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Immune profile modulation of blood and mucosal eosinophils in nasal polyposis with concomitant asthma

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ARTICLE INFO

Article history:

Received for publication October 22, 2014.

Received in revised form December 1, 2014.

Accepted for publication January 23, 2015.

ABSTRACT

Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is frequently associated with asthma. Mucosal eosinophil (EO) infiltrate has been found to correlate with asthma and disease severity but not necessarily in every patient. Other multifactorial immune processes are required to determine disease endotypes and response to treatment.

Objective: To evaluate EO immunomodulation for migration and survival in accordance with inflammatory protein profiles and asthmatic status in CRSwNP.

Methods: Ninety-three patients (47 with asthma) with CRSwNP were included. Each patient was staged clinically according to symptom severity and polyp size. Nasal secretions were collected to establish a cytokine profile. The EOs were purified from blood samples and nasal polyps to delineate specific immunophenotypes by flow cytometry and determine in vitro EO survival in relation to asthmatic status.

Results: The CRSwNP in patients with asthma was characterized by eosinophilia and a high level of interleukin (IL)-5 in nasal secretions. Although EOs exhibited activation profiles after mucosal migration, there was relative down-expression of IL-5 receptor- α (IL-5R α) on nasal EOs in patients with asthma. The EO culture with IL-5 and IL-9 showed an antiapoptotic effect in patients with asthma through IL-5R α modulation.

Conclusion: Mucosal eosinophilia seems to be induced by EO nasal trapping through modulation of adhesion receptors. In patients with asthma, EO involvement is enhanced by the antiapoptotic synergistic action of T-helper cell type 2 cytokines on IL-5R α expression. This study shows for the first time that IL-9 is involved in EO homeostasis in CRSwNP and could explain the low benefit of anti-IL-5 therapy for some patients with asthma and nasal polyposis.

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Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a clinical syndrome characterized by persistent symptomatic inflammation of the mucosa of the nose and paranasal sinuses. It affects 1% to 4.3% of the general population.^{1–3} The impact of the disease on quality of life is considerable, with chronic nasal obstruction and dysosmia.⁴

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Disclosure: Authors have nothing to disclose.

Funding: This work was supported by grants from the Otology Research Association (Lille, France) and the French National Institutes for Health and Medical Research (INSERM).

<http://dx.doi.org/10.1016/j.anai.2015.01.012>

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Functional endoscopic sinus surgery is required when symptoms persist despite optimal medical treatment, which is still based on topical and systemic corticosteroid.^{3,5} Medical failure is observed mainly in concomitant asthma or acetylsalicylic acid intolerance, which represents 42% or 15% of patients, respectively.^{6–10} Similarly, asthma associated with CRSwNP frequently accounts for patients with substantial residual disease despite contemporary airway treatment approaches.³

The pathogenesis of CRSwNP is still poorly understood, given that this syndrome encompasses major variants or endotypes. The impact of allergy as an excessive inflammation driven by aberrant T-helper cell type 2 (T_H2) immunity is controversial because allergy incidences in patients with CRSwNP and the general population are equal.³ Environmental factors such as fungal (*Alternaria* species) or

bacterial (*Staphylococcus aureus*) agents could act as triggers of nasal mucosa disruption and stromal inflammation through innate and adaptive immune responses.^{11–13} Whatever the hypothesis, the characteristic of CRSwNP inflammation in the white population is substantial eosinophil (EO) mucosal infiltrate.^{14–18}

Nasal infiltrate requires active blood EO recruitment and EO survival within the mucosa after transendothelial migration. Adhesion agents are described as pivotal in the interaction of EOs with nasal vessels and mucosal extracellular matrix components.^{19,20} Many of them, such as CD44, lymphocyte function-associated antigen-1 (LFA-1 or CD11a/CD18) β_2 -integrin, or very late antigen-4 (VLA-4 or CD49b/CD29) β_1 -integrin, have been studied in airway inflammation.^{21–23} The expression modulation of these interacting and homing receptors and their role in patients with CRSwNP with and without asthma require further investigations.

Concerning EO priming within the mucosa, many studies have emphasized the major role of proinflammatory cytokines by the epithelial thymic stromal lymphopoietin-dependent signaling pathway and innate lymphoid cell activation.^{24–26} Interleukin-5 (IL-5), mainly produced by activated T cells, mast cells, and EOs, is associated with the clinical severity of asthma.²⁷ In CRSwNP, high levels of IL-5 in nasal polyps have been proved to be associated with comorbid asthma.²⁸ Although anti-IL-5 therapies with mepolizumab or reslizumab are effective to improve quality-of-life scores and decrease exacerbation rates and blood and sputum EO counts in asthma,²⁹ only short-term efficacy has been observed on symptoms and polyp eosinophilia in patients with severe nasal polyposis.^{30–33} Among other cytokines produced by T cells and EOs, interleukin 9 (IL-9) has been studied in lower airway inflammation and has been shown to be involved in blood EO maturation and survival by enhancing IL-5 receptor- α (IL-5R α) membrane expression. Its interaction with IL-5 in CRSwNP has not yet been clearly evaluated.³⁴

To investigate the relation between asthma and CRSwNP further for EO involvement and disease variants, patients were compared for eosinophilia and levels of proinflammatory markers in blood samples and nasal secretions. Subsequently, patients' EO profiles were examined for adhesion and interleukin receptor expression. In patients with CRSwNP and asthma, a specific inflammatory endotype was delineated and potential additive effects of IL-5 and IL-9 on EO survival were found.

Methods

Patients

Ninety-three patients older than 18 years with CRSwNP (according to guidelines of the European Position Paper on Rhinosinusitis and Nasal Polyps³) were included. All patients provided written informed consent before participation. The study on EO sample collection was approved by local ethics review board (registration number 2009-A00314-53). The patients were initially referred to the authors' department for persistent symptoms despite optimal medical treatment (≥ 3 courses of 7-day oral corticosteroid and maximum dose of topical nasal corticosteroid spray). Concomitant asthma (diagnosed by spirometry), allergy (confirmed with skin prick testing), and history of acetylsalicylic acid intolerance were assessed. Patients were enrolled after maximum medical management failure and functional endoscopic sinus surgical indication. Patients with immunodeficiency, autoimmune disease, and/or cystic fibrosis were excluded.

The burden of sinonasal symptoms was measured by a visual analog scale (0 = no symptom). Polyp size was measured endoscopically using a 30° rigid nasal fiberoptic scope according to the grading system of Lidholdt et al³⁵ (maximum total score 3). A computed tomographic (CT) scan was performed to evaluate sinus

opacifications by the scoring system of Lund and Mackay³⁶ (maximum total score 24).

Patients with asthma ($n = 47$) were systematically compared with patients without asthma ($n = 46$; Table 1). These 2 groups were comparable in age, sex, duration of CRSwNP before surgery in the authors' institution, and tobacco use. For severity, mean visual analog scale score, polyp score, and CT scan opacification staging did not differ between these 2 groups of patients. However, prior surgery ($P = .036$) and allergy ($P = .027$) were more frequent in patients with asthma.

Samples Collection

Samples collected from the 93 patients were used for cytokine measurement, EO purification, and/or histopathologic analysis.

