

Distinctive Blood Eosinophilic Phenotypes and Cytokine Patterns in Eosinophilic Esophagitis, Inflammatory Bowel Disease and Airway Allergy

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Key Words

Cytokines · Eosinophils · Inflammatory bowel disease · Eosinophilic esophagitis · Flow cytometry

Abstract

Blood eosinophil numbers may be elevated in allergy, inflammatory bowel disease and eosinophilic esophagitis. The aim of this study was to examine whether circulating eosinophils display distinct phenotypes in these disorders and if different patterns of eosinophilic chemoattractants exist. Blood eosinophils from patients with symptomatic eosinophilic esophagitis (EoE; n = 12), ulcerative colitis (n = 8), airway allergy (n = 10) and healthy controls (n = 10) were enumerated and their surface markers analyzed by flow cytometry. Plasma levels of pro-eosinophilic cytokines were quantified in parallel. Data were processed by multivariate pattern recognition methods to reveal disease-specific patterns of eosinophil phenotypes and cytokines. EoE patients had higher numbers of eosinophils with enhanced expression of CD23, CD54, CRTH2 and CD11c and diminished CCR3 and CD44 expression. Plasma CCL5 was also increased in EoE. Although allergic patients had increased interleukin (IL)-2, IL-3, IL-5 and granulocyte macrophage colony-stimulating factor plasma

concentrations, their blood eosinophil phenotypes were indistinguishable from those of healthy controls. Decreased eosinophilic expression of CD11b, CD18, CD44 and CCR3, but no distinctive pattern of eosinophil chemoattractants, characterized ulcerative colitis. We propose that eosinophils acquire varying functional properties as a consequence of distinct patterns of activation signals released from the inflamed tissues in different diseases.

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Introduction

Eosinophilic granulocytes accumulate in the tissues in several inflammatory disorders, including allergies, inflammatory bowel disease [1, 2] and the recently described condition eosinophilic esophagitis (EoE) [3]. In EoE, eosinophils invade the mucosa of the esophagus, a part of the gastrointestinal tract that is normally devoid of these cells. Symptoms include difficulty in swallowing food, which may even result in food impaction, heartburn, abdominal pain, nausea and vomiting. Both children and adults are afflicted by the disease whose cause is unknown [4]. Many patients are sensitized to food and

Table 1. Patient characteristics

Diagnostic category	Patients n	Median age years	Females %	Disease-specific medication	Topical inhalant allergy medication	
					steroid	β_2 -stimulant
EoE	12	41 (17–62)	25	proton pump inhibitor 8% carbamazepine 8% no treatment 83%	17%	0%
UC	8	42 (19–76)	38	mesalazine (sulfa) 25% no treatment 62%	12%	12%
AA	10	30 (24–50)	100	sodium cromoglycate 10% oral antihistamine 20% no treatment 60%	0%	30%
HC	10	39 (22–61)	90	not applicable	0%	0%

Figures in parentheses are ranges.

inhalant allergens and children improve when started on hypoallergenic elemental diets; whether this holds true for adults is less clear [5].

Eosinophils mature and differentiate in the bone marrow under the influence of granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3 and IL-5 [6]. In the healthy state, newly produced eosinophils pass via the bloodstream to their final destination, primarily the gut mucosa, but also to the thymus, spleen and lymph nodes [7]. Eosinophils constitute <5% of the white blood cells in the circulation in healthy individuals, but this proportion may increase in parasite infections, as well as in allergic and other inflammatory conditions characterized by eosinophilic involvement.

Eosinophils are recruited from the bloodstream into the tissues by locally produced chemokines and other chemotactic factors. This traffic occurs under steady-state conditions and is further enhanced during inflammation. Factors that may attract eosinophils include the cytokines/chemokines eotaxin-1, -2 and -3, IL-5, CCL5 (RANTES), the tripeptide formyl-methionyl-leucyl-phenylalanine released by metabolically active bacteria and damaged mitochondria, and platelet-activating factor [8]. In the tissue, eosinophils may recognize and be activated by airborne allergens [9–11], bacteria [12] and necrotic epithelial cells [13]. Their role in the pathogenesis of asthma, inflammatory bowel disease and EoE is unclear, and both pathogenic [14, 15] and protective functions [16] have been suggested. In addition, eosinophils participate in tissue remodeling [17] and may be engaged in immune regulation [18].

As eosinophils appear to be able to fulfill several functions, it is possible that their phenotype in the blood may vary in different inflammatory disorders. This may, in turn, depend on the mixture of cytokines and other inflammatory mediators released from the afflicted tissues that regulate the production and release of eosinophils from the bone marrow, as well as the activation and trafficking of eosinophils in the bloodstream. Here, we have investigated the phenotype of blood eosinophils in patients with ongoing inflammatory diseases characterized by tissue eosinophil infiltration, namely allergic airway disease, ulcerative colitis (UC) and EoE. We also examined the blood levels of eosinophil chemoattractants and growth factors in the same individuals. The aim was to define disease-specific patterns of eosinophil activation, which could shed some light on the pathogenic processes characterizing these diseases and perhaps be used for diagnostic purposes.

Materials and Methods

Patients

We investigated 40 adult individuals: 12 with symptomatic EoE (n = 12), 8 with symptomatic UC, 10 with symptomatic airway allergy (AA; asthma and/or allergic rhinitis) and 10 healthy controls (HC) (table 1).

