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# Eosinophils Express Functional IL-13 in Eosinophilic Inflammatory Diseases<sup>1</sup>

Peter Schmid-Grendelmeier,<sup>2\*†</sup> Frank Altnauer,<sup>\*‡</sup> Barbra Fischer,<sup>\*§</sup> Christian Bizer,<sup>§</sup> Alex Straumann,<sup>¶</sup> Günter Menz,<sup>§</sup> Kurt Blaser,<sup>\*§</sup> Brunello Wüthrich,<sup>†</sup> and Hans-Uwe Simon<sup>\*‡</sup>

**IL-13 is an immunoregulatory and effector cytokine in allergic diseases such as bronchial asthma. A variety of immune and non-immune cells are known as IL-13 producers. In this study we investigated whether and under what conditions human eosinophils generate IL-13. Freshly isolated highly purified peripheral blood eosinophils from patients with several eosinophilic inflammatory diseases and from normal control individuals were investigated. We observed that blood eosinophils from patients suffering from bronchial asthma, atopic dermatitis, parasitic infections, hypereosinophilic syndrome, and idiopathic eosinophilic esophagitis expressed IL-13, as assessed by ELISA, ELISPOT assay, flow cytometry, and immunocytochemistry. By using nasal polyp tissues and immunohistochemistry, we demonstrated IL-13 expression in eosinophils under in vivo conditions. In contrast, blood eosinophils from control individuals as well as blood neutrophils from both eosinophilic and control patients did not produce detectable IL-13 levels. However, when blood eosinophils from control individuals were stimulated with GM-CSF or IL-5 in vitro, they generated IL-13 mRNA and protein, suggesting that IL-13 expression by eosinophils under inflammatory conditions is a cytokine-driven process. Stimulation of blood eosinophils containing IL-13 by eotaxin resulted in a rapid release of this cytokine. Eosinophil-derived IL-13 was functional, as it increased the surface expression of the low affinity IgE receptor (CD23) on purified B cells. In conclusion, human eosinophils are able to produce and release functional IL-13 in eosinophilic inflammatory responses. *The Journal of Immunology*, 2002, 169: 1021–1027.**

**E**osinophils predominate within cellular infiltrates of many parasitic and allergic diseases. Several mechanisms are involved in this process, such as increased eosinophil production in the bone marrow, preferential recruitment, chemotactic factors, and delayed apoptosis (1). At the site of inflammation, eosinophils release toxic cationic proteins upon stimulation, a process that has been thought to be important in host defense (2). Tissue damage caused by eosinophil granule proteins might also be important in the pathophysiology of asthma, atopic dermatitis, and other chronic allergic diseases (3). Besides their role as effector cells, eosinophils appear to be important immunoregulatory cells as they produce and release cytokines, at least under certain conditions (4, 5). The production of these cytokines might be important for the initiation and progression of eosinophilic inflammatory responses. The diversity of cytokine production by activated eosinophils is surprising, and it is possible that some in vitro findings may not be relevant under in vivo conditions (5).

IL-13 is a cytokine that regulates inflammatory and immune responses (6, 7) and shares many similarities with IL-4, which can be explained by the existence of a common receptor subunit ( $\alpha$ -

chain) of IL-13 and IL-4 receptors (8, 9). Therefore, IL-13 acts like IL-4 on B cells and stimulates both proliferation and IgE synthesis in these cells (10). However, there are also differences between the two cytokines. For instance, IL-4, but not IL-13, is able to differentiate naive T cells into Th2 cells (8). On the other hand, IL-13, but not IL-4, appears to be an effector cytokine that directly contributes to bronchial hyperreactivity and mucus overproduction in asthma (11–13). Besides the expression on B cells and structural bronchial cells, IL-13Rs have been observed on macrophages, dendritic cells, immature mast cells, basophils, and eosinophils (9, 14). The presence of IL-13Rs on human T cells is still under debate (8, 15).

IL-13 has been shown to be produced by T cells, mast cells, basophils, dendritic cells, and keratinocytes (16–18). In this study we investigated whether eosinophils and neutrophils are able to generate this cytokine. We obtained evidence that eosinophils, but not neutrophils, can generate functionally active IL-13 as a consequence of GM-CSF and/or IL-5 exposure under in vitro, ex vivo, and in vivo conditions.

## Materials and Methods

### Patients

We investigated blood eosinophils from a total of 80 patients and 9 control individuals. Groups of patients with bronchial asthma (BA),<sup>3</sup> atopic dermatitis (AD), idiopathic eosinophilic esophagitis (IEE), idiopathic hypereosinophilic syndrome (HES), parasitic infections associated with eosinophilia (parasitemia), and healthy control individuals were studied (Table I). The actual numbers of patients enrolled for the different assays are indicated in each case. All asthmatic subjects had a 20% decrease in forced expiratory volume 1 s after the inhalation of <8.0 mg histamine/ml, showed a reversibility of this obstruction after inhalation of salbutamol,

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<sup>3</sup> Abbreviations used in this paper: BA, bronchial asthma; AD, atopic dermatitis; APAAP, alkaline phosphatase-anti-alkaline phosphatase; C, healthy control donor; HES, hypereosinophilic syndrome; IEE, idiopathic eosinophilic esophagitis; PFA, paraformaldehyde.