To improve the homogeneity of the population, any kind of medical therapy (oral and topical corticosteroid) except nasal douching with saline solution was stopped at least 1 month before surgery. Blood samples for EO count and EO purification were obtained 3 weeks before surgery in Vacutainer tubes containing ethylenediaminetetraacetic acid (BD Medical, Franklin Lakes, New Jersey). Nasal secretions were collected during the surgical procedure. Two surgical patties measuring 1.27×2.54 cm (Codman, Raynham, Massachusetts) were placed in each nasal fossa for 5 minutes. Patties were put in 3 mL of phosphate buffered saline (PBS) and stored for 1 hour at 4°C. Nasal fluid was centrifuged at 1,400 rpm for 8 minutes to separate the cellular components. Then, supernatants were portioned according to the Bradford assay for protein concentration and stored at -70°C until cytokine determination. Nasal polyps were obtained at the beginning of the surgical procedure. Through an endoscope, 1 polyp was gently removed from each patient and kept for a maximum of 1 hour at room temperature (RT) in sterile saline before further procedures.

EO Purification from Blood Sample

The EOs were isolated as previously described³⁷ on Percoll gradient (Pharmacia Biotech, Uppsala, Sweden) followed by a negative immunomagnetic selection using anti-CD16-, anti-CD2- and anti-CD14-coated microbeads on a Cs column (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 minutes at 4°C. Purity was determined on cytospin preparations after RAL-555 staining and was greater 98%.

EO Purification from Nasal Polyp

The protocol was adapted from a previously published procedure.³⁸ Piecemeal tissue was subjected to a first mechanical dissociation with a GentleMacs Dissociator (Miltenyi Biotec). The fragments were subsequently digested by incubation for 1 hour at

Table 1
Demographic and clinical description of study population

	With asthma	Without asthma	P value
Population ($n = 93$)	47	46	NS
Age (y), mean	49.5	46.6	NS
Men/women	31/16	36/10	NS
Duration of CRSwNP (y)	12.4	10.4	NS
Prior surgery, %	53.2	30.4	.036 ^a
Allergy, %	44.7	21.7	.027 ^a
ASA intolerance, n	14	—	—
Tobacco use ($n = 9$)	2	7	NS
Polyp size score (1–3), mean	2.47	2.38	NS
CT scan score (1–24), mean	19.09	19.15	NS
VAS score (1–10), mean	7.3	7.1	NS

Abbreviations: ASA, acetylsalicylic acid; CRSwNP, chronic rhinosinusitis with nasal polyps; CT, computed tomographic; NS, nonsignificant; VAS, visual analog scale.

^aBy χ^2 test.

37°C with collagenase type II (2 mg/g of tissue) and *Dnase*I (80 µg/g of tissue; Invitrogen, Carlsbad, California) in RPMI-1640 culture medium without phenol red (supplemented with 2% heat-inactivated fetal calf serum, 25 mmol/L of HEPES buffer, 2 mmol/L of L-glutamine, 10 mmol/L of sodium pyruvate, and 10 µg/mL of gentamycin), hereafter referred to as complete medium. A second mechanical dissociation was performed and the digested tissue was filtered through a 300-µm and a 70-µm nylon mesh with PBS. Cell viability was determined on cytopspin preparations after RAL-555 staining and was greater 75%. After a negative selection procedure, cells were incubated with anti-CD326-, anti-CD105-, anti-CD2-conjugated magnetic beads (Miltenyi Biotec) and an Eosinophil Isolation kit (Miltenyi Biotec) for 30 minutes at 4°C with gentle tilting. The mixture was layered onto a steel wire column (MACS LD column; Miltenyi Biotec). The column was washed and the eluted CD236⁺ CD105⁺ CD2⁺ eosinophils were collected in PBS, 0.5% bovine serum albumin, and 2 mmol/L of ethylenediaminetetraacetic acid. Cell purity was measured using a Countess automated cell counter (Invitrogen) and was greater 80%. The mean EO purification yield was 48%.

EO Culture Procedure

Some purified EOs from blood were incubated at 37°C in 5% CO₂ in complete medium with IL-5, IL-9, or eotaxin (Peprotech, Rocky Hill, New Jersey). The best time to study receptor profile modulation and apoptosis in purified EO culture (18 hours) was determined by preliminary procedures.

Flow Cytometry

EO membrane staining

Antibodies and isotype controls used for flow cytometry (origin, clone) are listed in [eTable 1](#) (available online). Samples were analyzed using an Accuri C6 cytometer and software (BD Biosciences, San Jose, California). At least 10⁴ events were acquired per sample.

Membrane staining of EOs (2 × 10⁵ cells for each condition) was performed in PBS and 0.5% bovine serum albumin after blocking of nonspecific binding with 5 µL of mouse serum (Sigma-Aldrich, St Louis, Missouri) for 20 minutes. Cells were incubated with fluorescein isothiocyanate (FITC) with antibodies or FITC with control isotype antibodies for 25 minutes in the dark. For IL-9 receptor-α (IL-9Rα) staining, a primary goat biotin-conjugated antibody (Southern Biotech, Birmingham, Alabama) was used. Nonspecific binding was avoided with 10 µL of goat serum (Jackson ImmunoResearch, West Grove, Pennsylvania), and a subsequent step of incubation with FITC plus streptavidin (R&D System, Minneapolis, Minnesota) for 20 minutes was required. After washing in PBS, cells were subjected to C6Flow analysis (BD Biosciences).

To improve the accuracy of cell targeting, double staining was used for analysis of purified EOs from nasal polyps. After labeling for the membrane receptor with the relevant fluorochrome-conjugated antibodies for 25 minutes and washing in PBS, EOs were fixed for 10 minutes with 2% paraformaldehyde (ThermoScientific, Waltham, Massachusetts) at 4°C and made permeable for 10 minutes at RT with 0.01% saponin (Sigma-Aldrich) in PBS and 1% bovine serum albumin. Nonspecific binding was blocked with 5 µL of mouse serum for 10 minutes. Purified EOs were identified further by incubation with biotinylated anti-eosinophil peroxidase or isotype control for 30 minutes and streptavidin and phycoerythrin (1:500; Molecular Probes, Eugene, Oregon) for 20 minutes in the presence of saponin.

EO apoptosis

The EOs (1 × 10⁵ for each condition) were incubated on U-bottom plates at 37°C in 5% CO₂ in complete medium for 18 hours

without stimulation (medium alone), with IL-5 (10 and 100 nmol/L), IL-9 (1, 10, and 100 nmol/L), eotaxin (100 nmol/L), or IL-5 (10 nmol/L) and IL-9 (10 nmol/L; Peprotech). Apoptosis was measured after staining with annexin V and FITC (BD Pharmingen, San Diego, California) for 15 minutes at RT in the dark. Analysis was performed on an Accuri C6 cytometer and software (BD Biosciences).

IL-5Rα membrane expression modulation on EOs

The EOs (4 × 10⁵ for each condition) were incubated under the same conditions and IL-5Rα membrane staining was performed as described earlier.