Patients with EoE were recruited at the Head and Neck Surgery Department of either NÄL Hospital or Sahlgrenska University Hospital, Sweden. Ten out of 12 EoE patients had typical endoscopic features of EoE including trachealization, furrows, white specks, mucosal shredding and/or strictures and all had experienced dysphagia and/or food impaction. Biopsies were

Table 2. Monoclonal antibodies used for flow cytometric analyses

CD	Molecule	Clone	Isotype	Fluorochrome
CD4	co-receptor for MHC II	RPA-T4	IgG _{1, κ}	APC
CD9	binds integrins, triggers platelets	M-L13	IgG _{1, κ}	PE
CD11b	forms integrin Mac-1 (CR3) w CD18	D12	IgG _{2a, κ}	PE
CD11c	forms integrin p150,95 (CR4) w CD18	B-ly6	IgG _{1, κ}	APC
CD16	FcγRIIIb, IgG-R	3G8	IgG _{1a, κ}	FITC
CD18	integrin-β ₂ chain	6.7	IgG _{1a, κ}	FITC
CD19	part of BCR	HIB19	IgG _{1, κ}	PE
CD23	FcεRII, IgE-R	EBVCS-5	IgG _{1, κ}	APC
CD25	IL-2R α-chain	2A3	IgG _{1, κ}	APC
CD40	binds CD154 (CD40L) on T cells	5C3	IgG _{1, κ}	APC
CD44	binds hyaluronic acid	G44-26	IgG _{2b, κ}	APC
CD49d	VLA-4 α-chain	9F10	IgG _{1, κ}	PE
CD54	ICAM-1	HA58	IgG ₁	APC
CD66b	shed during granulocyte activation	80H3	IgG ₁	FITC
CD66c	granulocyte antigen	KORSA3544	IgG ₁	FITC
CD69	very early activation antigen	FN50	IgG _{1, κ}	APC
CD184	CXCR4	12G5	IgG _{2a, κ}	APC
CD193	CCR3	5E8	IgG _{2b, κ}	PE
CD294	CRTH2 (PGD ₂ receptor 2, DP2)	BM16	rat IgG _{2a}	Alexa Fluor 647
	formyl peptide receptor 2 (FPR2, FPRL1)	304405	IgG _{2b}	APC
	major basic protein (MBP P1)	AHE-2	IgG ₁	unconjugated

gathered from the distal, middle and proximal part of the esophagus and from the fundus ventriculi and the duodenum in all EoE patients. All EoE patients had a peak count of >20 eosinophils per microscopic high-power field in at least one biopsy [3]: the median peak number of eosinophils was 40/high-power field (range 20–80). Eosinophilic infiltration of the mucosa due to concurrent gastroesophageal reflux disease was ruled out by lack of response to proton pump inhibitor therapy and/or normal acid levels according to 24-hour pH monitoring. More than half (7/12) had a history of inhalant allergy; none were smokers.

UC patients (n = 8) were recruited at the Gastroenterology Unit at NÄL Hospital, Trollhättan, Sweden. Five of the patients were new cases, and 3 were previously diagnosed and displayed endoscopic signs of flare-up. The grade of inflammation was scored according to the Baron system (median score 2.0, range 1.5–3.0). The regional distribution in the intestine was as follows: segmental colitis (n = 1), proctitis (n = 2), proctosigmoiditis (n = 2), extensive colitis (n = 1) and total colitis (n = 2).

Patients with symptomatic AA were recruited at Norrmalm Primary Health Care Center, Skövde, Sweden, and had IgE-dependent allergy, as evidenced by Phadiatop (Phadia AB, Uppsala, Sweden) or skin prick tests. Patients taking systemic corticosteroids were excluded, and topical corticosteroids were discontinued prior to sampling (table 1). HC were recruited among friends and colleagues. No physical examination was done. The subjects declared themselves to be healthy and were not allowed to have any symptoms or inflammatory processes at the time of sampling. None were endoscoped. The study was approved by the Research Ethics Committee of the Medical Faculty at the University of Go-

thenburg, Gothenburg, Sweden, and written informed consent was obtained from all participants.

Study Setup

All study participants donated 12 ml of EDTA-anticoagulated venous blood on one occasion. The maximal time between drawing of blood and flow cytometry analyses was 24 h; blood samples were kept at room temperature, and control experiments revealed that this did not affect eosinophil phenotypes or cytokine levels in plasma (data not shown). Eosinophils were quantified using an automated cell counter (ADVIA 2120i; Siemens Medical Solutions Diagnostics, Tarrytown, N.Y., USA). After removal of erythrocytes by repeated hypotonic lysis, leukocytes were washed once in Krebs-Ringer-glucose buffer [19] and analyzed immediately by flow cytometry for eosinophil phenotypes. Plasma was stored at –20°C for later assessment of eosinophil-activating cytokines.

Flow Cytometry

Unfractionated leukocytes were incubated with panels of antibodies conjugated with FITC, PE, APC, or Alexa Fluor 647 (table 2) for 15 min at room temperature, washed and analyzed by 4-color fluorescence using a FACSCanto II™ Flow Cytometer (BD Biosciences, San Diego, Calif., USA). FluoroSpheres (DakoCytomation, Glostrup, Denmark) were employed as calibration controls. All antibodies were from BD Biosciences except for anti-KORSA-3544 (Beckham Coulter, Inc., Fullerton, Calif., USA) and anti-FPR2 antibody (R&D Systems, Minneapolis, Mich., USA). Intracytoplasmic staining of major basic protein and CXCR4 was performed using the IntraStain kit (DakoCytomation).

Eosinophils were identified as a population with high side scatter and low CD16 expression, as opposed to neutrophils that were CD16^{hi}. To confirm the absence of eosinophils in the CD16^{hi} population, CD16^{hi} cells were sorted (FACSAria cell sorter; BD Biosciences), cytopspins prepared (Cytospin; Shandon Scientific Co. Ltd., London, UK), stained by eosin and examined in the light microscope (100 cells counted). No eosinophils were found in this preparation.

