

IL-5 expression and release from human CD34 cells *in vitro*; *ex vivo* evidence from cases of asthma and Churg–Strauss syndrome

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Keywords

asthma; CD34⁺ progenitor cells; Churg–Strauss; eosinophils; hypereosinophilia; IL-5.

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Abstract

Background: Eosinophils develop from hematopoietic CD34⁺ progenitor cells in the bone marrow (BM) under the influence of Interleukin-5 (IL-5). The primary source of IL-5 is T-lymphocytes, although other sources may exist. The aims of this study were to determine whether CD34⁺ cells from human peripheral blood (PB) and BM have the capacity to produce IL-5 when stimulated *in vitro*, and secondly, whether an elevated number of IL-5-producing CD34⁺ cells can be found *in situ* in ongoing eosinophilic disease.

Methods: CD34⁺ cells from PB and BM were stimulated *in vitro*, and IL-5 production and release was assessed by ELISA, ELISPOT, flow cytometry and immunocytochemistry. Blood and BM from a patient with Churg–Strauss syndrome were analyzed by flow cytometry for CD34⁺/IL-5⁺ cells, and immunohistochemical staining of CD34⁺/IL-5⁺ cells in bronchial biopsies from an asthmatic patient was performed.

Results: Both PB and BM CD34⁺ cells can produce and release IL-5 when stimulated *in vitro*. In the Churg–Strauss patient, IL-5-producing CD34⁺ cells were found in PB and BM. Oral glucocorticoid treatment markedly decreased the number of IL-5-positive CD34 cells in the BM. CD34⁺/IL-5⁺ cells were present in a patient with asthma.

Conclusion: CD34⁺ cells in blood and BM are capable of producing IL-5 both *in vitro* and *in vivo* in humans, arguing that these cells may have the capacity to contribute to eosinophilic inflammation. Consequently, targeting CD34⁺ progenitor cells that produce and release IL-5 may be effective in reducing the mobilization of eosinophil lineage-committed cells in eosinophilic-driven diseases.

Eosinophils are known to develop from hematopoietic CD34⁺ progenitor cells, and interleukin-5 is a key cytokine involved in the differentiation, proliferation and survival of eosinophils. In eosinophilic diseases, the immune response is classically characterized by the development of CD4⁺ T-lymphocytes

producing Th2 cytokines such as IL-4, IL-5 and IL-13 (1–3). Bone marrow (BM) is constantly producing and releasing small numbers of CD34⁺ progenitor cells in the blood (4). In allergic disease, elevated numbers of IL-5 receptor-positive CD34⁺ cells has been found in the BM of both humans and mice (5, 6), in airways and lungs of mice (7, 8) as well as in human sputum (9).

CD34⁺ progenitor cells has the receptor for IL-5 which is a heterodimer consisting of an α -chain that specifically binds IL-5 and a signal-transmitting

Abbreviations

PB, peripheral blood; BM, bone marrow; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot assay.

β_c -chain, which is shared with the receptors for Interleukin-3 (IL-3) and the granulocyte-macrophage colony-stimulating factor (GM-CSF) (10, 11) triggering eosinophil development.

We have previously shown that murine CD34 have the capacity to produce and release IL-5 *in vitro* (12, 13), and an earlier study has demonstrated that blood CD34 cells from asthmatic subjects express more IL-5 mRNA than CD34 cells taken from normal controls (14).

Moreover, IL-5 seems to have its primary activity in the bone marrow, because systemic overexpression of IL-5 is more effective than airway overexpression of IL-5 in the enhancement of eosinophilia (12). Furthermore, anti-IL-5 treatment needs to reach the bone marrow before it can effectively reduce airway eosinophilia (8). Finally, the expression of the IL-5 receptor is downregulated on eosinophils as they enter the lung (15). IL-5 is produced by CD3⁺ lymphocytes in the bone marrow (16) although many other unidentified cells in the bone marrow also express IL-5, as only 20% of the IL-5 mRNA was co-localized to CD3⁺ T-lymphocytes in this study of human asthmatic bone marrow.

Although earlier studies have shown that both bone marrow and blood CD34 cells have the ability to produce and release IL-5, no studies have determined the relative release of IL-5 compared to the CD3 T-Lymphocytes from the same compartment. Thus, the aim of this study was to determine whether CD34⁺ progenitor cells from both bone marrow and blood express IL-5 to the same relative content as CD3⁺ cells. Furthermore, we also asked whether CD34⁺ cells are able to produce and release IL-5 in the blood and BM of a patient with Churg–Strauss syndrome as well as in the bronchial tissue of a patient with allergic persistent asthma.