Protein Assays

Stored nasal secretions and snap-frozen polyps were used. Polyps were subjected to mechanical dissociation with a Gentle-Macs Dissociator (Miltenyi Biotec). After centrifugation at 4,000 rpm, nasal polyp supernatants were collected. Eosinophil-derived neurotoxin (EDN), IL-5, and IL-9 levels were measured by specific enzyme-linked immunosorbent kits from MBL International (Nagoya, Japan), R&D System, and Bio-Rad (Hercules, California). The lower detection limits were 0.62 ng/mL for EDN, 0.29 pg/mL for IL-5, and 2.5 pg/mL for IL-9. Total IgE levels were measured by the Phadia ImmunoCAP test (ThermoScientific).

Immunohistochemistry and Immunofluorescence

Analyses were performed on purified blood EOs and nasal polyp sections. Detailed protocols are available online ([eMethods](#)).

Statistical Analyses

Data were input into Excel (Microsoft, Redmond, Washington) and statistical analysis was performed with SPSS 15.0 (SPSS, Inc, Chicago, Illinois). Means and standard deviations were reported for descriptive statistics. The χ^2 test was used for comparison of demographic data. The nonparametric Wilcoxon test was used to compare means of paired samples. The nonparametric Mann-Whitney and Kruskal-Wallis tests were used to compare means of unpaired samples. Correlation analyses were conducted with the Pearson test (using ρ as correlation coefficient). A 2-tailed *P* value less than or equal to .05 was considered statistically significant.

Results

Inflammatory Profiles of Patients with CRSwNP in Relation to Asthma

Eosinophilia and EDN as characteristics of patients with asthma

Blood samples (*n* = 55) and nasal polyps (*n* = 31) were collected for EO counting before cell purification. In patients with asthma, there were more EOs in the blood compartment (with asthma 759 ± 120 cells/mm³, without asthma 376 ± 48 cells/mm³; *P* < .01; [Fig 1A](#)). The rate of EOs in mucosa after polyp dissociation was higher in patients with asthma compared with others types of inflammatory cells (with asthma 21 ± 3.5%, without asthma 8.8 ± 3%; *P* < .01; [Fig 1B](#)). Nasal secretions were collected from patients with (*n* = 20) and without (*n* = 18) asthma. EDN, a cationic protein described as an alarmin present in EO granules, also was increased in patients with asthma (with asthma 489 ± 118 ng/mL, without asthma 200 ± 21 ng/mL; *P* < .05; [Fig 1C](#)). For the overall population, there was a positive correlation between EDN secretion levels and CT scan score (ρ = 0.47, *P* < .01; [Fig 1D](#)).

IL-5, IL-9, and IgE as markers of T_H2 polarization in patients with asthma

Nasal secretions were collected from patients with (*n* = 20 or 21) and without (*n* = 12) asthma. In patients with asthma, IL-5 (with asthma 37.5 ± 9 pg/mL, without asthma 12 ± 4.7 pg/mL;

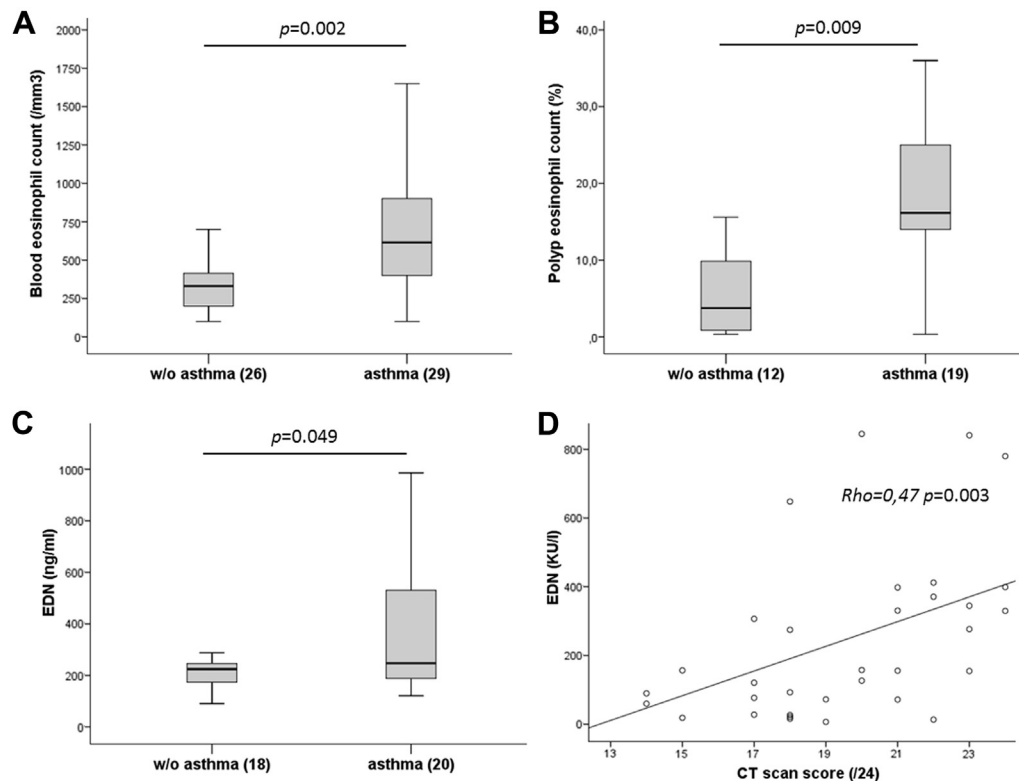


Figure 1. Eosinophilia and eosinophil-derived neurotoxin (EDN) as inflammatory markers in patients with chronic rhinosinusitis with nasal polyps and asthma. (A, B) For each sample, eosinophil counting was performed before cell purification. EDN was (C) increased in patients with asthma and (D) correlated to computed tomographic (CT) scan score.

$P < .05$; Fig 2A) and IgE (with asthma 63 ± 24.5 KUI/mL, without asthma 26.5 ± 12.3 KUI/mL; $P < .02$; Fig 2B) were statistically more expressed in nasal secretions. For the overall population, there was a positive correlation between IgE and IL-5 secretion levels ($\rho = 0.41$, $P < .05$; Fig 2C). More elevated levels of IL-5 (data not shown) and IL-9 (Fig 2D) were observed in polyps collected from patients with asthma.

Main Phenotypic Features in Blood and Polyp Purified EOs

EO adhesion marker modulations from blood to mucosal compartments

The expression of β -integrins (VLA-4, CD11a) and CD44 by EOs purified from blood samples ($n = 19$) and nasal polyps ($n = 20$) of patients with CRSwNP was studied by flow cytometry (Fig 3). Data represent the median channel fluorescence for each marker. The β_2 -integrin (CD11a) was expressed more than β_1 -integrin (VLA-4). A significantly decreased expression of VLA-4 (blood 52.5 ± 5 , polyp 32.8 ± 7) and CD11a (blood 517.3 ± 37.1 , polyp 299 ± 26.7) was observed between blood and nasal EOs ($P < .05$). CD44 also was decreased on the EO membrane in nasal polyps ($P < .001$). This down-expression of proteins involved in cell migration and interaction was observed in patients with and without asthma.