Multiple isotype controls are inadequate for multicolor staining, as true controls require that one parameter be changed at a time. Instead, the 'fluorochrome minus one' technique was used, in which positive events are discriminated from negative events not by a single isotype-based threshold or unstained cells, but by multiple controls characterized by sequential removal of each fluorochrome-labeled antibody used in the multicolor stain [20]. However, for intracellular stains, an irrelevant isotype-matched antibody (anti-CD19; BD Biosciences) was used to monitor background staining. Data were collected using FACSDiva™ Software 6.0 (BD Biosciences); 100,000 events were acquired and analyzed using the software Flow Jo 7.2.2 (Tree Star Inc., Ashland, Oreg., USA). Data were expressed as median fluorescence intensity.

Cytokines

Plasma levels of IL-2, IL-3, IL-5, GM-CSF, CCL5 (RANTES) and CCL11 (eotaxin-1) were determined using the Cytometric Bead Array (BD Biosciences) and analyzed by flow cytometry. Data were analyzed using the FCAP Array software (BD Biosciences). CCL26 was detected using Human Eotaxin-3 Duo Set ELISA (R&D Systems Ltd., Abingdon, UK). The limits of detection were: 11 pg/ml for IL-2, 0.3 pg/ml for IL-3 and 1.1 pg/ml for IL-5, 0.8 pg/ml for eotaxin, 0.002 pg/ml for CCL5, 0.2 pg/ml for GM-CSF and 30 pg/ml for CCL26.

Statistics

Multivariate analysis for pattern recognition was performed using the SIMCA-P statistical package (Umetrics, Umeå, Sweden). 'Partial least squares projections to latent structures' with discriminant analysis (PLS-DA) [21] was used to see if models could be created in which study persons clustered into their respective diagnostic categories based on eosinophilic activation markers in blood or plasma concentrations of cytokines. The fitness of the models was evaluated by cross-validation. Flow cytometry and cytokine data were log transformed prior to analysis when deemed appropriate by the software based on skewness of data. Cytokine values below the detection limit were set to 0.5 times this limit. Findings derived from the PLS-DA models were further analyzed using the nonparametric Kruskal-Wallis method (GraphPad Prism 5.0 software; GraphPad, San Diego, Calif., USA). Correlations were assayed using the Spearman rank correlation test (GraphPad Prism).

Results

To test the hypothesis that the various eosinophil-associated diseases differed with respect to eosinophil phenotypes and patterns of eosinophilic chemoattractants in peripheral blood, all data were analyzed by the multivar-

iate technique of PLS-DA. This method models the relationship between a large set of study variables (X), in this case eosinophilic activation markers or plasma cytokine levels, and outcome variables (Y), in this case, diagnostic categories. An advantage compared to traditional regression analyses is that all variables can be analyzed simultaneously since 'mass significance' is not a problem and variables are allowed to be interdependent.

We first examined eosinophil surface marker expression in the four diagnostic groups EoE, UC, AA and HC. However, no model could be obtained that was able to separate all four groups. This failure was the result of a large overlap between the HC and allergic groups (data not shown). Univariate analysis confirmed that eosinophils from controls and allergics did not differ significantly with respect to any of the measured parameters (data not shown). Therefore, we constructed a new model based on EoE, UC and HC, excluding AA study persons. This model showed a good fit (fig. 1a) and was able to separate the individuals into the three groups EoE, UC and HC, respectively (fig. 1b). Figure 1c shows which variables were responsible for this separation; variables situated close to the position of a certain diagnostic category tended to be higher in this group of patients, while variables distant from the diagnostic category tended to be lower, compared to the other disease categories. According to the obtained PLS-DA model, the following features characterized eosinophils retrieved from the blood of patients with EoE, relative to the other eosinophil-dominated inflammatory conditions and to HC: high absolute numbers of blood eosinophils, relative numbers of eosinophils and enhanced eosinophilic expression of CRTH2, CD11c, CD23 (low-affinity IgE receptor) and CD54 (ICAM-1) (fig. 1c). Conversely, the CCR3 and CD44 variables were positioned close to the category of HC, signifying that eosinophils derived from the two patient groups EoE and UC had reduced levels of these two receptors, compared with healthy persons (fig. 1c). Finally, CD11b and CD18 were positioned directly opposite to the UC category, indicating that these markers were relatively decreased in UC patients compared to EoE and HC (fig. 1c). Variables situated in the middle of the figure lacked the power to discriminate between the conditions included in the model and were not analyzed further (fig. 1c).

Variables identified by the pattern recognition method PLS-DA, important for the discrimination of EoE from UC and HC, respectively, were further selected for analysis using the univariate non-parametric Kruskal-Wallis test (fig. 2). These analyses confirmed that CD23,