Table I. Characteristics of patients and controls

Diagnosis	No. of Patients	Sex (male/female)	Age (years) <sup>a</sup>	Atopy (positive/negative) <sup>b</sup>	Total IgE (IU/ml) <sup>a</sup>	Blood Eosinophils (%) <sup>a</sup>
BA	23	6/17	43.3 (19–60)	13/10	197 (75–500)	11.9 (5–26)
BA and AD	24	7/17	25.3 (19–63)	15/3	858 (130–4010)	10.5 (6–22)
AD only	13	9/4	36.7 (20–52)	7/6	444 (130–1020)	7.5 (3–11)
HES	7	3/4	39.4 (21–63)	0/7	105 (30–185)	19.4 (13–33)
IEE	9	4/5	41.6 (25–53)	0/9	113 (20–145)	8.9 (6–11)
Parasitic infection	4	2/2	30.7 (22–39)	1/3	625 (295–980)	15.5 (12–21)
Healthy controls	9	3/4	38.8 (29–62)	0/9	71 (25–110)	4.08 (2–7)

<sup>a</sup> Mean value (range of patients).

<sup>b</sup> Positive subjects had at least one positive skin prick or radioallergosorbent/celluloseallergosorbent test.

and met the American Thoracic Society definition of asthma (19). All patients with AD fulfilled the diagnostic criteria of Hanifin and Rajka (20). Some patients with AD had also asthma (BA+AD). Patients with IEE were clinically, endoscopically, and histologically diagnosed (21). HES patients had idiopathic blood eosinophilia ( $>1500$  eosinophils/mm<sup>3</sup>); some of them had an underlying clonal T cell disease (22). All parasitemia patients suffered from infections with the helminth *Strongyloides stercoralis* (23). Controls were healthy, matched for age and sex, and had normal IgE levels and eosinophil numbers. At the time of the study neither patients nor control individuals received systemic corticosteroid treatment. Informed consent was obtained from both patients and controls, and the study was approved by the Swiss Academy of Medical Science represented by the medical ethics committee of Davos.

#### Collection of blood

Heparin anti-coagulated blood (50–100 ml) was collected under standard hospital-approved protocols for immunologic monitoring between 0700 and 0800 h.

#### Cell purification

PBMC were separated from peripheral blood cells by Ficoll Hypaque centrifugation (Seromed-Fakola, Basel, Switzerland). B cells were negatively isolated from PBMC (23) using MACS (B cell isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany). The resulting cell population contained 95–98% B cells as determined by two-color flow cytometry using anti-CD19 and anti-CD3 mAbs. For granulocyte isolation, the lower phase after Ficoll-Hypaque centrifugation was treated with erythrocyte lysis solution as previously described (24). The granulocyte population of normal control individuals contained  $>95\%$  neutrophils as determined by staining with DiffQuick (Baxter, Duding, Switzerland) and light microscopy. The granulocyte populations from eosinophilic patients used for the experiments had  $>90\%$  neutrophils with some eosinophil contaminations. These granulocyte populations were considered neutrophils. To purify eosinophils, the granulocyte population was incubated with anti-CD16 mAb microbeads (Miltenyi Biotec). CD16<sup>+</sup> neutrophils were depleted by passing the granulocytes through MACS (25). The resulting cell population contained  $>98\%$  eosinophils.

#### Eosinophil cultures

Eosinophils were cultured at  $1 \times 10^6$ /ml in the presence or the absence of GM-CSF (25 ng/ml), IL-5 (25 ng/ml), IL-4 (25 ng/ml), IFN- $\gamma$  (100 ng/ml), eotaxin (25 ng/ml), IL-13 (25 ng/ml; all from R&D Systems, Abingdon, U.K.), calcium ionophore A23187 ( $10^{-7}$  M), and C5a ( $10^{-8}$  M; all from Sigma, Buchs, Switzerland) for the indicated times. Except for the IL-13 release experiments, in which medium 199 (Life Technologies, Basel, Switzerland) was applied, complete culture medium (RPMI 1640 supplemented with 2 mM L-glutamine, 200 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10% FBS; all from Life Technologies) was used.

#### B cell cultures

B cells were cultured in complete culture medium in the presence or the absence of pooled eosinophil lysates ( $1 \times 10^7$  cells/500  $\mu$ l) derived from healthy controls (no detectable IL-13 levels) or asthmatic patients (containing IL-13 at 450 pg/ml, as assessed by ELISA) in two different concentrations (B cells/Eos lysates either 1/1 or 2/1 (v/v)) for the indicated times. As controls, 1 ng/ml recombinant human IL-13 or 100  $\mu$ g/ml mAbs against IL-4 (8 F12; anti-IL-4) and IL-13 (anti-IL-13; both from BD PharMingen, Heidelberg, Germany) or isotype-matched control mAb (also

from BD PharMingen) were added to B cell cultures. CD23 expression was measured in triplicate by flow cytometry.

