

Original article

Allergen stimulates bone marrow CD34⁺ cells to release IL-5 *in vitro*; a mechanism involved in eosinophilic inflammation?

The specific mechanisms that alter bone marrow (BM) eosinophilopoiesis in allergen-induced inflammation are poorly understood. The aims of this study were to evaluate (a) whether the number of BM CD34⁺ cells is altered due to allergen sensitization and exposure *in vivo* and (b) whether BM CD34⁺ cells produce and release interleukin (IL)-5, IL-3 and granulocyte macrophage-colony stimulating factor (GM-CSF) after stimulation *in vitro*. A mouse model of ovalbumin (OVA)-induced airway inflammation was used. Bone marrow CD34⁺ cells were cultured *in vitro* and the cytokine release was measured by enzyme-linked immunosorbent assay. The IL-5-production from CD34⁺ cells was confirmed by immunocytochemistry. Airway allergen exposure increased the number of BM CD34⁺ cells ($P = 0.01$). Bone marrow CD34⁺ cells produced IL-5 when stimulated with the allergen OVA *in vitro*, but not IL-3 or GM-CSF. Nonspecific stimulus with calcium ionophore and phorbol-myristate-acetate of BM CD34⁺ cells caused release of IL-5, IL-3 and GM-CSF. The induced release of IL-5 was increased in alum-injected *vs* naive mice ($P = 0.02$), but was not affected by allergen sensitization and exposure. The release of IL-3 and GM-CSF was increased after allergen sensitization and exposure ($P < 0.02$). In conclusion, allergen can stimulate BM CD34⁺ cells to produce IL-5 protein. It is likely that the CD34⁺ cells have autocrine functions and thereby regulate the early stages of BM eosinophilopoiesis induced by airway allergen exposure. Alum, a commonly used adjuvant, enhances the release of IL-5 and may thereby enhance eosinophilopoiesis.

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Eosinophils are the predominant cells found in allergic airway inflammation and are mainly produced in the bone marrow (BM) (1). They differentiate from CD34⁺ progenitor cells under the influence of interleukin (IL)-5, IL-3 and granulocyte macrophage-colony stimulating factor (GM-CSF) (2–4). The specific mechanisms that alter BM eosinophilopoiesis are poorly understood but T-lymphocytes have been implicated as a source of IL-5 that could act on differentiation of eosinophils (5, 6). A recent study of airway allergen exposure in T-lymphocyte deficient mice (Balb/c- nude) has shown a T-lymphocyte independent mechanism in regulating the increase of BM eosinophil progenitors (7). The increased number of BM eosinophil progenitors in response to airway allergen exposure was instead suggested to be a result of altered function of BM stromal cells, or perhaps developed immunocompetence within the CD34⁺ cell population.

Increased number of CD34⁺ progenitor cells in BM, blood and airways (8–10) as well as increased numbers of CD34⁺ cells expressing IL-5R α in bronchial biopsies (9)

have been found in atopic subjects. In asthma, increased expression of GM-CSF both at the RNA and protein level has been observed in bronchial epithelial cells (11), bronchial alveolar lavage fluid cells (BALf) (12, 13) and in antigen-stimulated peripheral blood mononuclear cells (14). Furthermore, increased number of cells expressing IL-3 in BALf and in bronchial biopsies has also been found in asthmatic subjects (15, 16). However, the mechanisms how these cytokines are regulated in BM and particular in CD34⁺ cells are poorly understood.

The aims of this study were to evaluate (a) whether the number of BM CD34⁺ cells is altered because of allergen sensitization and exposure *in vivo* and (b) whether BM CD34⁺ cells produce and release IL-5, IL-3 and GM-CSF after stimulation *in vitro*. To assess this, we cultured and stimulated BM CD34⁺ cells from Balb/c mice, either with allergen (ovalbumin, OVA) or a combination of calcium ionophore and phorbol-myristate-acetate. The animals used as cell donors were: naïve; injected with Al(OH)₃ and exposed to phosphate-buffered saline (PBS);

OVA-sensitized and PBS-exposed or OVA-sensitized and OVA-exposed. The release of IL-5, GM-CSF and IL-3 was measured by enzyme-linked immunosorbent assay (ELISA) and the production of IL-5 was further confirmed by intracellular immunocytochemical staining.

Methods

In vivo experiments

Animals. This study was approved by the Animal Ethics Committee at Göteborg University, Sweden. Balb/c mice were purchased from Banting–Kingman Universal AB (Sollentuna, Sweden) or Mollegaard-Bommice A/S (Ry, Denmark). The mice were 5–6 weeks old and were kept under conventional animal housing conditions, provided with food and water *ad libitum*.