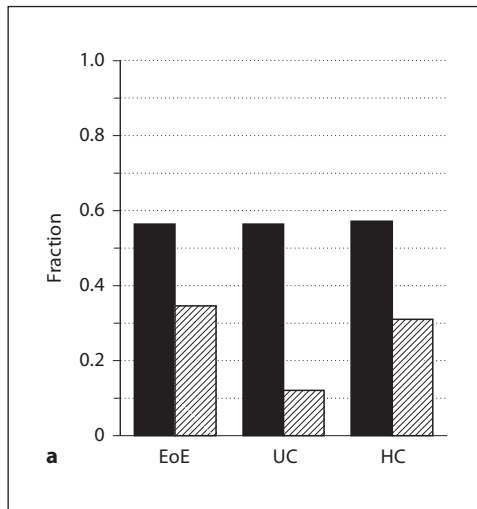
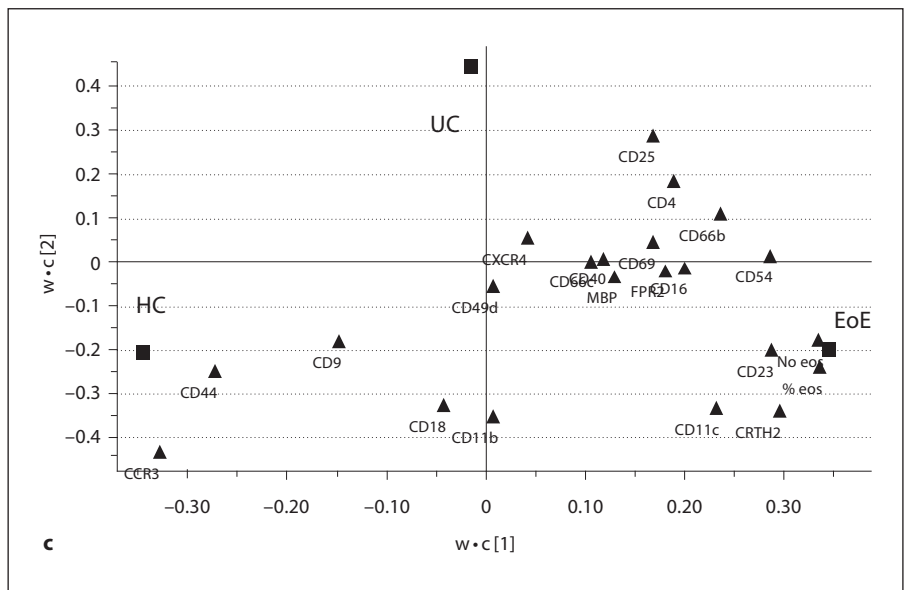
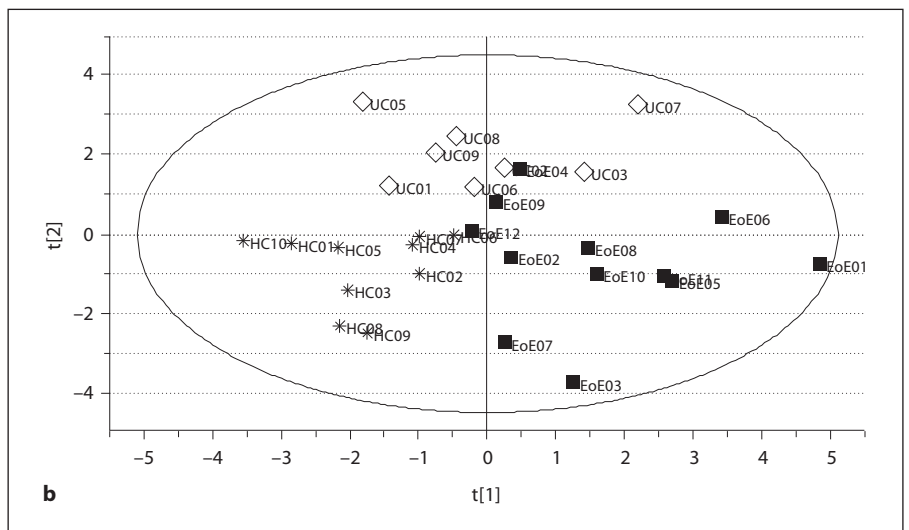


Fig. 1. Clustering of patients into clinical categories EoE, UC and HC based on eosinophil counts and surface markers, using ‘projection onto latent structures’ with ‘discriminant analysis’ (PLS-DA) modeling. **a** Fitness of model is indicated by $R^2 VY_{cum}$ (black columns), the cumulative sum of squares of all Y values (diagnostic categories) and X values (surface markers and eosinophil numbers) explained by the model, and $Q^2 VY_{cum}$ (hatched columns), the fraction of the total variation in the Y variable that can be predicted by the sum of the X variables. **b** Separation of patients according to diagnosis into HC (lower left quadrant, labeled by crosses), UC (upper part of diagram, open diamonds) and EoE (right part, black boxes). Axes are labeled by score vectors $t[1]$ and $t[2]$, synthetic variables constructed from the original X variables to contain as much information as possible of relevance for modeling the Y variables. **c** Eosinophil data (X variables) that contribute to the segregation of the clinical groups (Y variables). The position of the X variables corresponds to the position of the individuals in panel b, i.e., variables close to the EoE diagnosis marker (number of eosinophils, % eosinophils, CD23, CRTH2, CD11c, CD54) are higher in these patients, while variables close to the HC diagnosis marker (CCR3 and CD44) are generally higher in HC than in the two disease categories. Weight variables $w \cdot c[1]$ and $w \cdot c[2]$ show which variables contribute to the PLS-DA model; the weight for X variables is denoted ‘w’, while the weight for Y variables is denoted ‘c’, depicted in the same figure.