#### RT-PCR

RNA was isolated from  $10 \times 10^6$  eosinophils or neutrophils after culturing the cells for 3 and 6 h at the indicated conditions. As a positive control, we used PMA- and anti-CD28-stimulated PBMC (6). RNA was isolated using TRIzol solution (Life Technologies) according to the manufacturer's instructions. RT-PCR was performed using 2  $\mu$ l RNA and the Titan One-Tube RT-PCR System (Roche, Mannheim, Germany) in a final volume of 25  $\mu$ l. Primers for IL-13 were obtained from Stratagene (Amsterdam, The Netherlands): sense primer, 5'-GCATCCGCTCCTCAATCCTCT-3'; anti-sense primer, 5'-CCGTCCCTCGCGAAAAAGTTT-3'. RT was performed at 50°C for 30 min. The cycling parameters for IL-13 cDNA amplification were as follows: 35 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 90 s, followed by 7 min at 68°C. PCR products (430 bp) were separated on 1% agarose gels and visualized by ethidium bromide staining. Control amplifications were performed using primers for G3PDH (190 bp) (26).

#### Flow cytometry

Measurements of IL-13 expression in human peripheral blood granulocytes by flow cytometry was performed as previously described (27). Briefly,  $10^5$  eosinophils or neutrophils were fixed in 4% paraformaldehyde (PFA) solution (Fluka, Buchs, Switzerland) for 5 min, washed twice with HEPES-buffered saline solution, and subsequently permeabilized with Ortho-Permeafix (Ortho Diagnostic Systems, Raritan, NJ) immediately before staining. Cells were incubated with anti-IL-13 mAb (mAb 213, R&D Systems; diluted in HEPES-buffered saline solution-saponin; final concentration, 20  $\mu$ g/ml) or isotype-matched control mAb (DAKO, Zurich, Switzerland) for 20 min at room temperature. Cells were then incubated with R-PE-conjugated goat anti-mouse secondary Ab (BD PharMingen Europe, Basel, Switzerland) for 20 min at room temperature in the dark. Cells were washed and resuspended in 200  $\mu$ l 2% PFA solution and analyzed by flow cytometry in an EPICS XL (Coulter, Hialeah, FL). For CD23 expression on B cells, cells were incubated with RDE-conjugated anti-CD19 mAb and FITC-conjugated anti-CD23 mAb. RDE- and FITC-conjugated isotype-matched control mAb were used as negative controls (all from Beckman Coulter, Nyon, Switzerland).

#### ELISA and ELISPOT assay

IL-13 levels were measured in eosinophil and neutrophil lysates ( $1 \times 10^6$  cells/ml) and in eosinophil supernatants of activated eosinophils (release assay). To obtain cell lysates, cells went through two freeze-thaw cycles and microwave destruction at 4°C. To validate this system we treated eosinophils with 0.2% Triton X-100 (28) and observed that both lysate methods generated the same IL-13 levels. After centrifugation the supernatants were collected and frozen at  $-80^\circ\text{C}$  until analysis by ELISA (Endogen, Woburn, MA) according to the instructions of the manufacturer. The lower detection limit was  $<7$  pg/ml, and the assay range was from 0 to 1000 pg/ml. Total IL-13 contents were determined in eosinophils and neutrophils derived from 81 patients and nine healthy control individuals. To analyze IL-13 release, eosinophils were activated with several cytokines for 1 h, and IL-13 levels were measured in supernatants. All measurements were performed in duplicate. To visualize IL-13 release, the ELISA measurements were supplemented with a commercial ELISPOT kit (IL-13-ELISPOT, R&D Systems) under identical stimulation conditions. ELISPOTs were analyzed in triplicate using a video microscope equipped with special tailored software ( $\alpha$  Ease 5.5; Inotech, Wohlen, Switzerland). In

additional experiments. IL-4 was measured in supernatants from stimulated eosinophils (Endogen, Woburn, MA) (sensitivity, <2 pg/ml; standard curve range, 10.2–400 pg/ml).

#### Immunocytochemistry and immunohistochemistry

For immunocytochemistry,  $5 \times 10^5$  eosinophils were used per cytospin. Cytospins were air-dried for 1 min and immediately frozen at  $-20^\circ\text{C}$  in a dehumidified box. Slides were fixed in 4% PFA solution for 20 min and incubated with anti-human IL-13 mAb (BD PharMingen) or isotype-matched IgG1 control mAb (Coulter) in TBS plus 1% BSA for 1 h at room temperature. The alkaline phosphatase-anti-alkaline phosphatase (APAAP) method was performed using a commercial kit (DAKO) as previously described (29). Immunohistochemistry was performed on paraffin-embedded specimens from nasal polyps using anti-human IL-13 mAb (R&D Systems) and control mAb (DAKO), and the APAAP method was again applied (30).

#### Statistical analysis

Results are expressed as the mean  $\pm$  SEM. Statistical analysis was performed using the Mann-Whitney *U* test. A value of  $p < 0.05$  was considered statistically significant.