Allergen sensitization and exposure protocol. Balb/c mice were injected (i.p.) twice, 5 days apart, to (a) 8 µg of chicken OVA (Sigma-Aldrich, St Louis, MI) bound to 4 mg of aluminum hydroxide [Al(OH)₃, Sigma-Aldrich] in 0.5 ml of PBS or (b) only aluminum hydroxide in PBS. Eight days after the second injection, the mice were briefly anaesthetized using CO₂, and exposed intranasally to 100 µg of OVA in 25 µl PBS or PBS alone on three consecutive days. The animals were divided into four groups depending on sensitization and exposure: naïve (nonsensitized, nonexposed); Al(OH)₃/PBS (Al(OH)₃-injected, PBS-exposed); OVA/PBS or OVA/OVA.

Cell collection and sample processing. Samples were collected 24 h after the last exposure. The animals were anaesthetized with a mixture of xylazine (130 mg/kg, Rompun[®]; Bayer AB, Gothenburg, Sweden) and ketamine (670 mg/kg, Ketalar[®]; Pfizer AB, Täby, Sweden).

Bone marrow. The femurs were excised, cut at the epiphyses and flushed with 2 ml of PBS. For immunocytochemistry the samples were centrifuged at 350 *g* for 10 min at 4°C and diluted in 0.03% bovine serum albumin (BSA; Sigma-Aldrich) in PBS. Cytospins were prepared.

Immunocytochemistry. Cytospin preparations were fixed in 2% formaldehyde. All steps were performed in room temperature unless otherwise is stated.

CD34⁺/MBP⁺ cells. Endogenous biotin was blocked with biotin blocking system (Dako Corporation, Glostrup, Denmark). The preparations were incubated with a biotinylated rat anti-mouse CD34 monoclonal antibody (mAb) (clone RAM34; BD Biosciences, Erembodegem, Belgium) or its isotype control (biotinylated Rat IgG2a, BD Biosciences) followed by incubation with streptavidin-β-galactosidase (Roche Diagnostics GmbH, Mannheim, Germany). Bound antibodies (Ab) were visualized with β-gal staining set (Roche). For detection of major basic protein (MBP), saponin (Sigma-Aldrich) was added to all antibody dilutions. After blocking of endogenous biotin and unspecific staining with 10% swine serum (Dako) we stained for MBP using a rabbit anti-mouse MBP polyclonal antibody (pAb) (a gift from Dr James J Lee, Mayo Clinic, Scottsdale, USA) at 37°C. The preparations were thereafter incubated with a biotinylated F(ab)₂ swine anti-rabbit IgG mAb (Dako) and 2% mouse serum (Dako) followed by streptavidin alkaline-phosphatase (Dako). Bound Ab were visualized with vector red alkaline phosphatase substrate kit (Vector Laboratories Inc., Burlingame, CA).

In vitro experiments

Release of IL-5 from CD34⁺ cells and CD3⁺ lymphocytes. Bone marrow cell samples were mixed, filtrated, pooled and washed in 2 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich) in PBS before resuspension in 2 mM EDTA with 0.5% BSA (Sigma-Aldrich).

Bone marrow cells were labeled with a rat anti-mouse biotinylated mAb directed to CD34 antigen (clone RAM34, BD Biosciences). After washings, streptavidin microbeads (MACS, Miltenyl Biotec GmbH, Germany) were added, according to the manufacturer's instructions and CD34⁺ cells were enriched by magnetic separation. CD3⁺ lymphocytes were enriched by the same method, using a biotin conjugated hamster anti-mouse CD3e mAb (clone 145–2C11, BD Bioscience).

The CD34⁺ cell and CD3⁺ lymphocyte enriched fractions were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 µg/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (all from Sigma-Aldrich) in 1.0 × 10⁶ cells/200 µl. The cells were stimulated with either 1 µg/ml calcium ionophore (CI, batch A23187) and 2 ng/ml phorbol-12-myristate-13-acetate (PMA) or 5–500 µg/ml OVA (all from Sigma-Aldrich) for 20 or 96 h in 7% CO₂ at 37°C. The culture media was collected for measurement of IL-5, IL-3 or GM-CSF by ELISA (R&D Systems Inc., Abingdon, UK).

Immunocytochemical detection of intracellular IL-5 in CD34⁺ cells. Bone marrow cells from allergen sensitized mice were stimulated with CI and PMA as described above and the membrane transport of IL-5 was blocked by adding 10 µg/ml of Brefeldin A (Sigma-Aldrich) to the cell culture. The cells were cultured for 7 h, washed and fixed before cytospin preparations were made.