EO activation and survival receptor modulations from blood to mucosal compartments

The expression of CD69 and IL-5R α by EOs purified from blood samples ($n = 19$) and nasal polyps ($n = 20$) of patients with CRSwNP was studied by flow cytometry (Fig 4). Data represent the median channel fluorescence for CD69 and the percentage of EOs positive for IL-5R α . CD69 was significantly upregulated on nasal EOs in patients with and without asthma (blood 13.3 ± 5.5 , polyp 59.5 ± 15 ; $P < .05$). The IL-5R α membrane expression rate was low

but was slightly increased in nasal EOs (blood 2.1 ± 0.3 , polyp 6.4 ± 0.7 ; $P < .001$). There was a significant discrepancy between patients with and those without asthma. IL-5R α was expressed less on the EO membrane in nasal polyps for patients with asthma ($P < .05$). No difference was observed for IL-5R α expression on EOs collected from blood samples of patients with and without asthma.

To corroborate CD69 and IL-5R α presence on EOs from the 2 compartments, qualitative immunohistochemical and immunofluorescence analyses were performed. Further details are available online (eResults and eFig 1).

The expression of IL-9R α by EOs purified from blood samples of patients with ($n = 7$) and without ($n = 6$) asthma was determined by flow cytometry. IL-9R α was expressed more on blood EOs from patients with asthma (with asthma $4.4 \pm 0.9\%$, without asthma $1.7 \pm 0.9\%$; $P < 0.05$; data not shown).

Impact of IL-5 and IL-9 on IL-5R α Expression and EO Survival in Patients with CRSwNP and Asthma

In vitro IL-5R α membrane expression is upregulated in EOs of patients with asthma in the presence of IL-5 and IL-9

Purified blood EOs from blood samples of patients with ($n = 7$) and without ($n = 5$) asthma with CRSwNP were analyzed (Fig 5). Data represent the mean percentage of EOs positive for IL-5R α . Compared with nonstimulated cells, IL-5 (10 nmol/L), IL-9 (10 nmol/L), or eotaxin (100 nmol/L) did not induce IL-5R α expression by EOs of patients with or without asthma. IL-5 (100 nmol/L) increased IL-5R α expression in patients with asthma ($15.3 \pm 9\%$) compared with the other conditions, in particular IL-5 (10 nmol/L; $8.9 \pm 1.4\%$), although without reaching statistical significance. Co-incubation with IL-5 (10 nmol/L) and IL-9 (10 nmol/L) significantly increased the level of IL-5R α expression in EOs of patients

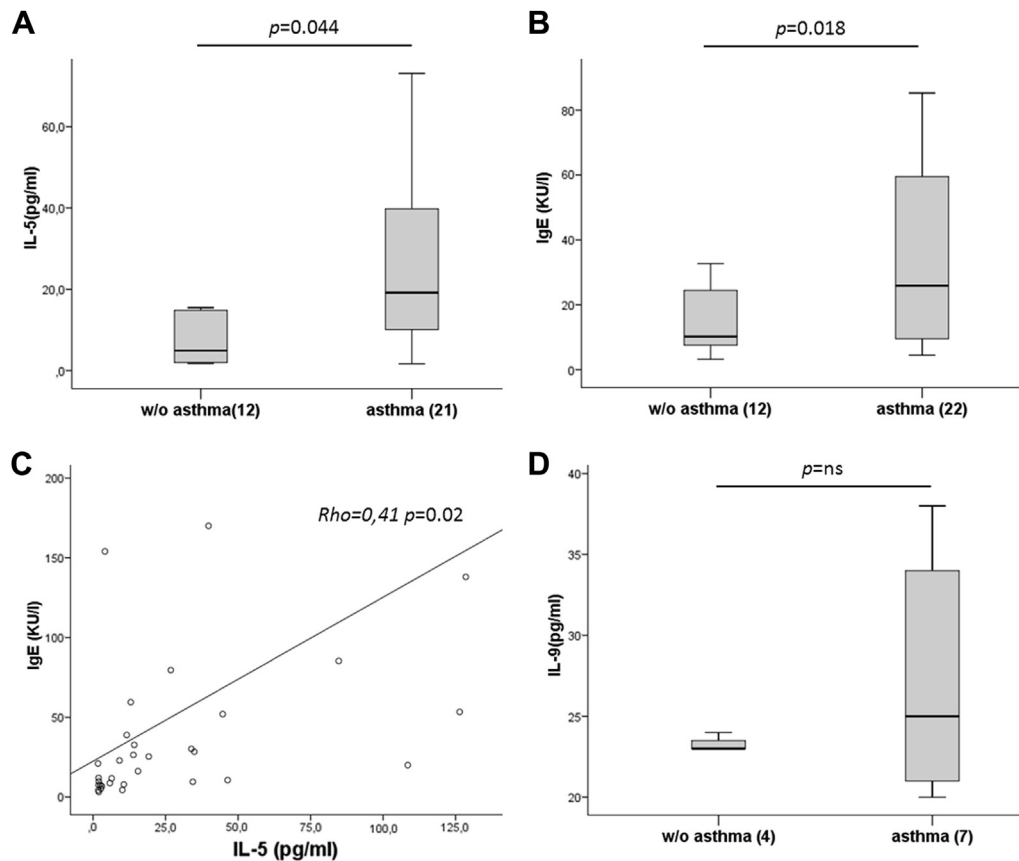


Figure 2. Inflammatory profile of chronic rhinosinusitis with nasal polyps with T-helper cell type 2 polarization in patients with asthma. (A) Interleukin-5 (IL-5) and (B) IgE nasal secretion levels were increased and (C) correlated in patients with asthma. (D) Interleukin-9 (IL-9) level was increased in the polyps of patients with asthma.

with asthma ($19.6 \pm 11\%$) compared with the medium alone ($12.9 \pm 4\%$; $P < .05$).

In vitro EO apoptosis is decreased in patients with asthma in the presence of IL-5 and IL-9

The role of IL-5 and IL-9 in EO survival was assessed (Fig 6). Apoptosis was studied by flow cytometric analysis on purified EOs from blood samples of patients with CRSwNP and asthma ($n = 5$) and those without asthma ($n = 5$). Data represent the mean percentage of EOs positive for annexin V. Compared with non-stimulated cells, EO apoptosis was decreased significantly in the presence of IL-5 in a concentration-dependent manner ($P < .05$) or in the presence of IL-5 and IL-9 ($P = .01$) in patients with and without asthma. IL-9 alone did not modify EO apoptosis whatever the concentration. Eotaxin had no influence on EO apoptosis. Co-incubation with IL-5 (10 nmol/L) and IL-9 (10 nmol/L) showed a decrease in EO apoptosis mean rate ($31 \pm 9.5\%$) compared with IL-5 alone (10 nmol/L; $46 \pm 19.7\%$) in patients with asthma (Fig 6), without reaching statistical significance.

Discussion

This report shows that eosinophilia, a major characteristic of CRSwNP, is mediated by the modulation of EO recruitment and survival factors. Adhesion receptor expression is modulated to favor EO migration to and trapping within the polyps. The present data show that an interactive downregulation of IL-5R α and IL-5 in patients with asthma might be thwarted by IL-9 and IL-5 enhancement of IL-5R α expression.