Methods

Sample collection

Peripheral blood (PB)

Human buffy coat preparations were obtained from healthy blood donors at the Department of Clinical Chemistry and Transfusion Medicine, Sahlgrenska University Hospital, Göteborg, Sweden. The peripheral blood mononuclear cell population was collected by density centrifugation over a Ficoll-PaqueTM Plus gradient (Amersham Biosciences, Uppsala, Sweden)

Bone marrow (BM)

After local anesthesia, 30 ml of BM was collected from the posterior iliac crest of four human subjects with no

known allergy, using a disposable BM aspiration needle. (Medical Device Technologies Inc. FL, USA). Mononuclear cells were collected as described earlier.

Clinical samples

Peripheral blood was donated by a 53-year-old female with Churg–Strauss syndrome (CCS) that was diagnosed and treated at the Sahlgrenska University Hospital in Gothenburg. The patient was referred to us from primary health care with persistent asthma that responded poorly to high dose of combined therapy with LABA/ICS [salmeterol/fluticasone propionate (50/500 ug) twice daily]. Upon admission, further evaluation revealed a history of sustained blood eosinophilia (>20%) in the last 2 years and chronic sinusitis, for which the patient had undergone surgery without significant improvement. Clinical examination revealed pain in both upper and lower extremities that was accompanied with purpura in one arm. Further laboratory evaluation showed FEV₁ of 2.5 l (92% of predicted) and a normal chest x-ray. Blood eosinophilia of 25% (WBC: $11.2 \times 10^9/l$, eosinophils: 2.8) was found.

Based on the earlier mentioned findings, a differential diagnosis of Churg–Strauss syndrome was suspected, as the patient fulfilled four of six 1990 ACR criteria (asthma, eosinophilia > 10%, neuropathy, nonfixed pulmonary infiltrates, paranasal sinus abnormality and extravascular eosinophils) (17, 18). However, as diagnosis of CSS is problematic, a biopsy of the affected skin tissue was also taken to examine the existence of an underlying vasculitis. Upon confirmation of vasculitis, a diagnosis of CCS was established, and the patient was called for a new visit. Anti-neutrophil cytoplasmic antibodies (ANCA) was not performed, as in two recent studies on large cohorts, ANCAs were present only in 38% of the patients (17, 18) while Churg–Strauss was mainly associated with vasculitis manifestations, which was already established in our patient.

On the second visit, the patient had blood eosinophilia of 22% (WBC: $8.7 \times 10^9/l$, eosinophils: $1.9 \times 10^9/l$). Furthermore, bone marrow aspiration was performed that demonstrated 25.5% eosinophils. At this point, a treatment with oral steroids was initiated. Because the patient had shown intolerance of such high doses on previous therapeutic occasions, she was administered 30 mg (0.5 mg/kg) prednisolone per day instead of the usual 1 mg/kg/day dosage. The patient continued the treatment for 6 weeks, after which a control visit was planned.

Upon visit three (after 6 weeks), the patient was clinically improved, and blood eosinophilia had disappeared. Therefore, a lower prednisone treatment was

administered for an additional 15-week period. At visit four, further evaluation showed no blood eosinophilia, and a new bone marrow aspiration demonstrated only 1% eosinophils. At this point, oral steroid treatment was stopped.

At visit two and four, as already mentioned, blood and bone marrow aspiration samples were obtained, and surface expression of CD34 and IL-5Ra and intracellular expression of IL-5 were evaluated by flow cytometry.

Bronchial biopsies were also obtained from a patient with moderate persistent allergic asthma. The patient had positive skin prick tests for 3 out of the 18 common aeroallergens tested (HAL Allergy, Benelux) and a current blood eosinophilia of 6.13% (WBC: $4.96 \times 10^9/l$, eosinophils: $0.3 \times 10^9/l$). The patient was recruited from the Athens Chest Hospital, and asthma severity was assessed according to GINA classification (19). Four endobronchial biopsies approximately 4–5 mm thick were obtained from various sites of the sub segmental carina of the right middle lobe, according to established guidelines (20).

Production and release of IL-5

Stimulation of CD34⁺ cells and CD3⁺ lymphocytes in vitro

Mononuclear cells from PB and BM were labeled with a hapten-modified anti-CD34 monoclonal antibody (mAb) (QBEND/10, MACS, Miltenyi Biotec GmbH, and Bergisch Gladbach, Germany). After washing, anti-hapten microbeads were added in accordance with the manufacturer's instructions, and CD34⁺ cells were enriched by magnetic separation. CD3⁺ lymphocytes from PB and BM were enriched from the CD34-depleted cell fraction using CD3 MicroBeads in accordance with the manufacturer's instructions (Miltenyi Biotec GmbH).