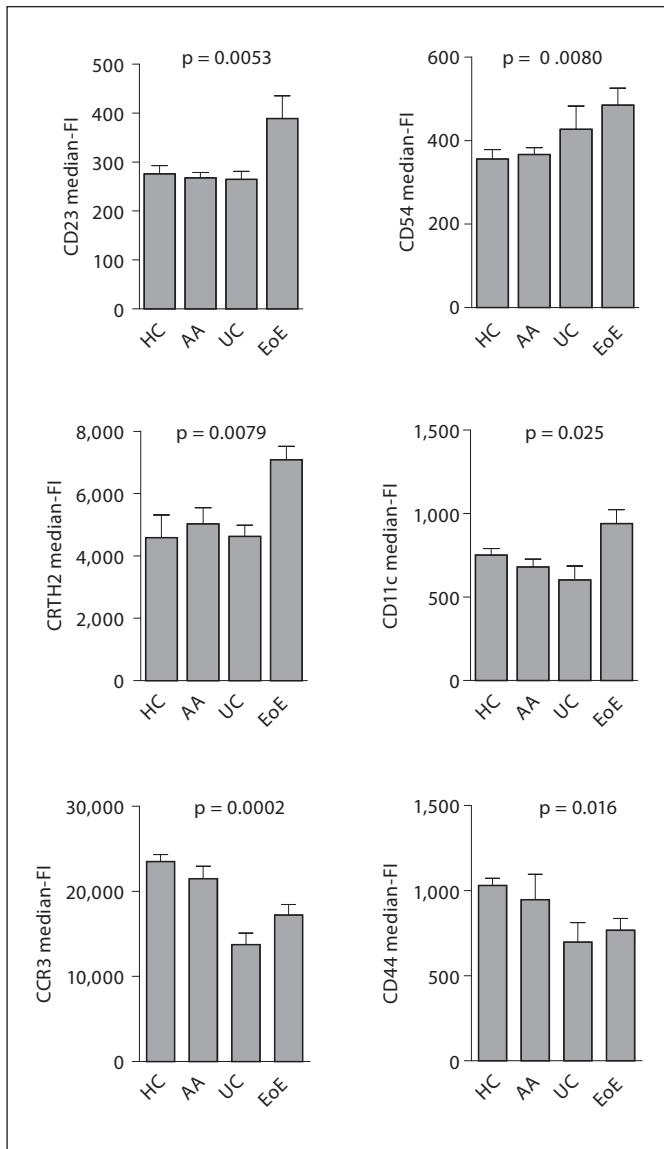


Fig. 2. Eosinophilic expression of surface markers. Expression of CD23, CD54, CRTH2, CD11c, CD44 and CCR3 was assessed by FACS and expressed as median fluorescence intensity (median-FI). Columns indicate the mean and SEM for HC, AA, UC and EoE patients. Differential expression of surface markers between the clinical groups was analyzed by the Kruskal-Wallis test; the p values are indicated in the figure.

CD54, CRTH2, CD11c, CCR3 and CD44 were differently expressed on eosinophils isolated from blood of patients belonging to the studied categories. Thus, eosinophils from EoE patients had increased expression of CD23, CD54, CRTH2 and CD11c, while CCR3 and CD44 were more strongly expressed on eosinophils from HC and AA patients (fig. 2). Moreover, EoE patients had significantly

Table 3. Absolute and relative eosinophil numbers in peripheral blood

	Eosinophils, $\times 10^9/l$		Eosinophils, %	
	median	25th–75th percentile	median	25th–75th percentile
EoE	0.34	0.17–0.51	4.6	2.3–7.9
UC	0.080	0.060–0.17	0.92	0.81–2.4
AA	0.15	0.10–0.34	2.1	1.5–3.6
HC	0.096	0.059–0.19	1.5	0.89–3.0
p value ¹	0.013		0.0074	

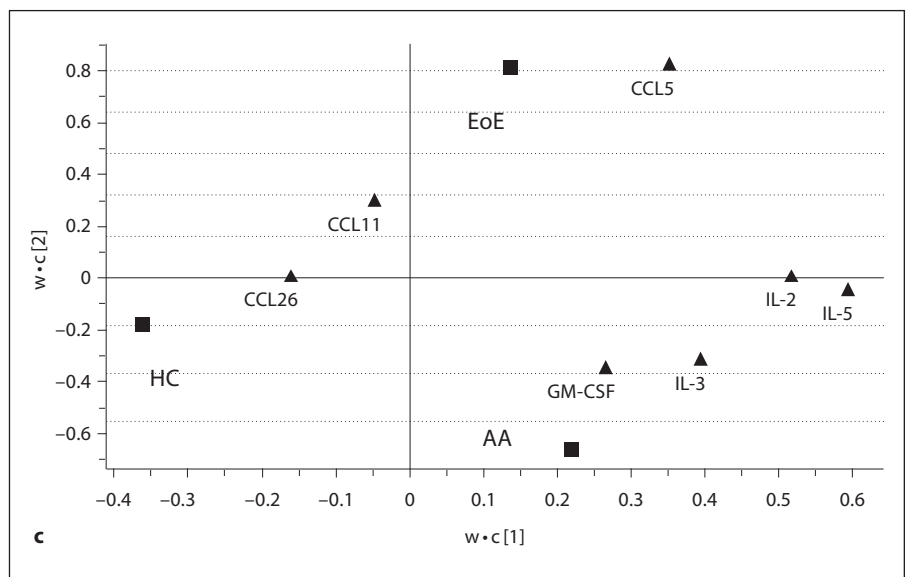
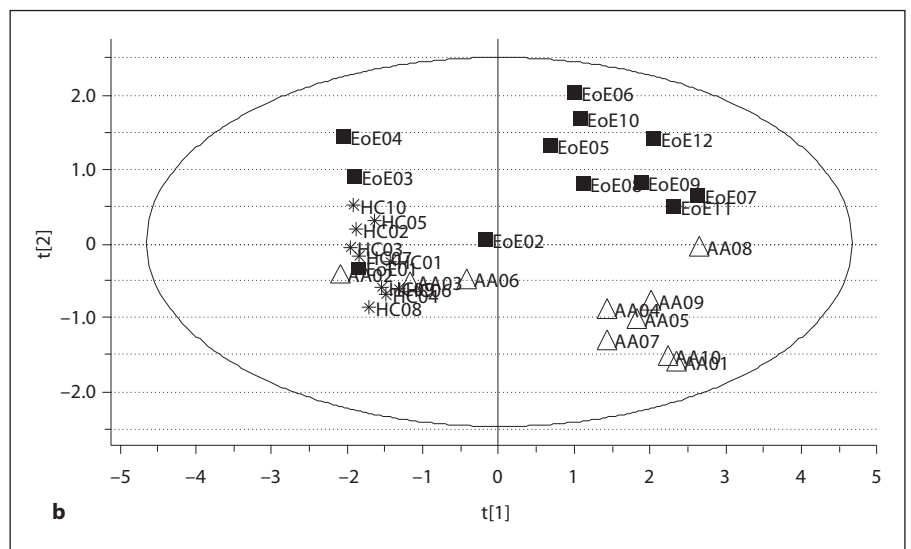
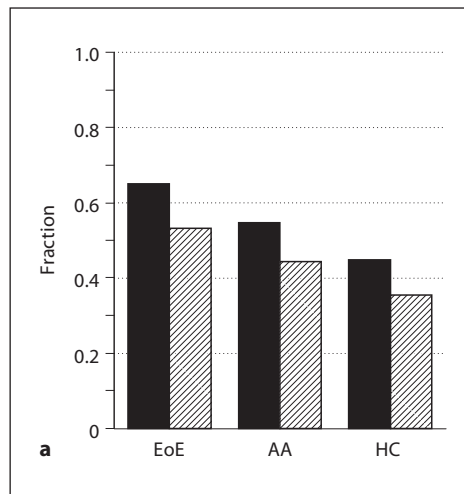
¹ Kruskal-Wallis test.