In white individuals, comorbid asthma is a well-described factor of CRSwNP severity.^{8,9} Patients with CRSwNP and asthma have a

poor medical and surgical response with a high recurrence rate.³⁰ Thus, there were more patients with prior surgical procedures in the asthmatic group in this study. Allergy also was more prevalent in this group. Although allergy is not clearly recognized as an etiologic factor in CRSwNP, its occurrence increases the disease burden.³

In most cases, CRSwNP is characterized by a prominent local eosinophilic inflammation with a high production of eosinophil cationic proteins, IL-5, and tissue IgE.^{12,39} In the spectrum of CRS diseases, those proteins have been cited as useful biological landmarks to delineate different endotypes.⁴⁰ In the present study, high expression levels of EDN, IgE, IL-5, and blood and mucosal eosinophilia in patients with CRSwNP could have been associated with comorbid asthma. Notably, these findings were unrelated to allergy diagnosed by skin prick tests (data not shown). With a positive correlation observed between IL-5 and IgE rates in this analysis, the present study corroborates recent evidence suggesting that local induction of polyclonal IgE formation is combined with severe eosinophilic local inflammation.⁴¹ Furthermore, involvement of EO degranulation in tissue remodeling is supported in the present study by a positive correlation between EDN levels in nasal secretions and CT scan score. EDN, known as an alarmin, is involved in the T_H2 polarized immune response.⁴²

Persistent mucosal eosinophilia requires blood EO recruitment and survival within the mucosa. The present study shows that EO migration into nasal polyps is associated with β -integrins and CD44 down-expression in patients with and without asthma. As with other leukocytes, CD44 could be a primary adhesion molecule required for EO migration.⁴³ In asthma, VLA-4 also appears to be involved in the initial adherence of activated EOs, when the cells slow down in the blood stream.²² The authors presume that, after a

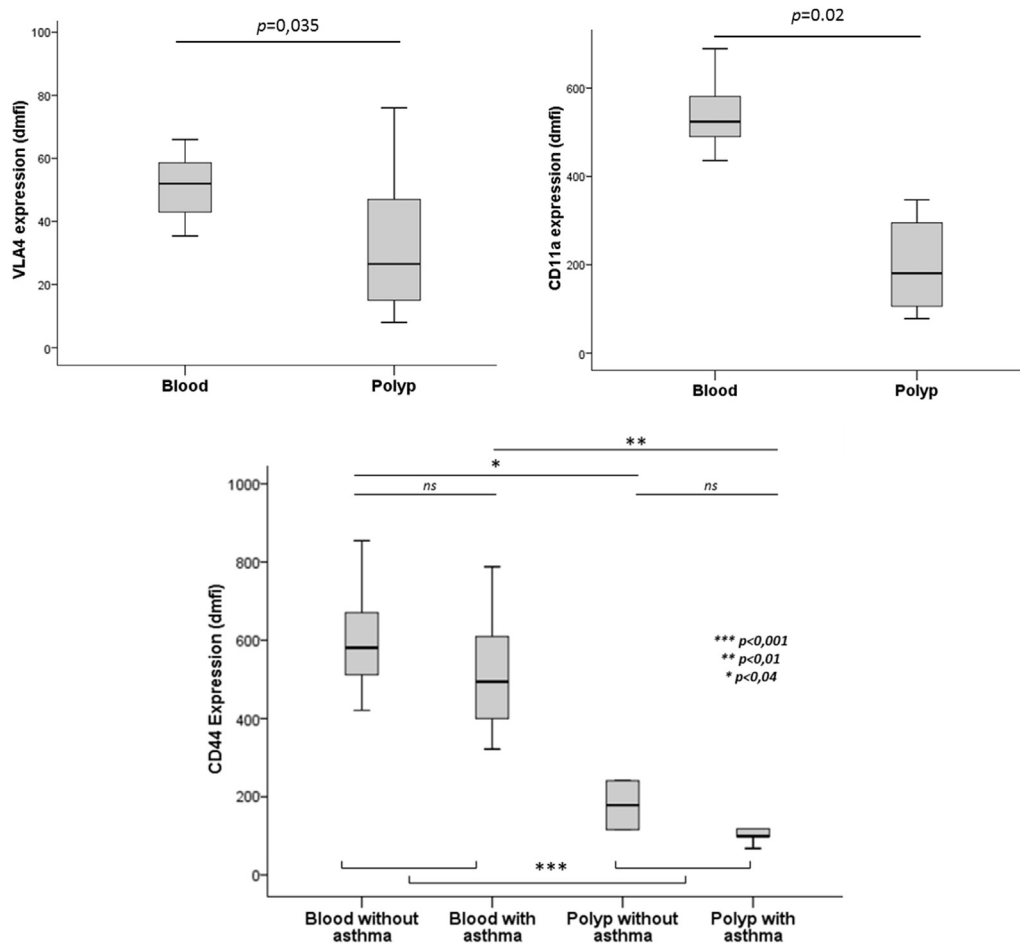


Figure 3. Adhesion receptor modulation in eosinophils between the blood ($n = 19$) and nasal mucosa ($n = 20$) compartments. Very late antigen-4 (VLA-4), CD11a, and CD44 expressions were decreased in nasal eosinophils. There was no difference between patients with and those without asthma for β -integrin (data not shown) and CD44 expression within each compartment.

priming useful for EO migration, CD44 and VLA-4 functions are decreased when EO mucosal migration is achieved. Another explanation could be a conversion of these proteins into a low avidity state in the polyp,²² decreasing their interaction with

specific antibodies used for cytometric analyses. The present study showed that β_2 -integrin LFA-1 (CD11a) was expressed more than β_1 -integrin VLA-4 on blood EOs. Because IL-5 is responsible for upregulation of β_2 -integrins on circulating EOs in asthma,⁴⁴ the