For intracellular staining of IL-5, all antibody dilutions were made with 1% saponin (Sigma-Aldrich). All incubations were performed at 37°C. Unspecific binding was blocked with 10% donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). The preparations were incubated with an anti-IL-5 mAb (clone TRFK-5, BD Biosciences) or isotype control (IgG₁, BD Biosciences). As a secondary step an alkaline phosphatase-conjugated donkey anti rat IgG (Jackson ImmunoResearch Laboratories Inc.) and 2% mouse serum (Dako) was used. Positive staining was visualized by vector red alkaline phosphatase substrate kit (Vector Laboratories Inc.). Endogenous biotin was blocked with biotin blocking system (Dako) before the preparations were incubated with a biotinylated rat anti-mouse CD34 mAb (clone RAM34; BD Biosciences) or isotype control (biotinylated Rat IgG2a; BD Biosciences). The slides were then treated with streptavidin-β-galactosidase (Roche) and bound ab were visualized with β-Gal staining set (Roche).

Immunocytochemical detection of CD34⁺ cell enrichment. Samples were incubated with a biotinylated rat anti-mouse CD34 mAb (clone MEC 14.7; Monosan, Uden, Netherlands) or its isotype control (Rat IgG2a; BD Biosciences) and subsequently incubated with streptavidin alkaline-phosphatase (Dako). Positive staining was visualized with fast red substrate according to the manufacturer's instructions (Dako).

Immunocytochemical detection of CD3⁺ lymphocyte enrichment. On day 1, nonspecific binding was blocked with 5% rabbit serum (Dako) followed by incubation with a rat anti-CD3 mAb (clone 012600030; Cedarlane Laboratories Ltd, Dakopatts, Sweden) or isotype control (Rat IgG1; BD Biosciences) overnight at 4°C. On day 2, the preparations were incubated with a rabbit anti-rat Ig (Dako) and 2% mouse serum (Dako) followed by rat APAAP mAb

(Dako). The two steps were repeated once. Bound ab were visualized with fast red alkaline phosphatase kit (Dako) or vector red alkaline phosphatase substrate kit (Vector Laboratories Inc.) before counterstaining with Mayer's hematoxylin (Sigma-Aldrich).

Immunocytochemical detection of IL-5R α cells in CD34⁺ cell population. Unspecific binding was blocked with 10% donkey serum (Jackson ImmunoResearch Laboratories Inc.). Slides were incubated with a rabbit anti-mouse IL-5R α pAb (Research Diagnostics Inc, Flanders, NJ) followed by an incubation with an alkaline phosphatase-conjugated donkey anti-rabbit mAb and 2% mouse serum (Dako). Bound ab were visualized with vector red alkaline phosphatase substrate kit (Vector Laboratories Inc.) before counterstaining with Mayer's hematoxylin (Sigma-Aldrich).

Characterization of CD34⁺ cells. The enriched CD34⁺ cell fractions were characterized by staining for eosinophils using luxol fast blue (17) or immunocytochemical staining of IL-5R α .

Statistical analysis. Data are presented as mean $\pm$ SEM. Statistical analysis was carried out using nonparametric analysis of variance (Kruskal-Wallis test) to evaluate the difference among more than one group. If a significant variance was found, the Mann-Whitney U-test was performed. $P < 0.05$ was considered statistically significant. Spearman rank correlation was used to determine if there was any correlation between the concentrations of OVA used for stimulation of CD34⁺ cells *in vitro*.

Results

In vivo experiments

Bone marrow CD34⁺/MBP⁺ cells. Allergen sensitization and exposure increased the relative number of CD34⁺ cells in BM compared with naïve mice ($12.8 \pm 1.9\%$ vs $3.2 \pm 1.0\%$, $P < 0.02$; Fig. 1). Sensitization and

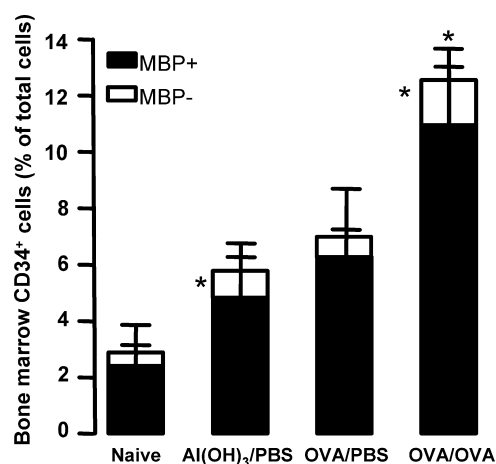


Figure 1. Relative number of bone marrow CD34⁺/MBP⁺ cells. Balb/c mice were; naïve (nonsensitized, nonexposed); Al(OH)₃/phosphate-buffered saline (PBS) (Al(OH)₃-injected, PBS-exposed); ovalbumin (OVA)/PBS (OVA-sensitized, PBS-exposed); OVA/OVA. Data are shown as mean $\pm$ SEM, $n = 5-9$. * $P < 0.05$ compared with naïve mice.