The CD34⁺- and CD3⁺-enriched and the CD3- and CD34-depleted cell fractions from PB and BM were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 µg/ml penicillin–streptomycin, 2 mM L-glutamine and 100 µg/ml sodium pyruvate (from Sigma–Aldrich, St Louis, MO, USA) at 0.2×10^6 cells/200 µl. Calcium ionophore (CI, 1 µg/ml, batch A23187, Sigma–Aldrich) and 2 ng/ml phorbol–12-myristate-13-acetate (PMA) (Sigma–Aldrich) were used for stimulation. The cells were incubated for 20 h in 5% CO₂ at 37°C before IL-5 protein measurement in the supernatant (IL-5 ELISA kit; R&D Systems, Inc., Abingdon, UK). An ELISPOT assay was performed using a reagent set for IL-5 (BD Bioscience) in accordance with the manufacturer's instructions.

CD3⁺ lymphocytes, CD34⁺ cells or the remaining cell fraction from BM and PB were seeded at a density of 0.2×10^6 cells to ImmunoSpot® plates (BD Biosciences). The spots were enumerated using a dissecting microscope at a magnification of 40× (CETI, Antwerpen, Belgium).

After magnetic separation of BM and PB, the enrichment of CD34⁺ cells was 63% in BM vs 60% in PB with the involvement of approximately 3% vs 9% of CD3⁺ lymphocytes. The purity of the BM and PB CD3⁺ lymphocyte fraction was 95% vs 96%, and no CD34⁺ cells were detected. The remaining cell fraction, CD34[−]/CD3[−], contained 3% CD3⁺ lymphocytes in BM and 10% in PB. No CD34⁺ cells were detected in this fraction as assessed by immunohistochemistry (See method below)

Flow cytometry analysis for IL-5

CD34⁺- and CD3⁺-enriched cell fractions (0.5×10^6) from PB were cultured as described earlier, and the monensin-based protein transport inhibitor (GolgiStop; BD Biosciences) was added during the final 6 h. Flow cytometry analysis was performed as previously described (21) with some minor modifications. The antibodies used were anti-CD8 (clone SK1), anti-CD4 (clone SK3) or anti-CD34 class III (clone 8G12) conjugated with peridinin chlorophyll protein (PerCP), anti-CDw125-IL5Ra (clone A14) conjugated with phycoerythrin (PE) for surface staining (from BD Biosciences) and anti-IL-5 carboxyfluorescein (CFS) conjugated for intracellular staining (clone 22 026, R&D Systems) or their isotype-matched control. The cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Ten thousand cells were computed in a list mode and analyzed using CELLQUEST software (BD Biosciences). Cell viability was assessed by staining with 7-amino-actinomycin D (7-AAD) (BD Biosciences). In one experiment, cells were stained with anti-CD69 PerCP (clone L78, BD Biosciences), to evaluate PMA/CI activation.

The purity of the gated magnetically separated unstimulated and stimulated CD3⁺ lymphocytes was 99.4% vs 93.4%, while for the gated CD34⁺ cells, the purity was 94.6% vs 95.9%.

The viability was 99% vs 94% in CD3⁺ unstimulated vs stimulated lymphocytes and 95% vs 92% in CD34⁺ unstimulated/stimulated cells. Stimulation with PMA/CI increased the activation level of the cells from 6% to 93%, as measured by the expression of CD69.

The donated PB and BM from the Churgh–Strauss patient were immediately stained for surface expression with anti-CD34 PerCP and anti-CDw125-IL5RA PE

and intracellular with an anti-IL-5 CFS conjugated as described earlier.

Immunocytochemical detection of CD34⁺ cell and CD3⁺ lymphocyte enrichment

Cytospin preparations from each cell fraction of CD34⁺, CD3⁺ and CD34[−]/CD3[−] were fixed in acetone, and endogenous peroxidase was blocked. Nonspecific binding was blocked with 5% rabbit serum (DAKO, Glostrup, Denmark), followed by incubation with mouse anti-human CD34 mAb (BIRMA-K3 and BI-3C5, DAKO) or mouse anti-human CD3 mAb (T3-4B5, DAKO) or isotype control mouse IgG₁ (MOPC-21, Sigma-Aldrich). As secondary antibody, peroxidase (HRP)-conjugated rabbit anti-mouse IgG₁ (Zymed Laboratories Inc, San Francisco, CA, USA) was added, and the bound antibody was visualized by Liquid DAB Substrate-Chromogen system (DAKO, Carpinteria, CA, USA) followed by counterstaining with Mayer's hematoxylin (Sigma-Aldrich).

Immunocytochemical detection of intracellular IL-5 in CD34⁺ cells

Bone marrow mononuclear cells obtained by density separation were stimulated as mentioned earlier, and a monensin-based protein transport inhibitor (Brefeldin A 10 µg/ml; Sigma-Aldrich) was added to the cell culture during the final 7 h. Cells were harvested, washed and fixed in 2% formaldehyde on ice for 30 min before cytopins were made.