higher levels of absolute as well as relative blood eosinophil counts compared to the other groups (table 3). CD11b and CD18 expression was lower in UC patients relative to the other categories but did not reach statistical significance (data not shown).

Next, we studied the plasma levels of cytokines of relevance for the maturation and release of eosinophils from the bone marrow into the bloodstream and their recruitment into the inflamed tissues. First, we examined whether PLS-DA could generate a model capable of discriminating between the four clinical groups. This was not possible; the UC group overlapped too much with the HCs to permit the construction of a valid model. Instead, we generated a model composed of HC, EoE and AA study persons. This model had sufficient explanatory capacity (fig. 3a) and was able to separate the three groups from one another reasonably well (fig. 3b). However, as seen in the observation plot, some patients with AA clustered together with the HC, and the EoE patients were widely spread out (fig. 3b). Figure 3c illustrates that CCL26 and CCL11 projected close to the HC, CCL5/RANTES was adjacent to the EoE disease category, and GM-CSF, IL-3, IL-2 and IL-5 were positioned close to the AA category. Univariate analyses largely confirmed these findings: EoE patients displayed higher average levels of CCL5 (RANTES) than all other groups, and elevated levels of GM-CSF typified airway allergics (fig. 4). IL-2 and IL-3 were similarly increased in the blood of EoE and AA subjects (fig. 4). Finally, although IL-5 was higher in airway allergics, it was elevated in all inflammatory conditions, e.g., in EoE and UC as well (fig. 4).

We also performed a series of univariate correlation tests (Spearman rank test) to unravel interdependence between the parameters identified to have power to dis-

Fig. 3. Clustering of patients by PLS-DA based on blood cytokine levels. The model permitted discrimination between EoE, AA and HC. **a** Model characteristics where R^2VY_{cum} (black columns) signifies the cumulative sum of squares of all the Y values (diagnostic categories) and X values (cytokines) explained by the model, and Q^2VY_{cum} (hatched columns) is the fraction of the total variation in the Y variable that can be predicted by the sum of the X variables. **b** Separation of patients according to diagnosis into HC (tight cluster on the left, labeled by crosses), AA (lower part of diagram, open triangles) and EoE (upper part of diagram, mainly to the right, black boxes). Axes are labeled by score vectors $t[1]$ and $t[2]$, synthetic variables constructed from the original X variables to contain as much information as possible of relevance for modeling the Y variables. **c** Cytokine data (X variables) that contribute to the segregation of the clinical groups (Y variables). The position of the variables corresponds to the position of the individuals in panel b, i.e., CCL5 adjacent to the EoE diagnostic category is higher in these patients, while GM-CSF, IL-2, IL-3 and IL-5 are close to the AA diagnosis, and hence, high in these patients. Weight variables $w \cdot c[1]$ and $w \cdot c[2]$ show which variables contribute to the PLS-DA model; the weight for X variables is denoted 'w', while the weight for Y variables is denoted 'c', depicted in the same figure.