exposure to allergen did not significantly change the relative number of CD34⁺ cells expressing MBP ($11.0 \pm 1.9\%$ vs $2.9 \pm 0.9\%$; Fig. 1).

In vitro experiments

Release of IL-5, IL-3 and GM-CSF from BM CD34⁺ cells after stimulation with allergen. Stimulation of BM CD34⁺ cells from allergen sensitized and exposed mice with (5–500 μ g/ml) OVA for 20 h did not influence the release of IL-5 (data not shown). However, a dose dependent increase of the IL-5 release was found when the CD34⁺ cells were exposed to OVA for 96 h ($Rho = -0.65$, $P < 0.001$; Fig. 2A). Enriched BM CD3⁺ lymphocytes released approximately three to four times the amount of IL-5 upon similar doses of allergen (data not shown). Stimulation with OVA did not in any way affect the release of IL-3 or GM-CSF (Fig. 2B,C).

Release of IL-5 from BM CD34⁺ cells and CD3⁺ lymphocytes after nonspecific stimulation. Stimulation of BM CD34⁺ cells and CD3⁺ lymphocytes with CI and PMA induced production of IL-5 (Fig. 3).

The production of IL-5 was significant higher in CD34⁺ cells from mice injected with Al(OH)₃ compared with cells from naïve mice (84 ± 31 pg/ml and 13 ± 4 respectively, $P = 0.02$; Fig. 4A), but was not further enhanced by allergen sensitization or allergen sensitization and exposure (84 ± 31 pg/ml vs 126 ± 20 pg/ml or 153 ± 7 pg/ml). Nonstimulated BM CD34⁺ cells did not release any detectable levels of IL-5.

Allergen sensitization increased the release of IL-5 from CD3⁺ lymphocytes compared with CD3⁺ lymphocytes from naïve mice (132 ± 7 pg/ml vs 86 ± 5 pg/ml, $P = 0.01$; Fig. 4B). Allergen exposure did not further enhance the release of IL-5 (126 ± 10 pg/ml vs 132 ± 7 pg/ml).

Release of IL-3 and GM-CSF from BM CD34⁺ cells after nonspecific stimulation. Allergen sensitization and exposure enhanced the release of IL-3 from CD34⁺ cells (167 ± 8 pg/ml vs 82 ± 7 pg/ml, $P = 0.01$; Fig. 5A) as well as GM-CSF (44 ± 9 pg/ml vs 10 ± 7 pg/ml, respectively, $P = 0.03$; Fig. 5B) compared with cells from naïve mice. The nonspecific stimulation did not *per se* affect the release of IL-3 (Fig. 2B).

Evaluation of CD34⁺ and CD3⁺ cell fraction. The enrichment of CD34⁺ cell fractions was 58–71% (Table 1). The CD34⁺ population contained 1.5–3.0% CD3⁺ lymphocytes and 3–12% eosinophils. IL-5R α was detected on 71–86% of the cells.

The purity of CD3⁺ lymphocytes was 90–92% according to immunocytochemistry (Table 1). Less than 1% CD34⁺ cells were found in the CD3⁺ lymphocyte fraction.

