-CFU. This suggests that, because the increase in bone marrow IFN- γ occurred at 48 hours, a time point that was coincident with a reduction in bone marrow IL-5 protein levels, IFN- γ may be suppressing eosinophil/basophil progenitors indirectly, via suppression of IL-5 production in the bone marrow. However, this must remain speculative, because we were unable to detect a difference in IFN- γ levels between isolated early and dual responders.

The role of the eosinophil in allergen-induced airway responsiveness is currently a subject of much debate. Leckie and coworkers (40) have suggested that IL-5 and eosinophils do not play an important role in allergen-induced airway hyperresponsiveness, although this study was not adequately designed to address this question (12). Murine models have also provided conflicting results (41–45). Foster and coworkers have suggested that these differences may be due to the presence of low levels of airway eosinophils in mouse models, which demonstrate persisting AHR (46). This suggestion is supported by Flood-Page and coworkers, who have shown that although an anti-IL-5 monoclonal antibody was able to reduce blood and bronchoalveolar lavage eosinophils, airway tissue and bone marrow eosinophils were reduced by only 50%. In addition, major basic protein (an

inflammatory mediator prominently produced by eosinophils) showed no significant reduction with treatment (47). In the current study, only dual responders, with significantly greater bone marrow and airway eosinophilic responses, developed airway hyperresponsiveness. These findings are therefore consistent with a role for eosinophils in the development of allergen-induced airway hyperresponsiveness.

In conclusion, this study has demonstrated that allergen-induced eosinophilopoiesis begins within 5 hours of allergen inhalation by upregulation of progenitor responsiveness to IL-3 and then increases in terminal differentiative responses to IL-5 over 48 hours. We have previously shown this is due to increases in progenitors expressing the IL-5 receptor and, in this study, increased levels of bone marrow IL-5. These changes occur only in dual responders who develop both a peripheral blood and airway eosinophilia and an associated prolonged airway hyperresponsiveness. The resolution of bone marrow eosinophilopoiesis may result from IFN- γ -mediated downregulation of IL-5 production. These results support a role for bone marrow eosinophilopoiesis and airway eosinophilia in allergen-induced airway hyperresponsiveness.

Conflict of Interest Statement: S.C.D. has no declared conflict of interest; R.S. has no declared conflict of interest; G.M.G. has no declared conflict of interest; R.M.W. has no declared conflict of interest; R.F. has no declared conflict of interest; G.L.J. has received honoraria from Actelion for presentations and from AstraZeneca for marketing research; J.A.D. has no declared conflict of interest; M.D.I. has no declared conflict of interest; P.M.O. has no declared conflict of interest.

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