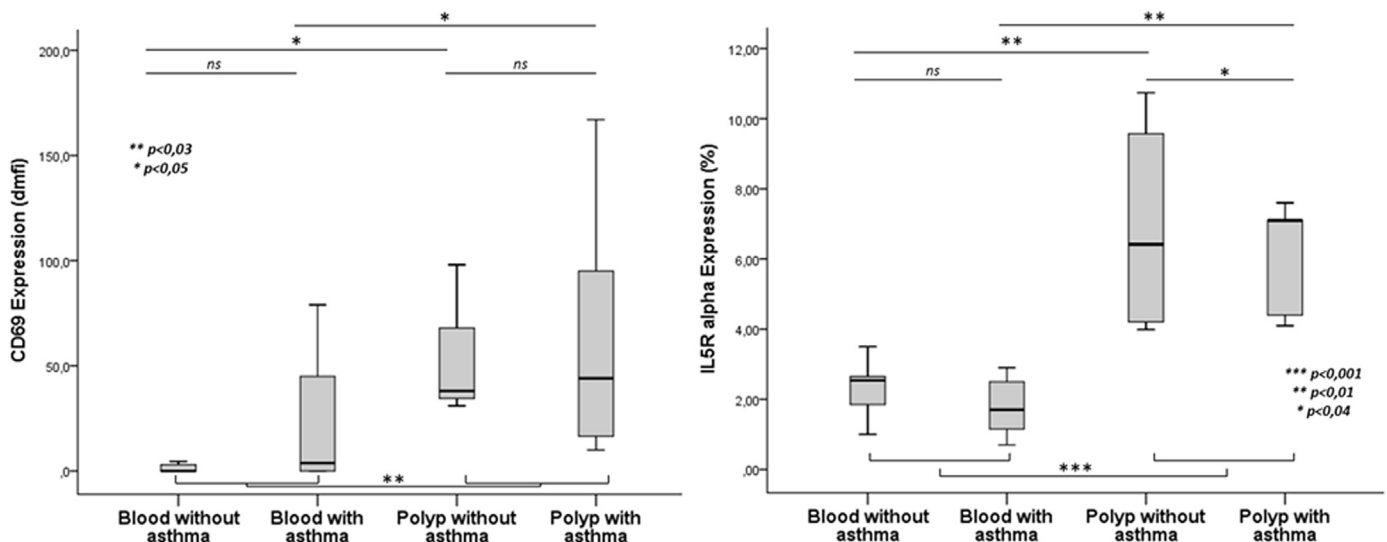


Figure 4. CD69 activation marker and interleukin-5 receptor- α (IL-5R α) modulation in eosinophils between the blood ($n = 19$) and nasal mucosa ($n = 20$) compartments. CD69 and IL-5R α were upregulated in nasal eosinophils in patients with and without asthma. IL-5R α was expressed less in the nasal polyps of patients with asthma. No difference was observed within each compartment.

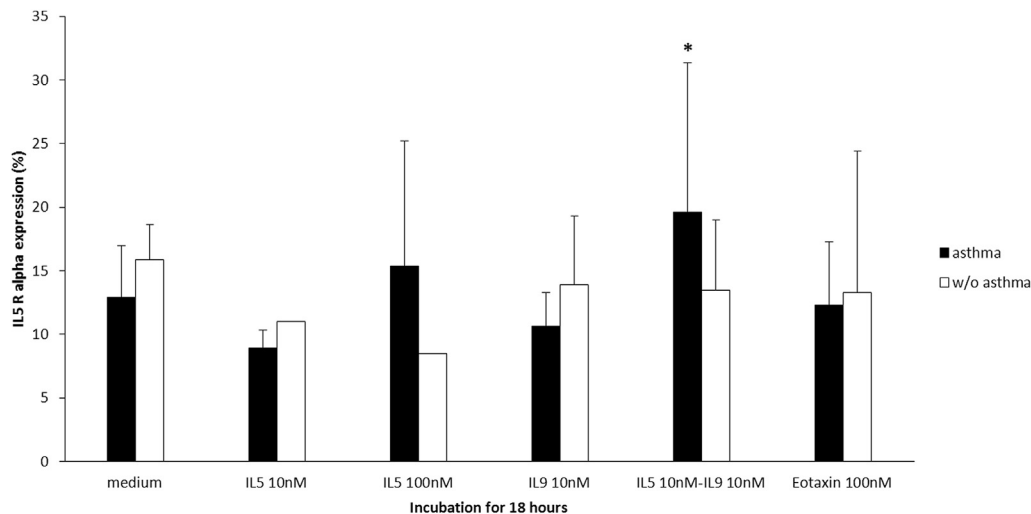


Figure 5. Interleukin-5 receptor- α (IL-5R α) expression modulation on eosinophils after 18-hour incubation under different cytokine conditions in patients with ($n = 7$) and without ($n = 5$) asthma. In patients with asthma, co-incubation of IL-5 and interleukin-9 (IL-9; 10 nmol/L) increased IL-5R α expression on eosinophils compared with the medium or IL-5 alone (10 nmol/L; * $P < .05$).

authors postulate that LFA-1 also is upregulated on blood EOs in CRSwNP. LFA-1 has been implicated in EO extravasation and can exist in bent, inactive conformations.⁴⁴ As is the case with CD44 and VLA-4, LFA-1 low expression levels in polyps could be related to EO mucosal trapping. Ultimately, the present results suggest that adhesion proteins have different levels of expression according to EO location in extra- or intra-tissue spaces.

This study showed using cytometry and immunohistochemistry that EOs in the polyps are activated (CD69⁺) and express IL-5R α and IL-9R α in patients with and without asthma. Such a result excludes the hypothesis of a possible EO membrane alteration through a purification protocol as might be postulated for adhesion receptor

down-expression. IL-5 plays a critical role in the lifespan of EOs by contributing to their activation state and by protecting them from apoptosis.⁴⁵ The authors observed that EO apoptosis was decreased in a concentration-dependent manner with IL-5 after an 18-hour incubation compared with unstimulated EOs in patients with and without asthma. Paradoxically, they observed a relative decrease of IL-5R α expression on mucosal EOs in patients with asthma. This result corroborates previous observations of a differential expression of IL-5R α isoforms in patients with asthma or nasal polypsis.^{33,46,47} Gevaert et al⁴⁵ showed that exposure of EOs to IL-5 decreased their responsiveness to IL-5 by downregulation of the IL-5R α membrane. Liu et al⁴⁶ observed a similar result after allergen