Double staining of CD34 antigen and intracellular IL-5 was based on two individually complete staining procedures. To detect CD34, the same protocol as mentioned earlier was used substituting the secondary step with an alkaline phosphatase-conjugated (19) rabbit anti-mouse IgG₁ (Zymed Lab) and using a Vector Red alkaline phosphatase substrate kit (Vector Laboratories Inc., Burlingame, CA, USA) for detection. For the intracellular staining of IL-5, all antibody dilutions were supplemented with 1% saponin (Sigma-Aldrich), and the incubations were performed at 37°C. Nonspecific staining was blocked by 5% donkey serum (Jackson Immuno Research Laboratories Inc. West Grove, PA, USA) followed by blocking endogenous biotin using Biotin blocking system (DAKO). The preparations were incubated with a rat anti-mouse IL-5 mAb (clone TRFK-5) or isotype control rat IgG₁ (both BD Biosciences), and as secondary step, a biotin-conjugated donkey anti-rat IgG antibody (Jackson IR) was used, followed by incubation with streptavidin-β-galactosidase (Roche Diagnostics GmbH, Germany) and visualization by a β-Gal staining set (Roche Diagnostics).

Detection of CD34⁺ cells and IL-5 in bronchial biopsies

Bronchial biopsies were fixed in 4% formaldehyde solution overnight and embedded in paraffin. Sections of 4–5 µm thickness were deparaffinized and rehydrated followed by antigen retrieval treatment using citrate buffer (pH 6) at 95°C. Endogenous peroxidase was quenched, and unspecific staining was blocked followed by incubation with two rat anti-human IL-5 antibodies (JES1-39D10 and TRFK5, BD Biosciences). Mouse anti-human CD34 monoclonal antibody (QBend10; DAKO) was added to the sections for 1 h followed by the secondary antibodies donkey anti-rat IgG-biotin and fluorescein isothiocyanate-conjugated (FITC) donkey anti-mouse IgG (Jackson IR). Sections were subsequently incubated with ExtrAvidin-HRP and rabbit anti-FITC-AP (Sigma-Aldrich), and antigens were visualized by Liquid DAB Substrate-Chromogen system and Liquid Permanent Red Chromogen (DAKO). Matched isotype controls were used at the same protein concentration as the respective antibodies. Finally, Mayer's hematoxylin was used for counterstaining.

Statistical analysis

Data are expressed either as mean ± SEM or as means with 95% confidence intervals (CI). Statistical analysis was carried out by using the nonparametric analysis Mann-Whitney *U*-test. *P*-value of <0.05 was considered statistically significant.

Results

IL5-producing CD34⁺ and CD3⁺ cells in peripheral blood

Peripheral blood CD34⁺ cells and CD3⁺ lymphocytes released IL-5 when cultured *in vitro* (Fig. 1A). Furthermore, stimulated CD34⁺ cells and CD3⁺ lymphocytes released more IL-5 protein than nonstimulated controls (774 ± 147 pg/ml vs 25 ± 24 pg/ml, *P* < 0.02 and 199 ± 25 pg/ml vs 2.0 ± 0.9 pg/ml, *P* < 0.003; Fig. 1A). CD34⁺ cells released significantly more IL-5 than the same number of CD3⁺ lymphocytes (774 ± 147 pg/ml vs 199 ± 25 pg/ml, *P* < 0.008; respectively). The cell fraction that had been depleted of CD34 and CD3 cells released minor quantities of IL-5 (10.8 ± 3.4 pg/ml vs 1.6 ± 1.2 pg/ml *P* < 0.01).

Magnetically separated CD34⁺ cells from blood donors by flow cytometry analysis showed the presence of CD34⁺/IL-5-producing cells. When stimulated with PMA and calcium ionophore, the CD34 cells produced significantly more IL-5 compared to unstimulated cells