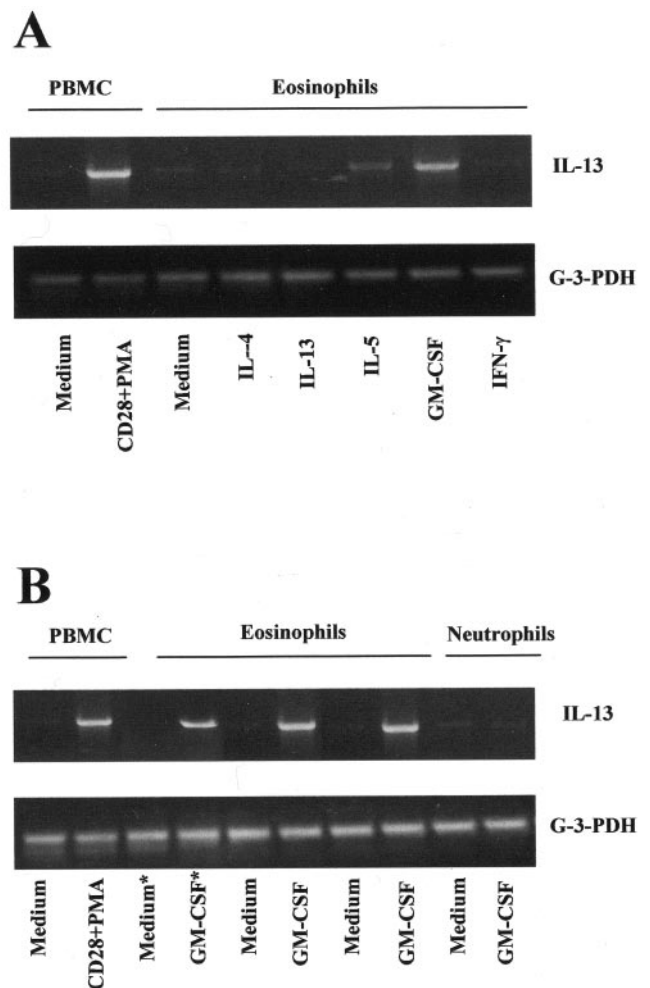
## Results

### *IL-13 mRNA is inducibly expressed in GM-CSF- or IL-5-stimulated eosinophils but not neutrophils*

As shown in Fig. 1A, eosinophils from normal control individuals did not express detectable levels of IL-13 mRNA. However, upon in vitro stimulation with GM-CSF or IL-5 for 3 h, eosinophils expressed IL-13 mRNA. In contrast, IL-4, IL-13, and IFN- $\gamma$  stimulation up to 6 h did not result in the induction of the IL-13 gene. Fig. 1B demonstrates three independent experiments using eosinophils from BA patients. As in the control individuals, freshly isolated blood eosinophils from most patients did not express detectable amounts of IL-13 mRNA, which, however, was inducible upon GM-CSF stimulation. In only a few cases were minimal amounts of IL-13 mRNA detectable. In GM-CSF-stimulated neutrophils, IL-13 gene activation was not observed. These data suggest that eosinophils from both control individuals and patients with asthma do not express significant levels of IL-13 mRNA. Moreover, and in contrast to neutrophils, eosinophils can be stimulated to express IL-13 mRNA by in vitro exposure to GM-CSF or IL-5.

### *IL-13 protein is expressed in eosinophils from eosinophilic patients but not control individuals*

To determine whether IL-13 protein is expressed in human blood eosinophils, we applied the following techniques: flow cytometry, immunocytochemistry, and ELISA. To verify ex vivo findings, we investigated eosinophil IL-13 expression by immunohistochemistry using nasal polyp tissue sections. In agreement with the data obtained at the mRNA level, blood eosinophils from normal donors did not demonstrate any evidence of IL-13 expression as assessed by flow cytometry (Fig. 2A). In contrast, a consistent increase in fluorescence indicative of intracellular IL-13 protein expression was observed in eosinophils from BA and AD patients. Neutrophils from control individuals or BA patients did not express detectable levels of IL-13. That BA eosinophils contain significant amounts of IL-13 was confirmed using immunocytochemistry (Fig. 2B). To better quantify IL-13 levels in blood eosinophils from control individuals and eosinophilic patients with different underlying diseases, we measured total IL-13 levels in eosinophil lysates by ELISA, allowing calculation of average cellular IL-13 concentrations. Eosinophils from BA and AD patients had increased IL-13 protein levels compared with normal eosinophils (Fig. 2C and Table II). Interestingly, IL-13 levels in BA patients were, on the average, higher than those in AD patients. If AD patients also suffered from BA, the intracellular IL-13 content was



**FIGURE 1.** Eosinophils express IL-13 mRNA upon activation with GM-CSF or IL-5. *A*, Eosinophils from healthy controls were cultured in the absence or the presence of the indicated cytokines for 3 h. The same data were observed in 6-h cultures (not presented). As a positive control, activated PBMC were used. mRNA expression was assessed by RT-PCR. One representative of three independent experiments is shown. *B*, Eosinophils from three BA patients were cultured in the absence or the presence of GM-CSF for 3 h. Neutrophils from normal controls and BA patients did not express detectable IL-13 mRNA after GM-CSF stimulation.