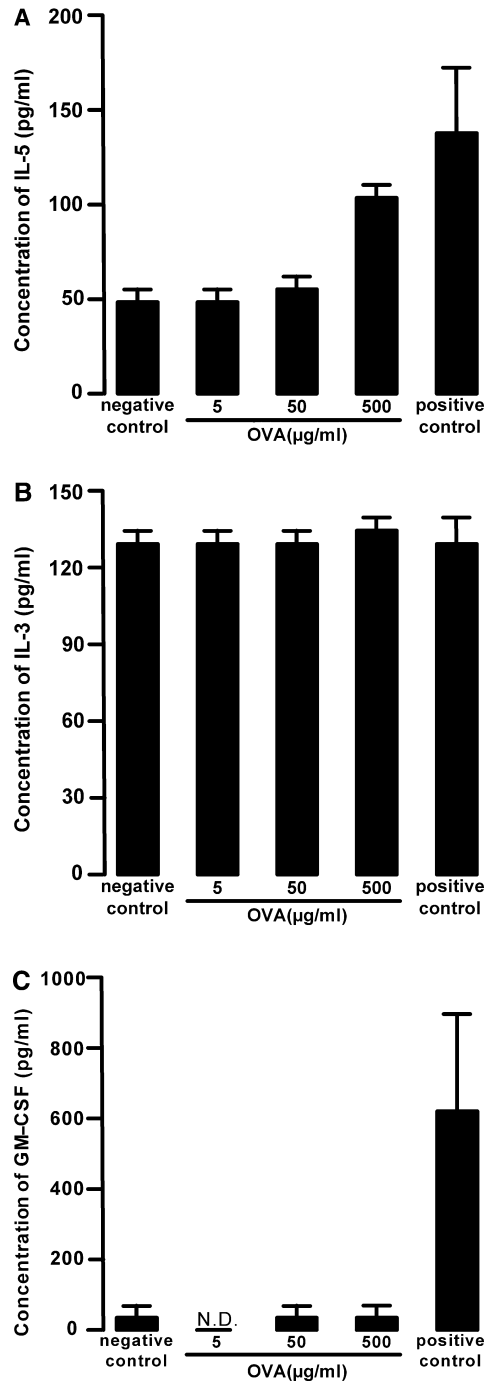


Figure 2. Release of (A) IL-5, (B) IL-3 and (C) GM-CSF from bone marrow CD34⁺ cells (10^6 cells/well) stimulated with ovalbumin (5–500 µg/ml) for 96 h. Stimulation with 1 µg/ml calcium ionophore and 2 ng/ml phorbol-myristate-acetate served as positive control. Nonstimulated cells served as negative control. The cells were obtained from allergen sensitized Balb/c mice. Data are shown as mean $\pm$ SEM, $n = 7$ –10. Dose dependent release of IL-5 (RhO = -0.65 , $P < 0.001$; Spearman Rank Correlation). Detection limit was 5 pg/ml for IL-5, 7.8 pg/ml for IL-3 and 1.0 pg/ml for GM-CSF.

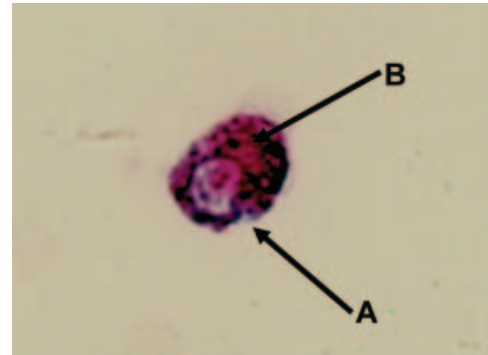


Figure 3. Photomicrograph of ICC staining for CD34⁺ cell (A) expressing intracellular interleukin (IL)-5 (B) in mouse bone marrow. The cells were stimulated with calcium ionophore and phorbol-myristate-acetate. Membrane transport of IL-5 was blocked with 10 µg/ml Brefeldin A for 7 h.

Discussion

In this study, we have shown for the first time that BM derived CD34⁺ cells can produce and release IL-5 protein when stimulated *in vitro* with allergen. Nonspecific stimulation of BM CD34⁺ cells caused release of IL-5, IL-3 and GM-CSF. The induced release of IL-5 was increased in alum-injected vs naive mice but was not affected by allergen sensitization and exposure. In contrast, the release of IL-3 and GM-CSF were increased in allergen sensitized and exposed mice.

It is known that airway allergen exposure affect the BM eosinophilopoiesis (18, 19). The eosinophilia in asthma has recently been suggested to be a result of two distinct hematopoietic regulatory mechanisms (7). The increased number of eosinophil progenitors found within the BM in response to airway allergen exposure has been shown to be T-lymphocyte independent, as T-lymphocyte deficient mice (Balb/c nude) also respond to airway allergen exposure with increased numbers of eosinophil progenitors. However, the increased number of BM eosinophil progenitors in these allergen sensitized T-lymphocyte deficient mice was not associated with terminal differentiation of these progenitors to eosinophils. The mechanism of this effect in the progenitors was not determined, but it cannot be excluded that the CD34⁺ cells themselves responded to allergen. In the present study, we have shown that BM CD34⁺ cells have capacity to produce IL-5 when stimulated with OVA *in vitro*. We can only speculate whether there is an allergen-specific release of IL-5 from BM CD34⁺ also *in vivo*. In a previous study, we have shown that allergen sensitized and exposed mice not only have increased number of CD3⁺ lymphocytes expressing intracellular IL-5 in BM but also increased number of non-CD3 lymphocytes expressing intracellular IL-5 as compared with PBS-exposed mice (5). This data indicates for additional cell types besides CD3⁺ lymphocytes are