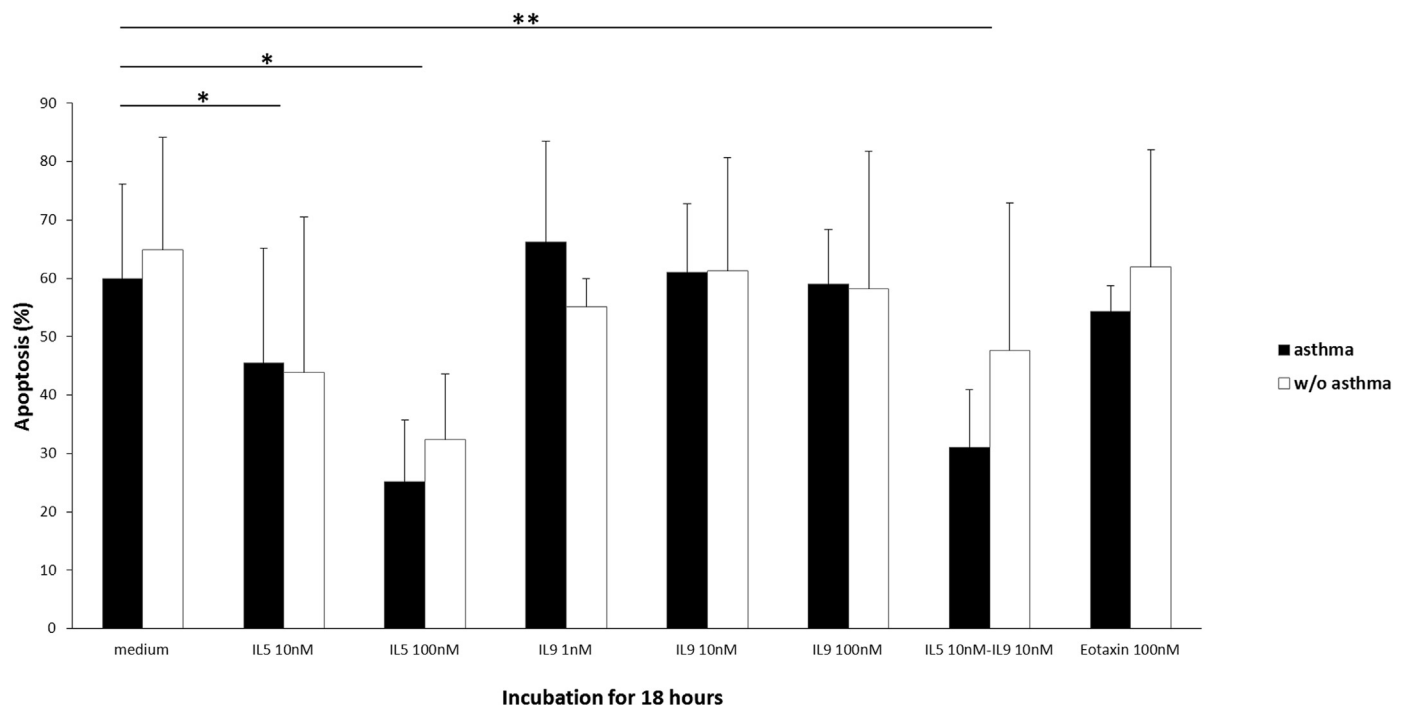


Figure 6. Apoptosis assessment of purified eosinophils after 18-hour incubation under different cytokine conditions in patients with ($n = 5$) and without ($n = 5$) asthma. Compared with medium alone, interleukin-5 (IL-5) in a concentration-dependent manner (* $P < .05$) or co-incubation with IL-5 and interleukin-9 (IL-9; ** $P = .01$) decreased the apoptosis rate. With co-incubation, IL-9 improved the antiapoptotic action of IL-5 in patients with asthma.

challenge of patients with asthma whose EOs were collected from bronchoalveolar lavage. The investigators assumed that metalloproteinase-mediated receptor cleavage⁴⁷ or mobilization-internalization processes⁴⁸ could be involved in an IL-5–dependent manner. Immunofluorescence polyp section analyses showed positive staining for IL-5R α in the stromal compartment. The authors postulate that a steady state of IL-5R α recycling and cleavage on the EO membrane could corroborate published data and the present findings. From this perspective, it was pertinent to evaluate a potent local effect of IL-9 on IL-5R α regulation.

The authors found that IL-9R α was expressed more (with asthma $4.4 \pm 0.9\%$, without asthma $1.7 \pm 0.9\%$; $P < .05$) on purified EOs of patients with asthma. IL-9 has been described as a T_H2 antiapoptotic cytokine on T-cell lines⁴⁹ and as a factor of EO maturation in patients with asthma.⁵⁰ As such, despite similar concentrations of IL-9 in the polyps of the 2 groups of patients, reactivity to this cytokine might favor EO persistence in CRSwNP in an IL-5–dependent manner. The present data show that IL-9 alone did not affect EO survival. In contrast, as previously described in asthma,⁵⁰ the supplementation of IL-5 with IL-9 sustained the antiapoptotic effect on EOs compared with IL-5 alone. Moreover, co-incubation with IL-5 and IL-9 increased IL-5R α expression on EOs in patients with asthma. The synergistic action of IL-5 and IL-9 is corroborated by published data on induced eosinophilia in IL-9 transgenic mice.⁵¹ Produced by EOs,³⁴ IL-9 also could enhance this potential additive interaction by an autocrine process. In patients with concomitant asthma and CRSwNP characterized by high IL-5 levels in nasal polyps, IL-9 could refine interactions between IL-5 and IL-5R α by promoting IL-5R α expression on the EO membrane. Eotaxin, an important activator of EOs,⁵² had no effect on EO apoptosis and IL-5R α expression in the present study. Eotaxin activity could require environmental cofactors that were absent in the present in vitro culture conditions.

Ultimately, the authors believe that a functional redundancy exists among T_H2 cytokines to allow persistent and severe disease in patients with asthma and CRSwNP. Downregulation of IL-5R α on the EO membrane seems to be offset by IL-9 promotion of IL-5R α expression. Taken together, the present results suggest that eosinophilia in CRSwNP is achieved in part by the modulation of adhesion agents on the EO membrane. With concomitant asthma, intricate T_H2 cytokine involvement in EO survival could contribute to a specific inflammatory endotype. This new insight needs to be corroborated by in vivo models to confirm the potential impact of IL-5 and IL-9 co-targeting in new therapeutic approaches.

Taken together, the present results indicate that mucosal eosinophilia in CRSwNP requires a combination of recruitment and survival processes whose modulations can be observed in patients with vs without asthma. With a specific approach to cell purification, the authors showed that blood EO homing into the nose and T_H2 cytokine crosstalk are cornerstones of CRSwNP inflammation in patients with asthma.

For the first time in nasal polyposis, these results point to an involvement of IL-9 in EO survival, especially in patients with asthma. Understanding the mechanisms of IL-9–dependent IL-5R α upregulation on the EO surface needs further investigations.

Future targeted immune therapies for patients with medically and surgically resistant CRSwNP will have to consider these intricate inflammatory pathways simultaneously.

Acknowledgments

The authors thank Ms Virginie Lefèvre (EA2686) and Dr Bénédicte Vanteghem, PhD (EA2686) for their expert technical assistance in data collection and Dr Lennart T. Mars, PhD (U995) for proofreading this report.

Supplementary Data

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.anai.2015.01.012>.

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Supplementary Data

eMethods

Immunohistochemistry and Immunofluorescence

Purified blood EOs were cytocentrifuged on slides for 2 minutes at 300 rpm. Cells were fixed with a 1:1 methanol and acetone mixture for 2 minutes at 4°C.

Nasal polyp staining required 2 types of preparation. For CD69 staining, samples were fixed in 10% formalin, embedded in paraffin, sectioned 10 µm thick with an RM-2145 microtome (Leica Biosystems, Nussloch, Germany), and mounted on Superfrost Plus-coated slides (VWR, Radnor, Pennsylvania). For IL-5Rα staining, samples were frozen in dry ice, cryo-protected by immersion in cold 2-methylbutane, and stored at –80°C before being sectioned further with a cryostat (Leica Biosystems) at 12 µm thick.