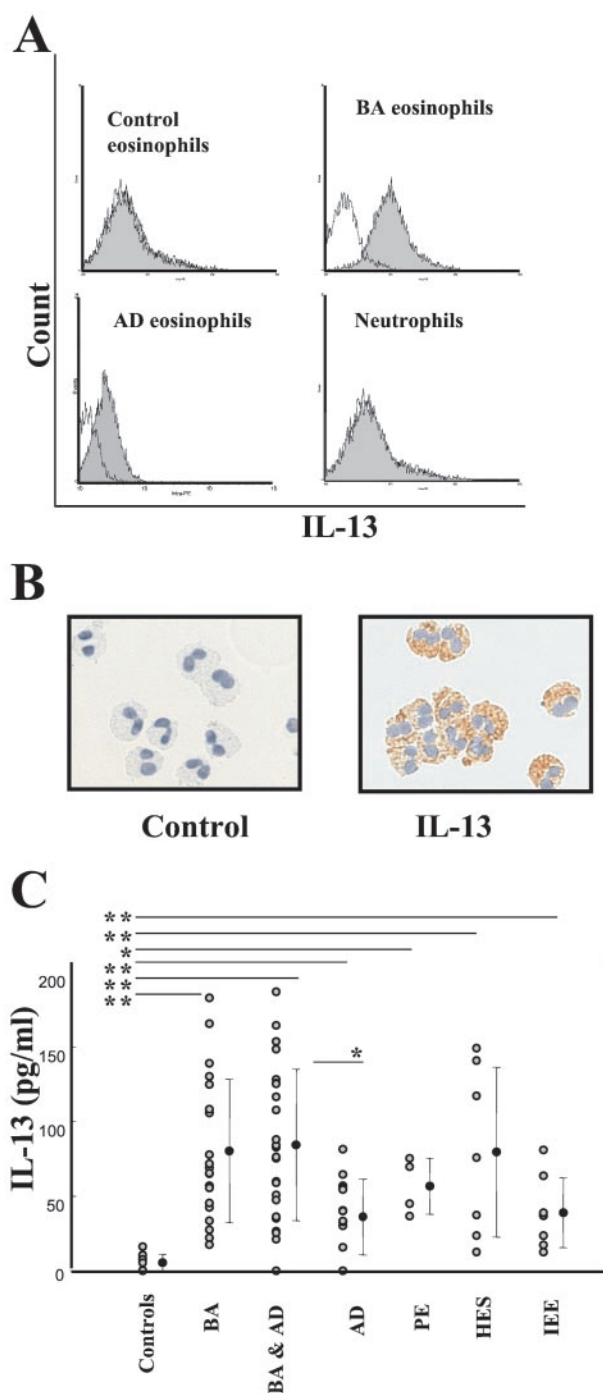
increased and reached the same levels as observed in BA patients. There was also clear evidence for increased IL-13 levels in blood eosinophils from patients with parasitemia, HES, and IEE (Fig. 2C and Table II). IL-13 levels in neutrophil lysates were below the detection limit in both healthy controls and eosinophilic patients (not presented).

Eosinophils in nasal polyp tissues are a target of IL-5 (30), implying that these cells might also express IL-13 protein. Indeed, immunohistochemical examination of this tissue provided evidence for IL-13 expression by eosinophils under in vivo conditions (Fig. 3).

### *Eosinophils synthesize IL-13 protein in the presence of GM-CSF or IL-5*

We cultured eosinophils from normal donors and BA patients in the absence and the presence of IL-5 or GM-CSF and determined their total IL-13 content as a function of time. Freshly purified blood eosinophils from control individuals expressed little or no IL-13 protein (Fig. 4). However, when exposed to IL-5 or GM-CSF, we obtained evidence for IL-13 synthesis by eosinophils after





**FIGURE 2.** Eosinophils from eosinophilic patients express IL-13 protein. *A*, Freshly purified eosinophils from healthy controls, BA patients, and AD patients were stained with control (open histograms) and anti-IL-13 mAb (filled histograms) and analyzed by flow cytometry. One representative of at least three independent experiments for each cell population is shown. *B*, Freshly purified eosinophils from three BA patients and three AD patients were stained with control (left) and anti-IL-13 mAb (right) and analyzed by immunocytochemistry (APAAP). One representative experiment is shown. *C*, Freshly purified eosinophils from healthy controls and from patients with several eosinophilic diseases were used to obtain cell lysates, in which the IL-13 concentration was measured by ELISA. Single data as well as the mean  $\pm$  SEM are presented. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

12 h of stimulation. Maximal levels were observed in 18- to 24-h cultures. In contrast to blood eosinophils from normal donors, BA eosinophils contained significant IL-13 protein levels between 60–

110 pg/ml/ $10^6$  eosinophils. IL-5 and GM-CSF increased IL-13 content only in those eosinophils with relatively low basic IL-13 levels. In eosinophil populations with  $>100$  pg/ml IL-13/ $10^6$  eosinophils, no further cytokine-mediated increase in IL-13 levels was observed. However, whereas BA eosinophils cultured without cytokine support decreased their IL-13 levels after 24 h, both IL-5 and GM-CSF were able to maintain high IL-13 levels in these cells (Fig. 4).

#### *Eosinophils release IL-13 protein upon activation*

As eosinophils from eosinophilic patients contain significant amounts of intracellular IL-13, we were interested in determining whether in vitro activation of these cells leads to IL-13 secretion. Under the conditions used, eotaxin was identified as a strong IL-13 releaser (Fig. 5A). Although IL-5 and GM-CSF were also able to release small amounts of IL-13, the levels did not reach statistical significance. C5a did not stimulate IL-13 release. Simultaneous stimulation with different agonists resulted in significant IL-13 release in those combinations where eotaxin had been used. Small additive effects were seen under these conditions. The calcium ionophore A23187 was used as a positive control. The same results were observed when we analyzed IL-13 release in single eosinophils with the ELISPOT assay (Fig. 5B). Eosinophils from healthy control individuals did not release IL-13 under any stimulatory condition used. Significant IL-4 release was seen under the same conditions in which we observed IL-13 release. Eotaxin was also the most potent releasing factor for IL-4 (mean, 18 pg/ml/ $10^6$  eosinophils; SEM, 2.69 pg/ml).