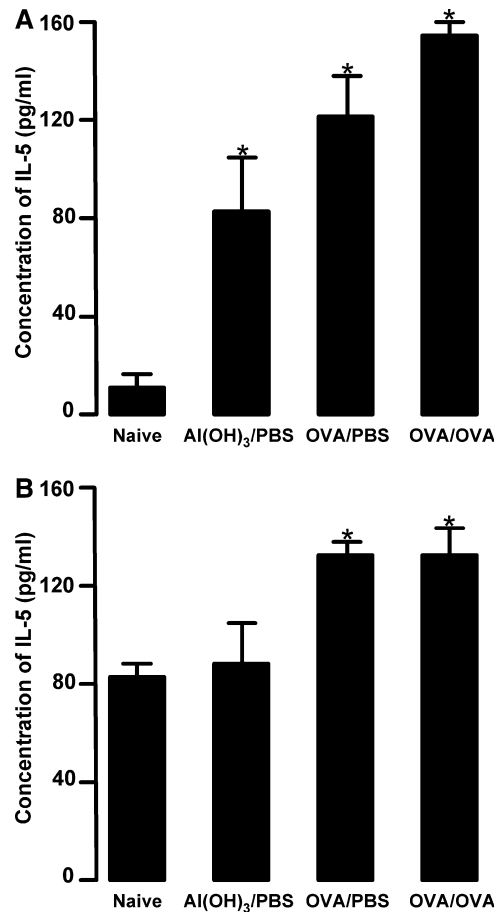


Figure 4. Release of IL-5 from 10⁶ cells/well of bone marrow (A) CD34⁺ cells and (B) CD3⁺ lymphocytes stimulated with 1 µg/ml calcium ionophore and 2 ng/ml phorbol-myristate-acetate for 20 h. The cells were obtained from Balb/c mice that were naïve, injected with aluminum hydroxide (Al(OH)₃) and exposed to phosphate-buffered saline (PBS), ovalbumin (OVA)-sensitized and exposed to either PBS or OVA. Data are shown as mean ± SEM, *n* = 5–12, **P* < 0.05 as compared with naive mice (Mann–Whitney *U*-test).

capable of expressing IL-5 in BM in response to airway allergen exposure. One such non-CD3 lymphocyte cell could be the CD34⁺ cell. However, the fact that CD34⁺ cells can be stimulated with allergen to produce IL-5 *in vitro* argues for these cells having some degree of immunocompetence, and may imply an autocrine mechanism of these cells to stimulate to eosinophilopoiesis. It has previously been shown that CD34⁺ cells do express receptors for IL-3 and GM-CSF, but in low density on the most immature subsets of CD34⁺ cells (20). In this study, allergen stimulation of BM CD34⁺ cells did not cause release of any detectable levels of IL-3 or GM-CSF. However, when these cells were stimulated with a nonspecific stimulus they did show the capacity to release IL-3 and GM-CSF. This data, taken together with our data on IL-5, further support the hypothesis that