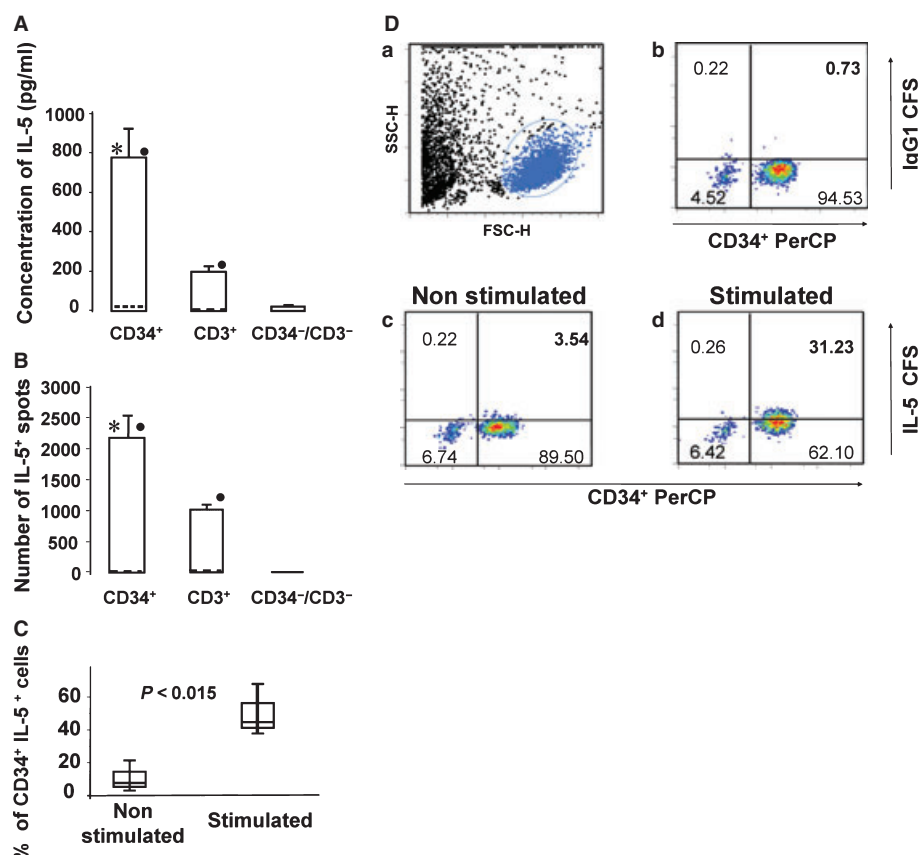


Figure 1 Expression of IL-5 in enriched CD34⁺ cells from PB after stimulation of Calcium Ionophor and PMA *in vitro*. (A) Secretion of IL-5 measured by ELISA. (B) IL-5-positive ELISPOTS after stimulation. Broken lines indicate release from nonstimulated cultures. Data is expressed as mean $\pm$ SEM ($n = 4-8$). * $P < 0.05$ CD34⁺ vs CD3⁺ $P < 0.05$ stimulated vs nonstimulated cells (Mann-Whitney U -test). (C) Percentage of IL-5-positive CD34⁺ cells detected by flow cytometry. Data is shown as med-

ian $n = 3$ $P < 0.01$. (D) Representative plots of intracellular staining of IL-5 in PB CD34⁺ (expressed as %) as detected by flow cytometry. (a) The gate was set on intact cells by forward- and side-scatter light emission; (b) plotted by CD34⁺/isotype control IgG1- CFS (c) unstimulated CD34⁺ cells cultured with medium as a control and (d) CD34⁺ cells stimulated with PMA/Ci. The numbers in the quadrants represent percentage of gated cells in plot 4a.

50.4% (95%CI 32.6–68.2) vs 11.1% (95% CI 0.3–21.9) $P < 0.05$ Fig. 1C,D). CD3 lymphocytes had increased expression of IL-5 after the same stimulation (77.6% vs 59.1%). Analysis of expression of IL-5 in CD3 cell subset, demonstrated an enhancement of IL-5 in stimulated CD4⁺ lymphocytes vs unstimulated 34.6% (95% CI 13–56.2) vs 23.8% (95% CI 2–45.6) $P < 0.27$. Interestingly, CD8⁺ lymphocytes produced only baseline IL-5 and could not be stimulated to express more IL-5 in two out of three buffy coats 35.4% (95% CI 13.857.0) vs 43.1%. (95% CI 29.3–57.1) $P < 0.7$

In the ELISPOT assay, the PB CD34⁺ cell fraction produced IL-5 when stimulated, as shown by 2130 ± 158 positive spots after seeding 2×10^5 cells vs 6 ± 2 spots in nonstimulated controls, $P < 0.01$; (Fig. 1B). The CD3⁺ stimulated lymphocyte fraction

formed 1006 ± 42 positive spots after seeding 2×10^5 cells vs 12 ± 1.5 positive spots in nonstimulated controls ($P < 0.001$). The CD34⁻/CD3⁻ cell fraction produced very few spots in this assay (3 ± 0.3 vs 3 ± 1.2 , $P < 0.3$).