#### *Eosinophil-derived IL-13 induces CD23 surface expression on B cells*

For functional analysis, eosinophil-derived IL-13 was added to normal B cells, and CD23 expression was analyzed. Since T cells are potential sources of IL-13, the B cells used here were highly purified to exclude the possibility that any T cell-derived IL-13 is in the system. The eosinophil-derived IL-13 was generated from  $10^7$  BA eosinophils/ml, resulting in a lysate containing 450 pg IL-13/ml. Recombinant human IL-13 (1 ng/ml) induced significant CD23 expression in 24-h B cell cultures (not presented). Maximal effects were observed after 48-h stimulation (Fig. 6), whereas CD23 levels declined in 72-h cultures (not presented). CD23 expression was also observed when supernatants of cultivated eosinophils with IL-13 concentrations  $>30$  ng/ml were used for B cell activation. Eosinophil-derived IL-13 also induced, in a dose-dependent manner, significant CD23 surface expression on B cells, suggesting functional activity. Neutralizing anti-IL-13 mAb (anti-IL-13) almost completely blocked the effect of the eosinophil lysates, demonstrating that the effect on CD23 expression was due to IL-13 and no other factor. Since the blocking effect was not complete, a neutralizing anti-IL-4 mAb (anti-IL-4) was added in combination with the anti-IL-13, resulting in complete prevention of increased CD23 levels (Fig. 6). This suggests that the eosinophil lysates contained, besides IL-13, some small amounts of IL-4 that contributed to increased CD23 expression on B cells in this in vitro system.

## **Discussion**

This study was undertaken to determine whether eosinophils are able to synthesize and release IL-13, which seems to play a central role in the pathogenesis of asthmatic inflammation. We studied IL-13 expression by blood eosinophils derived from patients with several different eosinophilic diseases and from healthy controls.

Table II. Mean IL-13 protein content in eosinophils of patients with atopic diseases and controls

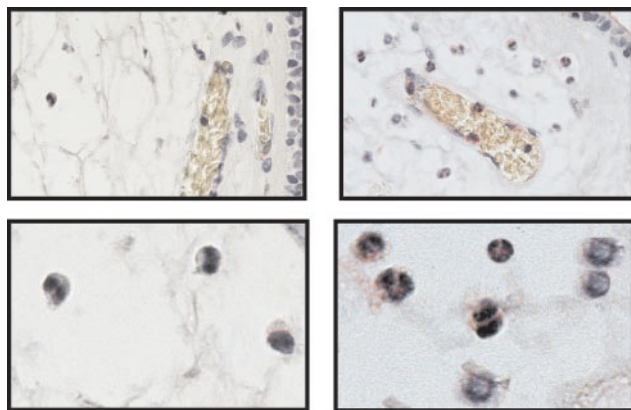
Patient Group	No. Included	IL-13 Protein/10 <sup>6</sup> Cells (pg/ml) (mean ± SEM)	IL-13 Protein Range (pg/ml)	% Eosinophils (mean (minimum–maximum))	Absolute Eosinophil No. (mean (minimum–maximum))
BA all	47	82.9 ± 5.4	0.0–187.1	10.8 (4.5–26.2)	0.801 (0.205–3.162)
BA only	23	81.4 ± 9.4	16.56–182.9	11.2 (4.5–26.2)	0.866 (0.205–3.162)
BA and AD	24	84.3 ± 10.4	0.0–187.1	10.5 (5.2–23.1)	0.739 (0.338–2.793)
AD all	37	66.8 ± 7.9	0.0–187.1	10.3 (2.0–23.1)	0.804 (0.132–2.793)
AD only	13	36.1 ± 6.8	0.0–81.3	7.5 (2.0–16.1)	0.601 (0.132–1.474)
All atopics (BA and AD)	60	72.9 ± 6.1	0.0–187.1	10.6 (2.0–26.2)	0.803 (0.132–3.162)
All patients	80	61.88 ± 7.3	0.0–187.1	11.4 (3.0–29.6)	0.907 (0.132–4.813)
Healthy	9	5.32 ± 1.9	0.0–5.232	3.3 (1.0–4.3)	0.176 (0.051–0.201)
All donors	89	60.8 ± 10.9	0.0–187.1	10.4 (1.0–33.2)	0.722 (0.051–4.813)

We observed that eosinophils produced IL-13 upon in vitro stimulation with IL-5, which is known to be a specific eosinophil differentiation factor (31). It was therefore not surprising that all eosinophil populations from different eosinophilic diseases demonstrated evidence of increased IL-13 protein expression, although mRNA expression was not detectable in these cells. One possible explanation for this observation might be that IL-13 mRNA and protein have different half-lives. During isolation the cells are not exposed to cytokines, explaining the reduced mRNA expression. The fact that GM-CSF was also able to induce the IL-13 gene in eosinophils was not unexpected, since IL-5 and GM-CSF receptors share a common β-subunit, which is essential for signal transduction. In contrast, other cytokines that act via different surface receptors on eosinophils had no effect on IL-13 production, suggesting some specificity in the signal transduction pathway required for this functional response.