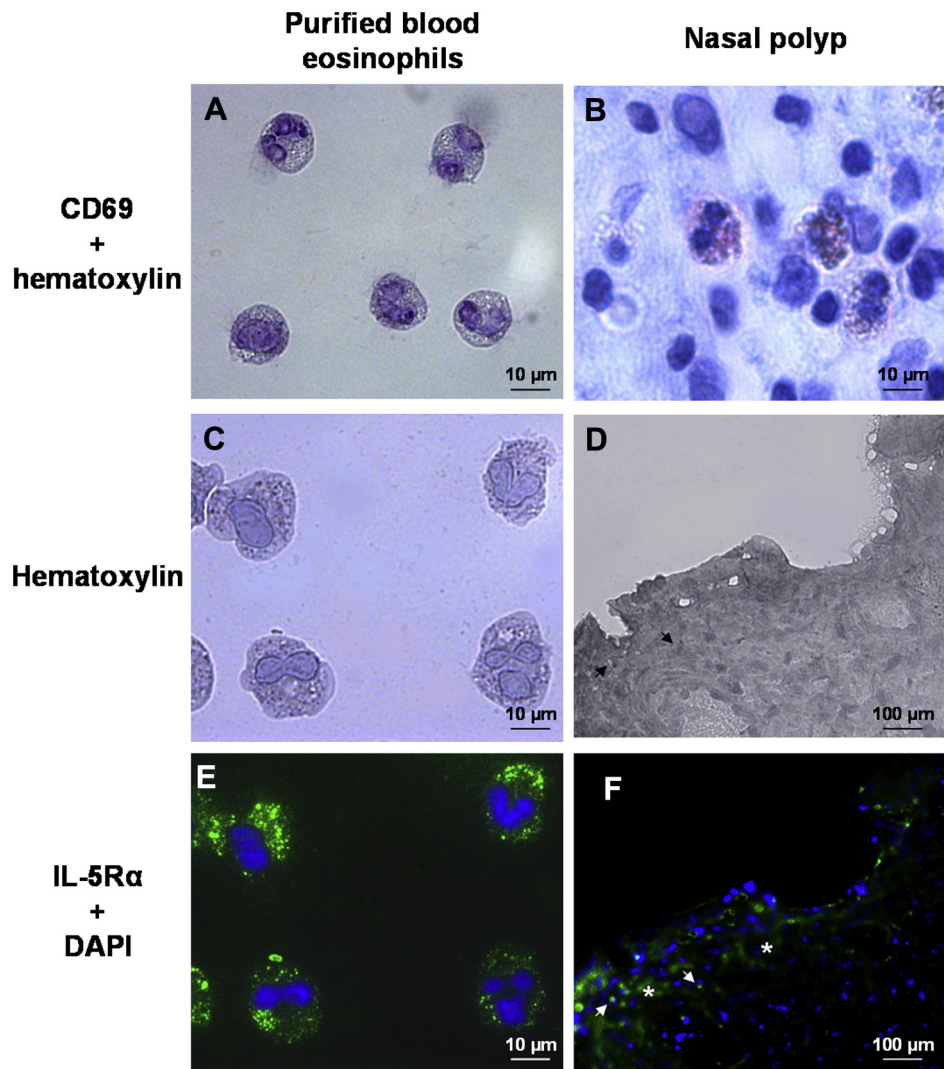
Paraffin-embedded polyp slides were cleared in xylene and rehydrated by a graded series of alcohol. For the heat-mediated antigen retrieval step, slides were subsequently boiled in 10 mmol/L of sodium citrate buffer (pH 6.0), maintained at a sub-boiling temperature for 10 minutes, and cooled for 30 minutes. Cytospin blood EO slides previously stored and polyp slides washed with Tris buffered saline and 0.05% Tween were incubated with blocking buffer (PBS and 5% de-complemented goat serum; R&D System) for 30 minutes. Then, they were incubated with monoclonal mouse anti-CD69 (1:25; Abcam, Cambridge, Massachusetts) for 30 minutes at RT. After washing with Tris buffered saline and 0.05% Tween, slides were incubated with biotinylated goat anti-mouse IgG (1:100; Vector Laboratories, Burlingame, California) for 30 minutes at RT. Incubation with mouse IgG (Jackson ImmunoResearch) or secondary antibody alone was used as a negative control. After washing unbound secondary antibodies (twice with Tris buffered saline and 0.05% Tween), Vectastain ABC-AP reagents (Vector Laboratories) were used for subsequent incubation with alkaline phosphatase substrate solution for 20 minutes in the dark. Endogenous peroxidase activity was quenched by levamisole. Slides were washed with distilled water and counterstained with Harris hematoxylin solution (Sigma-Aldrich). Lithium carbonate was used to obtain a blue stain. After mounting, slides were observed with an AxioImager D1 light microscope (Carl-Zeiss AG, Oberkochen, Germany).

Frozen sections were thawed before IL-5Rα immunofluorescence staining. Centrifuged blood EO slides and polyp slides were stained with hematoxylin and washed 3 times in PBS. After incubation with blocking buffer (PBS and 5% de-complemented goat serum; R&D System) for 30 minutes at RT, polyclonal rabbit anti-IL-5Rα (1:100; Abcam) was added for overnight incubation at 4°C in a humid chamber in the dark. After washing of unbound antibodies (PBS, 3 times), goat antirabbit coupled with FITC and F(ab')₂ (1:100; Jackson ImmunoResearchPA) was added for 2 hours in the dark at RT. Incubation with a secondary antibody alone was used as a negative control. Slides were washed (PBS, 3 times) immediately before mounting with Fluoroshield with 4',6-diamidino-2-phenylindole dihydrochloride (1:1,000; Sigma-Aldrich). Slides were analyzed with fluorescent microscopy (Leica Microsystem, Wetzlar, Germany).

eResults

CD69 and IL-5Rα expression on blood and polyp eosinophils

Immunohistochemical and immunofluorescence analyses were performed on purified EOs from blood samples (eFig 1A, C, E) and on nasal polyps (eFig 1B, D, F). Positive staining for CD69-labeled samples (eFig 1A, B) was detected with an avidin-biotin peroxidase staining system that results in a red-brown reaction product. Sections were counterstained with hematoxylin (blue) for contrast. IL-5Rα-positive EOs were located on sections with hematoxylin (blue) and detected with FITC fluorescence (green). The nucleus was counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (blue). Immunohistochemical analysis for CD69 showed no positive EOs in blood samples (eFig 1A). The EOs in nasal polyps showed positive staining (red-brown) for CD69 within inflammatory cell stromal infiltrate (eFig 1B). In EOs collected from blood samples (eFig 1C), IL-5Rα fluorescence staining (green) was detected on the cell surface and mainly in cytoplasm (eFig 1E). Beneath the epithelial layer of nasal polyps (eFig 1D), a weak positive staining for IL-5Rα (green) was observed in EOs and in the extracellular space (eFig 1F).



eFigure 1. . CD69 and interleukin-5 receptor- α (IL-5R α) staining of purified eosinophils from (A, C, E) blood sample and (B, D, F) nasal polyp eosinophils. Depicted are relevant qualitative findings from patients with and without asthma. (A) No CD69⁺ cells were detected in the blood sample. (B) In the nasal polyps, CD69⁺ eosinophils (red-brown) were observed within inflammatory cell stromal infiltrate. (C–E) In the blood sample, IL-5R α fluorescence staining (green) was detected on the cell surface and mainly in cytoplasm. (F) Beneath the epithelial layer of the nasal polyp (D), positive staining for IL-5R α (green) was observed in eosinophils (arrows) and in the stroma (asterisks). DAPI, 4',6-diamidino-2-phenylindole dihydrochloride.

eTable 1
Antibodies and isotype controls for flow cytometric and immunohistochemical analyses

Name	Supplier	Clone	Isotype
FITC–isotype mouse IgG1	BD Pharmingen	555748	
FITC–monoclonal mouse anti-CD44	BioLegend (San Diego, California)	BJ18	mouse IgG1
FITC–monoclonal mouse anti-CD69	BioLegend	FN50	mouse IgG1
FITC–monoclonal mouse anti–IL-5R α	R&D System	26815	mouse IgG1
FITC–monoclonal mouse anti–VLA-4	BD Pharmingen	9F10	mouse IgG1
FITC–monoclonal mouse anti-CD11a	Bender MedSystems (Vienna, Austria)	BMS102	mouse IgG1

Abbreviations: FITC, fluorescein isothiocyanate; IL-5R α , interleukin-5 receptor- α ; VLA-4, very late antigen-4.