IL5-producing CD34⁺ and CD3⁺ cells in bone marrow

Bone marrow CD34⁺ cells and CD3⁺ lymphocytes released IL-5 when cultured *in vitro*. Stimulated CD34⁺ cells and CD3⁺ lymphocytes released significantly more IL-5 than nonstimulated controls (101 ± 16 pg/ml vs 22 ± 3 pg/ml, $P < 0.005$ and 184 ± 26 pg/ml vs 11 ± 3 pg/ml, $P < 0.0001$; respectively, Fig. 2A). No significant difference was observed in the amount of IL-5 being released from the stimu-

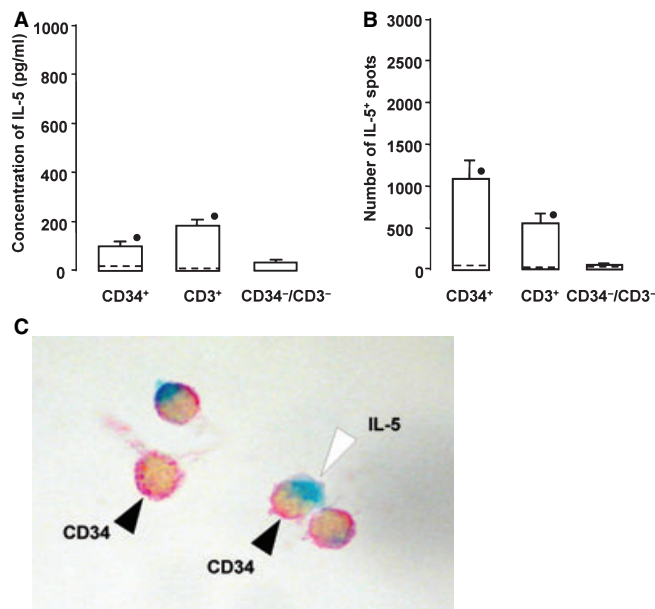


Figure 2 Expression of IL-5 in enriched CD34⁺ cells from BM after stimulation by PMA and CI *in vitro*. (A) Secretion of IL-5 measured by ELISA. (B) IL-5-positive ELISPOTS after stimulation. Broken lines indicate release from nonstimulated cultures. Data expressed as

mean $\pm$ SEM ($n = 4$). * $P < 0.05$ CD34⁺ vs CD3⁺ $P < 0.05$ stimulated vs nonstimulated cells (Mann–Whitney U test). C. Photomicrograph of BM CD34⁺ cells with expression of intracellular IL-5 (closed arrow). Magnification $\times 1000$.

lated CD34⁺ cells vs the CD3⁺ lymphocytes $P < 0.12$ (Fig. 2A). The CD34⁻/CD3⁻ cell fraction released small concentrations of IL-5 (25 ± 5 pg/ml vs 11 ± 3 pg/ml, $P < 0.09$).

In the ELISPOT assay, the BM CD34⁺ cell fraction released IL-5 when stimulated, as shown by 1091 ± 365 positive spots after seeding 2×10^5 cells vs 35 ± 21 spots in nonstimulated controls ($P < 0.006$; Fig. 1D). The stimulated CD3⁺ lymphocyte fraction formed 553 ± 94 spots after seeding 2×10^5 cells vs 21 ± 10 in nonstimulated controls ($P < 0.01$). The stimulated CD34⁻/CD3⁻ cell fraction produced 45 ± 5 positive spots vs 31 ± 10 in nonstimulated controls $P < 0.31$ (Fig. 2B).

The capability of BM CD34⁺ cells to express IL-5 protein was confirmed by immunocytochemistry using double staining with CD34 and IL-5 (Fig. 2C).

CD34 cells and IL-5 production in a patient with Churg–Strauss syndrome

Flow cytometry analysis demonstrated that the mononuclear CD34⁺ progenitors represent 0.42% of total leukocytes in blood and 0.7% of total cells in BM, which was reduced to 0.25% and 0.35%, respectively, during oral prednisolone treatment. Importantly, 49.9% of blood CD34⁺ cells and 30.5% of BM CD34⁺ cells expressed intracellular IL-5, which was reduced to 8.68% and 4.17%, respectively, during oral prednisolone

treatment (Fig. 3). Furthermore, 19.2% of blood CD34⁺/IL-5⁺ cells and 16.94% of BM CD34⁺/IL-5⁺ cells also expressed the IL-5Ra, reduced to 3.85% and 2.08%, respectively, after steroid treatment.

CD34⁺ cells and IL-5 in an asthmatic patient

Immunohistochemical staining of CD34 and IL-5 of biopsies from a patient with asthma demonstrate cells that are double positive (Fig. 4)

Discussion

Using several approaches, this study shows that human blood and bone marrow CD34⁺ progenitor cells have the ability to produce and release IL-5 protein *in vitro* and that a patient with Churg–Strauss syndrome had IL-5-positive CD34 cells in both blood and bone marrow. Furthermore, we have documented, by immunohistochemistry, that CD34 cells in bronchial mucosa can express IL-5. It seems that the production of IL-5 per cell can be as high in CD34 cells as in CD3 cells, under stimulated conditions.

We have previously shown that CD34 cells from mice have the capacity to produce and release IL-5 *in vitro* (13), and the current study supports the notion that human CD34 cells can behave in a similar manner.