There have been previous reports suggesting that human eosinophils do not express IL-13 (18, 32). The observations in these studies were made either using eosinophils from non-eosinophilic donors (32) or using supernatants from IL-5-stimulated eosinophils (18). Therefore, both earlier reports are in agreement with our data, since we also observed no significant IL-13 expression in control eosinophils, and IL-5 (in contrast to eotaxin) was unable to stimulate IL-13 release from eosinophils. The demonstration of IL-13 expression in eosinophils associated with several allergic, infectious, and idiopathic eosinophilic diseases, however, is a new finding. It is possible that the previously observed correlations be-

tween eosinophil number and IL-13 mRNA expression in asthmatic airways (33, 34) were largely due to the production of IL-13 by eosinophils themselves.

The amount of IL-13 produced by eosinophils appears to be 5- to 10-fold lower than that in basophils, in which IL-13 levels of 200–2000 pg/10<sup>6</sup> cells have been reported (18, 32, 35). However, it should be noted that eosinophil numbers are much higher compared with those of basophils, suggesting that eosinophils might represent important sources of IL-13 at inflammatory sites. Moreover, compared with other eosinophil-derived cytokines, the quantity of IL-13 generated per cell is high. We observed IL-13 levels in BA eosinophils between 80 and 180 pg/10<sup>6</sup> cells, whereas IL-4 concentrations were between 50 and 100 pg/10<sup>6</sup> eosinophils (36) (37). Other Th2-type cytokines released by eosinophils are IL-5 and IL-10 (36, 38). Furthermore, the proinflammatory cytokine IL-12 has been reported to be expressed by eosinophils and may promote a switch from a Th2-like to a Th1-like immune response in allergic skin reactions (39). Most of the cytokines are stored in crystalloid granules of eosinophils. Further studies are required to determine the exact intracellular localization of IL-13 in eosinophils (40).



Control

IL-13

FIGURE 3. Eosinophils express IL-13 protein in nasal polyp tissues. Immunohistochemical staining with control (left) and anti-IL-13 mAb (right) is shown (APAAP). Eosinophils were identified by morphology. The upper panel (×400) gives an overview. The lower panel (×1000) shows more details on a cellular level. One representative of three independent experiments is shown.

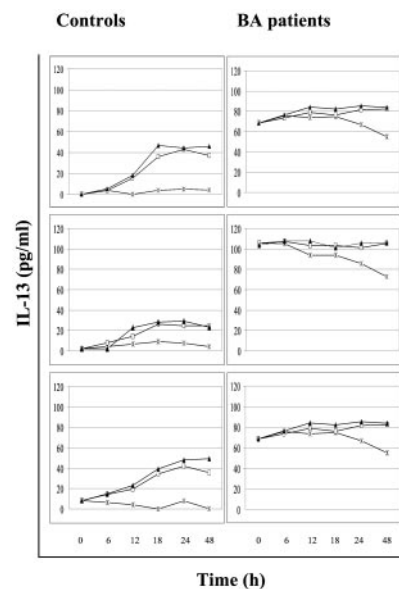
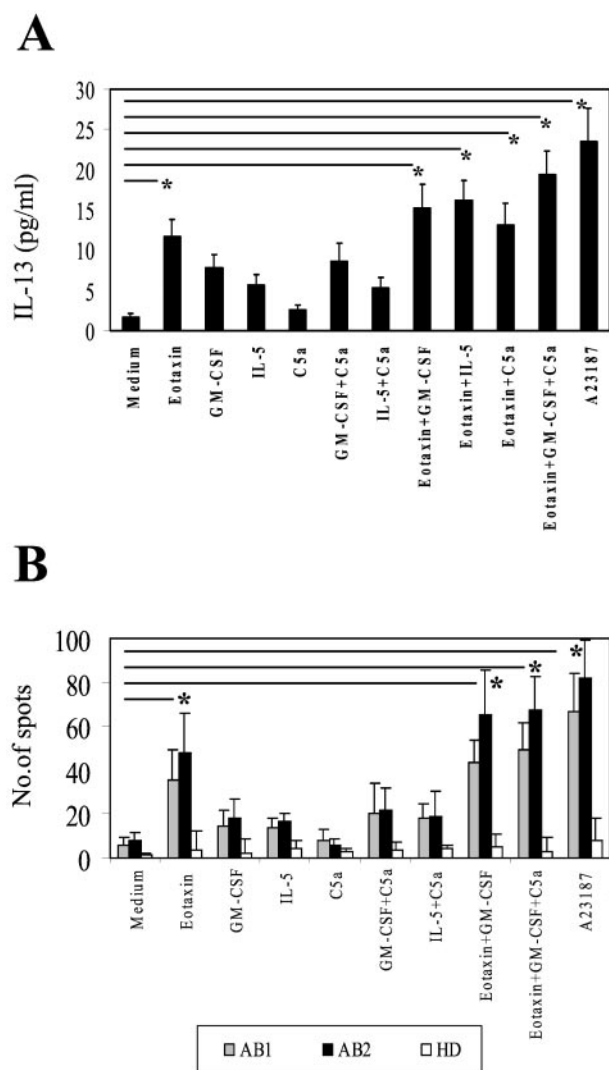