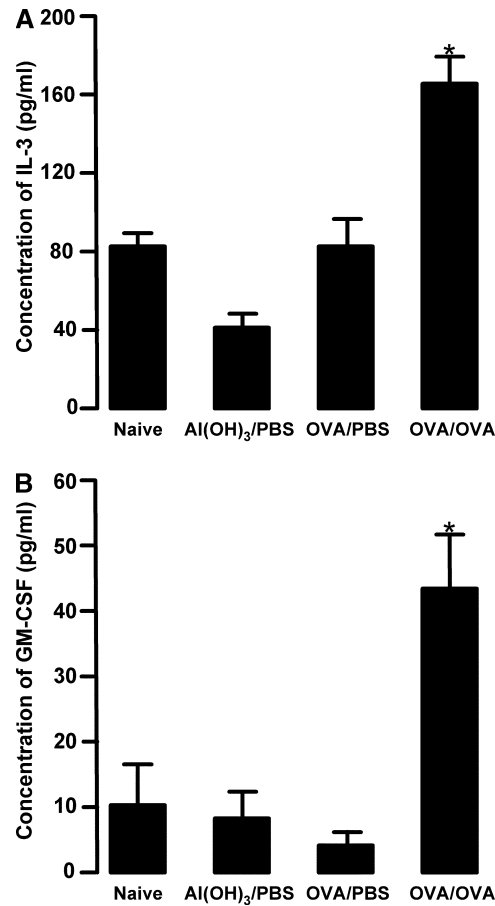


Figure 5. Release of (A) interleukin (IL)-3 and (B) granulocyte macrophage-colony stimulating factor (GM-CSF) from bone marrow CD34⁺ cells (10⁶ cells/well) stimulated with 1 µg/ml calcium ionophore and 2 ng/ml phorbol-myristate-acetate for 20 h. The cells were obtained from Balb/c mice that were naïve, injected with aluminum hydroxide (Al(OH)₃) and exposed to phosphate-buffered saline (PBS), ovalbumin (OVA)-sensitized and exposed to either PBS or OVA. Data are shown as mean ± SEM, *n* = 4–7, **P* < 0.05 as compared with naive mice (Mann–Whitney *U*-test).

Table 1. Evaluation of CD34⁺ and CD3⁺ cell fraction

	CD34 ⁺ (%)	CD3 ⁺ (%)
CD34 ⁺	58–71	<1
CD3 ⁺	1.5–3	90–92
Eosinophils	3–12	1–2
IL-5Rα	71–86	–

Relative number of BM CD34⁺ cells, CD3⁺ lymphocytes, eosinophils and IL-5Rα expressing cells in CD34⁺ cell and CD3⁺ lymphocyte fractions.

CD34⁺ cells may act in an autocrine way in eosinophilopoiesis, but that IL-3 and GM-CSF may be released by allergen-independent mechanisms.

Interestingly, we have shown that BM CD34⁺ cells from mice injected with alum have enhanced production of IL-5, when stimulated in a nonspecific way, as compared with cells from naïve mice. Sensitization and exposure to OVA did not further enhance the induced production of IL-5 but IL-3 and GM-CSF. Other studies have shown a general immunostimulatory effect of alum, inducing T_{H2} cytokine production (21, 22), although the mechanisms by which this occurs is unclear. Alum is commonly used as an adjuvant in immunotherapy and also in animal models of allergy, and it is therefore of importance to understand the effects of alum in immune responses.

Immunocytochemical staining of the CD34 antigen together with staining for intracellular IL-5 confirms the capability of CD34⁺ cells to produce IL-5, which is important to show, as the enrichment with magnetic beads is not 100%. Thus, it is unlikely that the CD3 cells contaminating the CD34 cell fraction contributed extensively to IL-5-release.

This study shows for the first time that BM CD34⁺ cells can express and release IL-5. Importantly, IL-5 can be released from CD34⁺ cells after allergen-specific stimulation. This argues for an autocrine function of BM CD34⁺ cells in allergen-induced airway inflammation, driving also the early events of BM eosinophilopoiesis.