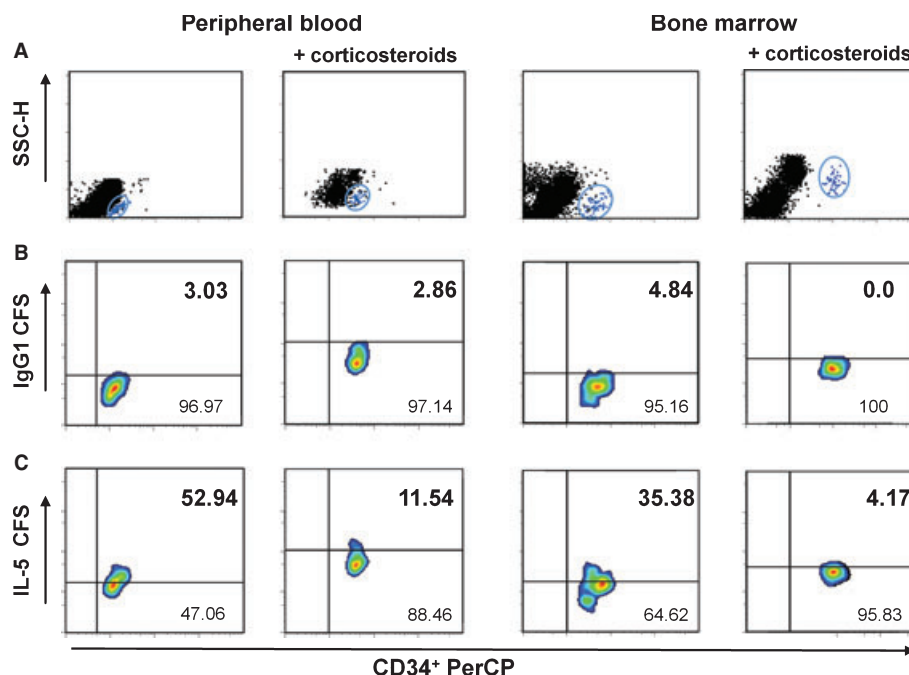


Figure 3 Representative plots of intracellular staining of IL-5 in PB and BM CD34⁺ in Churg–Strauss patient, before and after steroid treatment. (A) CD34⁺ cells (blue) were gated in mononuclear fraction against isotype control (black). (B) CD34⁺ cells were plotted by

CD34⁺/isotype Control IgG1-CFS, and finally (C) CD34⁺ cells were stained for IL-5 expression. The numbers in the quadrants represent percentage of gated CD34⁺ cells in plot 3A.

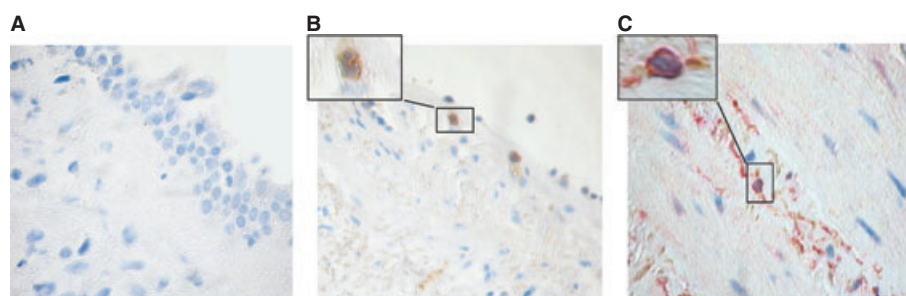


Figure 4 Bronchial biopsies obtained from a patient with moderate persistent asthma and prominent blood eosinophilia. The biopsies were immunohistochemically stained for IL-5 and CD34⁺ cells (A)

Isotype control (B) CD34⁺ progenitor cell. (C) CD34⁺/IL-5-positive cell. Magnification $\times 100$ and 400 .

Moreover, we have taken the finding of IL-5 release from blood CD34 cells to show that also bone marrow CD34 cells from healthy individuals can release IL-5 when stimulated. In general, the baseline expression of IL-5 in CD34⁺ cells was not prominent (11.1%), but had a potential to be substantially increased after non-specific stimulation. The ability of blood and bone marrow CD34 cells to produce and release IL-5 was strongly documented, by *in vitro* cell culture experiments showing release of protein, as well as by flow cytometry showing upregulation of protein expression intracellular. Lastly, the presence of IL-5 in unstimulated blood and bone marrow samples from a patient

with hypereosinophilia confirmed that the CD34 cells could produce IL-5 *in vivo* in humans.

When comparing the capacity of blood and bone marrow CD34 cells to produce and release IL-5, it seems that a larger relative number of blood CD34 cells have the capacity to produce and release IL-5 compared to bone marrow CD34 cells, as documented by both ELISPOT analysis and protein release assays. Furthermore, when analyzing the same number of CD34 cells from blood and bone marrow taken from healthy individuals, it was evident that the blood CD34 cells released more IL-5 per cells *in vitro*. Importantly, it has to be understood that the relative ability of the CD34

cells to produce IL-5 may be different in different diseases, and in fact, we could document that a significant proportion of BM CD34⁺ cells taken from a Churg–Strauss patient do express IL-5. To our knowledge, this is the first study directly comparing the ability of human CD34 cells from blood and bone marrow to release IL-5.