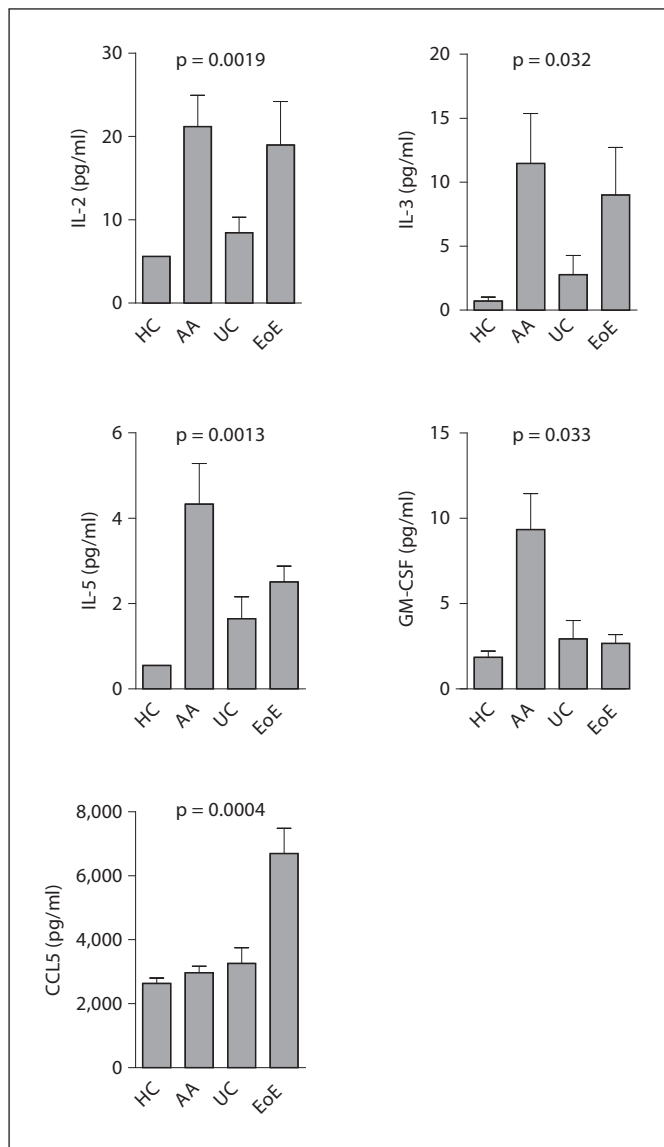


Fig. 4. Plasma levels of cytokines involved in eosinophil production, maturation and chemotaxis in HC, AA, UC and EoE patients. Difference in cytokine levels according to clinical group was tested using the Kruskal-Wallis test. Arithmetic means + 1 SEM are shown.

criminate between the studied clinical categories. Each diagnostic group was analyzed separately to avoid the creation of false correlations that depended on differences between the groups. First, we examined whether the surface markers that were relatively increased in EoE, e.g., CRTH2, CD23, CD11c and CD54, or relatively decreased in EoE, CCR3 and CD44, were correlated. A strong positive correlation was found between CD23 (low-affinity IgE receptor) and CD54 (ICAM-1; $r = 0.82$,

$p = 0.001$), while none of the other markers were significantly correlated to one another. Next, we examined correlations between absolute or relative eosinophil numbers in blood and the identified eosinophil markers and cytokines. The percentage of eosinophils in the circulation of EoE patients correlated positively with the expression of CD9 on eosinophils ($r = 0.59$, $p = 0.041$) and negatively with the levels of CCL11 (eotaxin-1) in plasma ($r = -0.61$, $p = 0.037$). In fact, a highly significant negative correlation was seen between the percentage of eosinophils in the circulation and plasma CCL11 levels when EoE and UC patients were analyzed jointly ($r = -0.60$, $p = 0.0049$), but not when AA patients and/or HC were included in the analysis. No other significant correlations were uncovered between eosinophil numbers, surface markers and cytokines for the other clinical conditions.

Discussion

Here, we studied surface marker expression on circulating eosinophils as well as eosinophil maturation factors and chemoattractants in the blood of patients with three different diseases characterized by eosinophilic tissue infiltration: EoE, UC and AA. Surface marker expression was analyzed by flow cytometry using nonfractionated leukocytes in order to avoid spurious activation caused by immunomagnetic purification of eosinophils. All data were analyzed using the multivariate 'pattern recognition' method PLS-DA to reveal disease-specific eosinophilic phenotypes and/or cytokines, followed by univariate analysis of variables identified to have discriminatory power. Our findings indicate that blood eosinophil phenotypes could be used to distinguish patients with EoE and UC from one another and from HC. In contrast, plasma cytokine patterns discriminated between EoE patients, allergics and healthy persons.

Of the studied diseases, EoE was characterized by the highest absolute and relative eosinophil numbers in the blood. Eosinophils are produced in the bone marrow in response to GM-CSF, IL-3 and IL-5 [6]. An elevated plasma GM-CSF level was restricted to airway allergics, whereas IL-3 and IL-5 were raised in the plasma of EoE patients and airway allergics alike. IL-2 was increased in the blood of both allergics and EoE patients, suggestive of T-cell involvement in these conditions. Unexpectedly, correlation analyses failed to demonstrate any significant associations between the levels of bone marrow stimulatory cytokines and absolute numbers of eosinophils in the blood of any patient category. This lack of association

may depend on the presence of additional substances that promote the production of eosinophils and their release from the bone marrow. A candidate chemoattractant is prostaglandin D₂, whose receptor CRTH2 was overexpressed by eosinophils in EoE. Activation of CRTH2 by prostaglandin D₂ boosts the release of eosinophils from the bone marrow and primes the cells to become more sensitive to the effects of other chemoattractants [22]. Mast cells are the main producers of prostaglandin D₂ and appear in greater numbers in the esophagus of EoE patients [3]. An alternate explanation for our inability to demonstrate a positive association between eosinophil numbers and cytokines is that an eosinophilopoietin such as IL-5 primarily exerts its effects on eosinophil progenitor cells that express the IL-5 receptor, and not on mature eosinophils that have lost the receptor [6]. In fact, anti-IL-5 therapy of EoE patients has been tested and resulted in much reduced blood eosinophil levels but only halved tissue eosinophil counts, which may explain the modest clinical improvement that was achieved [23].