References

1. SERGEJEVA S, JOHANSSON AK, MALM-HALL C, LOTVALL J. Allergen exposure-induced differences in CD34⁺ cell phenotype; relationship to eosinophilopoietic responses in different compartments. *Blood* 2004;**103**:1270–1277.
2. CLUTTERBUCK EJ, HIRST EM, SANDERSON CJ. Human interleukin-5 (IL-5) regulates the production of eosinophils in human bone marrow cultures: comparison and interaction with IL-1, IL-3, IL-6, and GM-CSF. *Blood* 1989;**73**:1504–1512.
3. YAMAGUCHI Y, SUDA T, SUDA J, EGUCHI M, MIURA Y, HARADA N et al. Purified interleukin 5 supports the terminal differentiation and proliferation of murine eosinophilic precursors. *J Exp Med* 1988;**167**:43–56.
4. SHALIT M, SEKHSARIA S, MALECH HL. Modulation of growth and differentiation of eosinophils from human peripheral blood CD34⁺ cells by IL5 and other growth factors. *Cell Immunol* 1995;**160**:50–57.
5. TOMAKI M, ZHAO LL, LUNDAHL J, SJOSTRAND M, JORDANA M, LINDEN A et al. Eosinophilopoiesis in a murine model of allergic airway eosinophilia: involvement of bone marrow IL-5 and IL-5 receptor alpha. *J Immunol* 2000;**165**:4040–4050.
6. WOOD LJ, SEHMI R, DORMAN S, HAMID Q, TULIC MK, WATSON RM et al. Allergen-induced increases in bone marrow T lymphocytes and interleukin-5 expression in subjects with asthma. *Am J Respir Crit Care Med* 2002;**166**:883–889.
7. HOGAN MB, WEISSMAN DN, HUBBS AF, GIBSON AF, PIKTEL D, LANDRETH KS. Regulation of eosinophilopoiesis in a murine model of asthma. *J Immunol* 2003;**171**:2644–2651.
8. SEHMI R, HOWIE K, SUTHERLAND DR, SCHRAGGE W, O'BYRNE PM, DENBURG JA. Increased levels of CD34⁺ hemopoietic progenitor cells in atopic subjects. *Am J Respir Cell Mol Biol* 1996;**15**:645–655.
9. ROBINSON DS, DAMIA R, ZEIBECOGLOU K, MOLET S, NORTH J, YAMADA T et al. CD34(+) /interleukin-5Ralpha messenger RNA⁺ cells in the bronchial mucosa in asthma: potential airway eosinophil progenitors. *Am J Respir Cell Mol Biol* 1999;**20**:9–13.
10. ZEIBECOGLOU K, YING S, YAMADA T, NORTH J, BURMAN J, BUNGRE J et al. Increased mature and immature CCR3 messenger RNA⁺ eosinophils in bone marrow from patients with atopic asthma compared with atopic and nonatopic control subjects. *J Allergy Clin Immunol* 1999;**103**(1 Pt 1):99–106.
11. MARINI M, VITTORI E, HOLLEMBORG J, MATTOLI S. Expression of the potent inflammatory cytokines, granulocyte-macrophage-colony-stimulating factor and interleukin-6 and interleukin-8, in bronchial epithelial cells of patients with asthma. *J Allergy Clin Immunol* 1992;**89**:1001–1009.
12. BODEY KJ, SEMPER AE, REDINGTON AE, MADDEN J, TERAN LM, HOLGATE ST et al. Cytokine profiles of BAL T cells and T-cell clones obtained from human asthmatic airways after local allergen challenge. *Allergy* 1999;**54**:1083–1093.
13. SHAVER JR, ZANGRILLI JG, CHO SK, CIRELLI RA, POLLICE M, HASTIE AT et al. Kinetics of the development and recovery of the lung from IgE-mediated inflammation: dissociation of pulmonary eosinophilia, lung injury, and eosinophil-active cytokines. *Am J Respir Crit Care Med* 1997;**155**:442–448.
14. NAGATA MYH, TABE K, SAKAMOTO Y, MATSUO H. Eosinophil-adhesion-inducing activity produced by antigen-stimulated mononuclear cells involves GM-CSF. *Int Arch Allergy Immunology* 2000;**122**(Suppl. 1):15–19.
15. ACKERMAN V, MARINI M, VITTORI E, BELLINI A, VASSALI G, MATTOLI S. Detection of cytokines and their cell sources in bronchial biopsy specimens from asthmatic patients. Relationship to atopic status, symptoms, and level of airway hyperresponsiveness. *Chest* 1994;**105**:687–696.
16. ROBINSON DS, YING S, BENTLEY AM, MENG Q, NORTH J, DURHAM SR et al. Relationships among numbers of bronchoalveolar lavage cells expressing messenger ribonucleic acid for cytokines, asthma symptoms, and airway methacholine responsiveness in atopic asthma. *J Allergy Clin Immunol* 1993;**92**:397–403.
17. PHILLIPS PG, CHIKKAPPA G, BRINSON PS. A triple stain technique to evaluate monocyte, neutrophil, and eosinophil proliferation in soft agar cultures. *Exp Hematol* 1983;**11**:10–17.
18. OHKAWARA Y, LEI XF, STAMPFLI MR, MARSHALL JS, XING Z, JORDANA M. Cytokine and eosinophil responses in the lung, peripheral blood, and bone marrow compartments in a murine model of allergen-induced airways inflammation. *Am J Respir Cell Mol Biol* 1997;**16**:510–520.
19. GASPAR ELSAS MI, JOSEPH D, ELSAS PX, VARGAFTIG BB. Rapid increase in bone-marrow eosinophil production and responses to eosinopoietic interleukins triggered by intranasal allergen challenge. *Am J Respir Cell Mol Biol* 1997;**17**:404–413.

20. WOGNUM AW, DE JONG MO, WAGEMAKER G. Differential expression of receptors for hematopoietic growth factors on subsets of CD34⁺ hemopoietic cells. *Leukemia and Lymphoma* 1996;**24**:11–25.
21. BREWER JM, CONACHER M, SATOSKAR A, BLUETHMANN H, ALEXANDER J. In interleukin-4-deficient mice, alum not only generates T helper 1 responses equivalent to Freund's complete adjuvant, but continues to induce T helper 2 cytokine production. *Eur J Immunol* 1996;**26**:2062–2066.
22. BREWER JM, CONACHER M, HUNTER CA, MOHRS M, BROMBACHER F, ALEXANDER J. Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4 or IL-13-mediated signaling. *J Immunol* 1999;**163**:6448–6454.