In the current study, we carefully compared the ability of CD34 and CD3 cells to produce and release IL-5. Interestingly, the blood CD34 cells produced more IL-5 per cell than the CD3 cells from the same location. By contrast, bone marrow CD3 and CD34 cells produced similar amounts of IL-5 per cell. Conceptually, the T-lymphocyte is a major source of IL-5 in humans and mice, but the current study argues that the CD34 cell may be an important source as well. The pivotal role of CD4⁺ T-lymphocytes is well established, and as expected, we were able to enhance the IL-5 production in this subpopulation with stimulation. Interestingly, in the same experiment, the unstimulated CD8⁺ T-lymphocyte population in PB contained even more IL-5-producing cells, but this expression could not be further enhanced. CD8⁺ T-lymphocytes have recently been shown to have the capacity to secrete Th2 cytokines in allergic airway inflammation (22, 23). In addition, CD8⁺ T-lymphocytes from mild asthmatic patients can be stimulated to produce IL-5 *in vitro* (22). Furthermore, we recently showed in a mouse model that CD8⁺ T-lymphocytes can regulate BM eosinophilopoiesis, both at baseline and after allergen exposure, suggesting that CD8⁺ T-lymphocytes partly regulate eosinophil production in the bone marrow (24). However, further investigation is required to determine the direct role of IL-5-producing CD8⁺ T-lymphocytes in human eosinophilopoiesis.

To further determine any ability of CD34 cells to produce IL-5, we analyzed blood and bone marrow CD34⁺ cells from a patient diagnosed with Churg–Strauss syndrome. In flow cytometric analysis, we were able to confirm that approximately half of the CD34⁺ cells in peripheral blood were indeed expressing IL-5 protein. In addition, approximately 30% of the patient's bone marrow CD34⁺ cells expressed IL-5 protein. These two findings together argue that CD34⁺ cells can be an important source of IL-5 also in humans. The possible role of the IL-5 production by CD34⁺ cells in the Churg–Strauss patient is supported by the data showing a strong reduction in CD34/IL-5 expression when treated with oral glucocorticoids. Mepolizumab, an anti-IL-5 monoclonal antibody, has been tested in patients with hypereosinophilic syndrome (25), but is not yet available to be tested in patients with Churg–Strauss syndrome. However, as this data is based only on one patient,

further research is needed to determine whether overexpression of IL-5 in patients with different hypereosinophilic diseases may be an important cause of the disease.

As CD34 cells in sputum from asthmatic individuals have been shown occasionally to express IL-5 (9), we performed immunohistochemistry of CD34 and IL-5 in bronchial biopsies from a patient with allergic asthma, in fact documenting the presence of double-positive cells in the mucosa. Thus, the local IL-5 production in the airways may in part be attributed to local CD34 cells. Growing evidence argues that CD34⁺ progenitor cells are important in allergic inflammation, as eosinophil progenitors have increased numbers in nasal mucosa, peripheral blood and bone marrow in patients with allergic rhinitis and asthma (26–29). Furthermore, it has recently been shown that CD34 cells present in the sputum of patients with asthma express both IL-5 and IL-13 and increase in numbers after airway allergen exposure (29). Revealing the exact interaction between the CD34⁺ and other inflammatory cells in the airways during allergic inflammation may further improve our understanding of the mechanisms of the disease, as it seems possible that CD34 cells may act as inflammatory effector cells by themselves, directly contributing to allergic inflammation (29). Several studies have proposed that eosinophilic progenitor cells can also mature, and perhaps proliferate, within the site of allergic inflammation (9, 27–31). Thus, local tissue CD34⁺ progenitor cells can complete their maturation into eosinophils following *ex vivo* stimulation with IL-5 or allergen, suggesting the possibility of local eosinophil production (9, 31). This can occur in parallel with enhanced BM eosinophilopoiesis, followed by the release of newly produced eosinophils from the bone marrow into the circulation, allowing them to traffic into the lungs and airways. It is likely that this is a major source of eosinophils (5, 6, 21, 32–35).

Taken together, we found that CD34 cells from different sources can produce and release IL-5, and we could determine CD34 cells that express IL-5 in both a Churg–Strauss patient as well as in a patient with allergic persistent asthma. However, the importance of these observations in the understanding of the fundamental mechanisms regulating eosinophil progenitors and hematopoiesis *in situ* and the prospect of potentially beneficial therapies of eosinophilic-driven diseases needs to be further investigated.

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