Among EoE patients, we found raised levels of a single chemokine in the blood relative to healthy persons and the other disease categories, namely CCL5 (RANTES). CCL5 is a strong eosinophil chemoattractant [24], which is produced in the esophagus in EoE [25]. CCL26 (eotaxin-3) has been regarded as a signature chemokine of EoE, previously found in both the esophagus and the circulation [26]; however, in our patient material, we found neither CCL26 nor CCL11 (eotaxin-1) to be increased in the blood of EoE subjects or any of the other patient groups. Actually, an inverse correlation was documented regarding the percentage of circulating eosinophils and CCL11 levels in the blood for EoE and UC patients, indicative of consumption of this chemokine. Moreover, we observed lower surface expression of CCR3, the receptor for both CCL11 and CCL26 in these same patient groups, which we interpret to reflect internalization of the receptor following activation by ligand(s) [27]. Our data also suggest that CCR3-CCL11 appear to be of importance in the eosinophilic disorders affecting the gastrointestinal tract, e.g., the esophagus and colon, but not the airways, since CCR3 expression and CCL11 levels were similar in allergics and in HC. Lastly, we uncovered a positive correlation between raised percentage of blood eosinophils and upregulated CD9 expression in EoE, which may be linked to the capacity of CD9 activation to prolong eosinophilic survival [28].

A large number of surface molecules on blood eosinophils were upregulated in EoE. CD23, the low-affinity IgE receptor, whose two isoforms a and b are expressed by eosinophils [29], was expressed in increased amounts.

This is interesting in view of recent findings indicating that local production of IgE may occur in the esophagus of patients with EoE [30]. CD23 positively regulates IgE production [31] but may also facilitate antigen presentation of allergen complexed to IgE [32], which may contribute to the perpetuation of chronic inflammation to minute amounts of allergen or other exogenous antigen implicated in the pathogenesis of EoE. ICAM-1 was also upregulated on EoE eosinophils. The ligands for ICAM-1 are the β_2 -integrins Cd11aCD18 (LFA-1) and Cd11bCD18 (Mac-1) found on many types of leukocytes. Importantly, we discovered an unexpected and strong correlation between ICAM-1 and CD23 on EoE eosinophils. ICAM-1 expression by eosinophils has been shown to promote their interaction with T cells [33]. Close cellular encounters are required for immunomodulation, and eosinophils are able to skew T cells towards Th1 and Th2 types of responses [34]. Moreover, eosinophils are endowed with antigen-presenting ability [34]. This might indicate an immunoregulatory role for eosinophils in the chronic inflammatory condition of EoE, whose cause is presently unknown, although a triggering allergen or other exogenous agent may be at the root of it.

Blood eosinophils also bore markers of activation in UC. Eosinophils derived from the intestinal mucosa of patients with UC live longer and release eosinophil peroxidase more readily than do eosinophils from Crohn's disease patients [35]. We found some resemblance between blood eosinophils from UC and EoE patients, e.g., a shared diminished expression of CCR3 and CD44, the receptor for hyaluronic acid. In accordance, Lampinen et al. [35] have described increased surface expression of CD44 by eosinophils in the quiescent phase of UC. Maybe downregulation of CCR3 and CD44 is a pattern characteristic of eosinophils homing to inflamed gastrointestinal mucosa. Decreased binding to hyaluronic acid in the tissue might endow eosinophils with greater migratory potential. A tendency towards decreased expression of the β_2 -integrin Mac-1 (CD11bCD18) has previously been reported in UC patients compared to HC [36]; a similar but nonsignificant tendency was noted here. The infiltration of eosinophils into the intestinal wall in UC has been considered to be a negative prognostic factor [37] as well as a favorable one [16]. The eosinophil does have destructive potential since it can release potent granule proteins that can catalyze the creation of toxic metabolites [18]. On the other hand, we have shown that eosinophils exposed to damaged intestinal cells *in vitro* produce fibroblast growth factor 2 [13], suggesting that they may be involved in the healing process.

None of our AA patients had severe allergic manifestations at the time of sampling, which may explain why we could not document an allergy-specific phenotype. Our results are in agreement with those of Bochner [38], who emphasizes the difficulty of defining unique eosinophilic phenotypes in allergic diseases. This finding was quite surprising, considering that the AA patients exhibited the highest plasma levels of all but one (CCL5) of the eosinophil-related cytokines in our patient material, namely IL-2, IL-3, IL-5 and GM-CSF. All these cytokines have been shown to enable diapedesis of eosinophils across endothelial layers and prime eosinophils to become more responsive to other stimuli [38]. However, we were unable to unmask any correlations between any of these cytokines and any of the examined eosinophil activation markers. Inhibition of IL-5 abrogates the release of eosinophils into the blood and reduces their extravasation into the bronchial tree in patients with AA [39]. In fact, anti-IL-5 treatment has been shown to improve asthma control in selected patients who are resistant to corticosteroids [14, 40]. Although the use of systemic corticosteroid was not allowed in our study, a few patients used topical steroids (inhaled or nasal); the limited number of patients did not permit us to analyze the impact of topical steroids on eosinophil phenotypes. Hence, we cannot exclude that topical steroid treatment caused the relative inertness of blood eosinophils in patients with AA.

A limitation of our study was that we did not examine eosinophil phenotypes in the inflamed tissues, but only the phenotypes of eosinophils 'en route' to the tissues. However, our hypothesis was that eosinophil phenotypes would vary, depending on the underlying disease, already

in the circulation before having reached the tissues and become modulated by tissue-specific factors. We were able to confirm that blood eosinophil phenotypes differed according to the associated disease, which suggests that eosinophils receive disease-specific signals during their differentiation in the bone marrow and/or in the blood, presumably to meet specific demands of the particular condition. Furthermore, our findings might also be used as an aid in the diagnosis of inflammatory diseases in the future, e.g., to indicate which patients need more invasive diagnostic procedures such as biopsies.

In summary, eosinophils displayed diverse immunophenotypic profiles in patients with EoE, UC and AA, respectively. This suggests that eosinophils receive different signals of activation in these diseases. Hopefully, these findings can be exploited to facilitate the diagnosis of eosinophil-related diseases and increase the knowledge regarding their pathophysiology.

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Disclosure Statement

The authors declare that they have no competing commercial or financial interests.

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