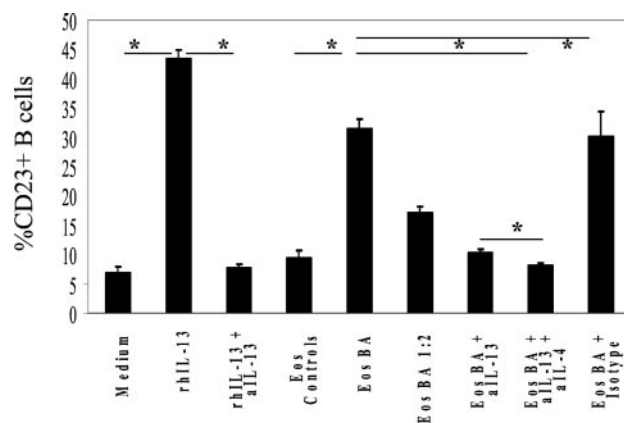
FIGURE 4. Eosinophils synthesize IL-13 protein upon activation with GM-CSF or IL-5. Eosinophils from three healthy controls and three BA patients were cultured in the absence or the presence of GM-CSF and IL-5 for the indicated times. In eosinophils from normal donors IL-13 levels were increased due to cytokine exposure. Eosinophils from BA patients had higher levels immediately after isolation compared with controls. GM-CSF and IL-5 maintained these high IL-13 levels, whereas in cells without cytokine support IL-13 levels declined.



**FIGURE 5.** Eosinophils release IL-13 upon stimulation with eotaxin. *A*, Eosinophils from six BA patients were stimulated with the indicated agonists for 60 min, and IL-13 levels were measured by ELISA. The mean  $\pm$  SEM are presented. \*,  $p < 0.05$ . *B*, IL-13 was measured in single cells by ELISPOT. Eosinophils from two BA patients were stimulated with the indicated agonists for 60 min. Triplicates were measured in each case, and the mean  $\pm$  SEM were calculated. As a negative control, one eosinophil population from a normal individual was used.

This study demonstrated that the IL-13 produced can be released from eosinophils by eotaxin stimulation. Indeed, eotaxin has been shown to be an important activator of eosinophils (41). We observed detectable IL-13 levels 60 min after stimulation of freshly isolated eosinophils, as assessed by ELISA and ELISPOT. However, the amount of IL-13 released was low (3–5%) compared with the total cellular IL-13 content. Similar data were reported by other groups that investigated the release of other cytokines from eosinophils (42). For instance, although eosinophils seem to express IL-4, no evidence was obtained that IL-4 is released from eosinophils (36). Whether repetitive stimulation (28) would result in greater IL-13 releases remains to be investigated.

Eosinophils might be not only a source but also a target of IL-13. For instance, IL-13 was suggested to be a chemotactic factor, an activator, and a survival factor for eosinophils (11, 43). Therefore, we investigated whether IL-13 would be able to stimulate its own expression. The results of these experiments suggested that it is unlikely that IL-13 can induce its own synthesis. In



**FIGURE 6.** Eosinophil (Eos) derived IL-13 increases CD23 expression on purified B cells. Purified B cells from normal controls were cultured with eosinophil lysates from normal individuals (no IL-13) and from BA patients containing 450 pg/ml IL-13. The latter was used in 1/1 and 1/2 dilutions as well as in combinations with neutralizing anti-IL-13 and anti-IL-4 mAb. Recombinant IL-13 (1 ng/ml) and isotype-matched Abs served as controls. CD23 expression was measured by flow cytometry. aIL-4 and -13, anti-IL-4 and -13.

addition, we found no increased expression of IL-13 mRNA upon stimulation with IL-4, which acts partly through the same receptor subunit (9).

The role of eosinophils in asthma has recently been challenged, since treatment of mild asthmatics with anti-IL-5 mAb, while decreasing eosinophil number, had no effect on airway hyper-reactivity or the late phase response following provocation with allergen (41). However, it should be noted that this study did not exclude the possibility that treatment with anti-IL-5 mAb might have a clinical benefit in more severely asthmatic patients. The current study supports the hypothesis that eosinophils play an important role as effector cells in BA together with T cells (3, 44). As an important source of IL-13, eosinophils might significantly contribute to mucus secretion and airway hyper-responsiveness (11–13). Thus, blocking of IL-5 appears to be a reasonable strategy to reduce eosinophil-derived IL-13, which is considered a key cytokine in asthma pathogenesis. Although the first clinical trials have been disappointing (45), further clinical trials are required to evaluate the clinical and immunological effects of anti-IL-5 mAb therapy in asthma.

In contrast to eosinophils, we did not obtain evidence of IL-13 production by neutrophils. This was surprising, because neutrophils have been reported as a source for many cytokines, including IL-4 (46). On the other hand, our data might support the view that eosinophils and not neutrophils are important effector cells in most cases of asthmatic inflammation. Moreover, the inability of neutrophils to produce IL-13 upon GM-CSF stimulation suggests that either a signaling molecule(s) required for IL-13 gene expression is missing or the pathway leading to transcriptional activation is somehow blocked. Further experimentation is required to understand the differences in GM-CSF signal transduction between eosinophils and neutrophils.

In summary, our study confirms previous findings describing the ability of human eosinophils to synthesize and release cytokines. Eosinophils do not constitutively express IL-13, but inducibly synthesize this cytokine upon stimulation with IL-5 and GM-CSF. The fact that eosinophils express IL-13 in several eosinophilic inflammatory diseases suggests that these cytokines might also be responsible for induction of the IL-13 gene under in vivo conditions.



Further work is required to better understand the role of eosinophils and its IL-13 production under inflammatory conditions.

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