

BRIEF COMMUNICATION

CD8⁺ T cells producing IL-3 and IL-5 in non-IgE-mediated eosinophilic diseases

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Abstract

The cytokines IL-5, IL-3, and GM-CSF are crucial for eosinophil development, survival, and function. To better understand their role in non-IgE-mediated eosinophilic diseases, we investigated plasma levels of these cytokines as well as cytokine expression in peripheral blood T cells. While we did not find any evidence for an involvement of T-cell-derived GM-CSF, some of these patients did show an increased proportion of IL-5- or IL-3-producing CD4⁺ T cells. However, in a significant proportion of patients, IL-5-producing CD8⁺ T cells, so-called Tc2 cells, which in healthy donors can only be detected at very low levels, were prominent. Furthermore, increased IL-3 production by CD8⁺ T cells was also observed, strongly supporting the notion that CD8⁺ T cells, not just CD4⁺ T cells, must also be considered as a potential source of the cytokines promoting eosinophilia.

Eosinophils are multifunctional effector cells that normally constitute less than ~5% of peripheral blood leukocytes with absolute counts below 500 cells/ μ l in healthy individuals. Eosinophilia is defined as an absolute eosinophil count >500 and hypereosinophilia as >1500 eosinophils/ μ l blood. Persistent peripheral (hyper)eosinophilia may result in eosinophil infiltration with tissue damage, thus requiring monitoring or treatment (1).

The cytokines IL-3, IL-5, and GM-CSF, also termed eosinophil hematopoietins, are the most important activators and growth factors for eosinophils. Activated CD4⁺ Th2 cells have been considered to be the main source for these cytokines. However, within the CD8⁺ T-cell compartment, there is also a small Tc2 population that can produce IL-5 (2).

Peripheral eosinophilia might be owing to several causes, the most common of which include helminthic infections and allergic diseases, such as atopic dermatitis (3), bronchial asthma (4, 5), rhinosinusitis (6), or eosinophilic esophagitis (7). However, a number of other conditions can also be associated with persistent eosinophilia, such as paraneoplastic eosinophilia, and various skin diseases (1). (Hyper)eosinophilia can also be caused by increased IL-5 production by aberrant T-cell clones (8), and patients with hypereosinophilic syndromes can exhibit increased serum levels of IL-3 and/or IL-5, suggesting that eosinophilia in these cases might be reactive (9).

To better define potential causes of unexplained eosinophilia in non-IgE-mediated diseases and to elucidate the

source of possible increases in cytokine levels, we performed intracellular cytokine staining (ICC) of patient-derived T cells together with determinations of plasma cytokine levels.

Materials and methods

Patients

Blood of 25 patients with persistent blood eosinophilia not related to IgE-mediated diseases and negative for the FIP1-like 1/platelet-derived growth factor receptor- α fusion mutation (10) as well as of 17 healthy controls was prospectively investigated between February 2010 and June 2013. Patient characteristics are summarized in the Online Supplement.

Intracellular cytokine staining and ELISA

Intracellular cytokine expression in stimulated peripheral blood mononuclear cells (PBMCs) and plasma cytokine levels were determined as outlined in the Online Supplement.

Statistics

Groups were compared using the two-tailed Mann–Whitney *U*-test. *P* values <0.05 were considered to be statistically significant.

Results and discussion

To determine whether eosinophilia in our patients was reactive, we employed two complimentary approaches. Determining cytokine levels in plasma is informative for identifying cytokines potentially responsible, but does not allow conclusions about the source of these cytokines. Furthermore, because of a time lag between production of the respective cytokine and an eosinophilic response, cytokine levels might already be diminishing at the time of sampling. Thus, intracellular cytokine staining of T cells provides an approach to recognize T cells as the potential cellular source for cytokines, or to exclude their contribution, and, consequently, to

provide information about the pathomechanism. Increased plasma cytokine levels together with very low levels of cytokine-producing T cells may suggest that the respective cytokine might be derived from a different source. Strong increases in numbers of cytokine-producing T cells, on the other hand, can pinpoint the cause of the eosinophilia even when plasma cytokine levels are low.

T-cell-mediated reactive eosinophilia and hypereosinophilia are typically thought to be a result of Th2 activation and eosinophil hematopoietin production by CD4⁺ T cells or aberrant T-cell clones (1, 8, 11). To investigate the potential involvement of T-cell-derived cytokines in (hyper)eosinophilia in our patients, we performed ICC for IL-5, IL-3, and

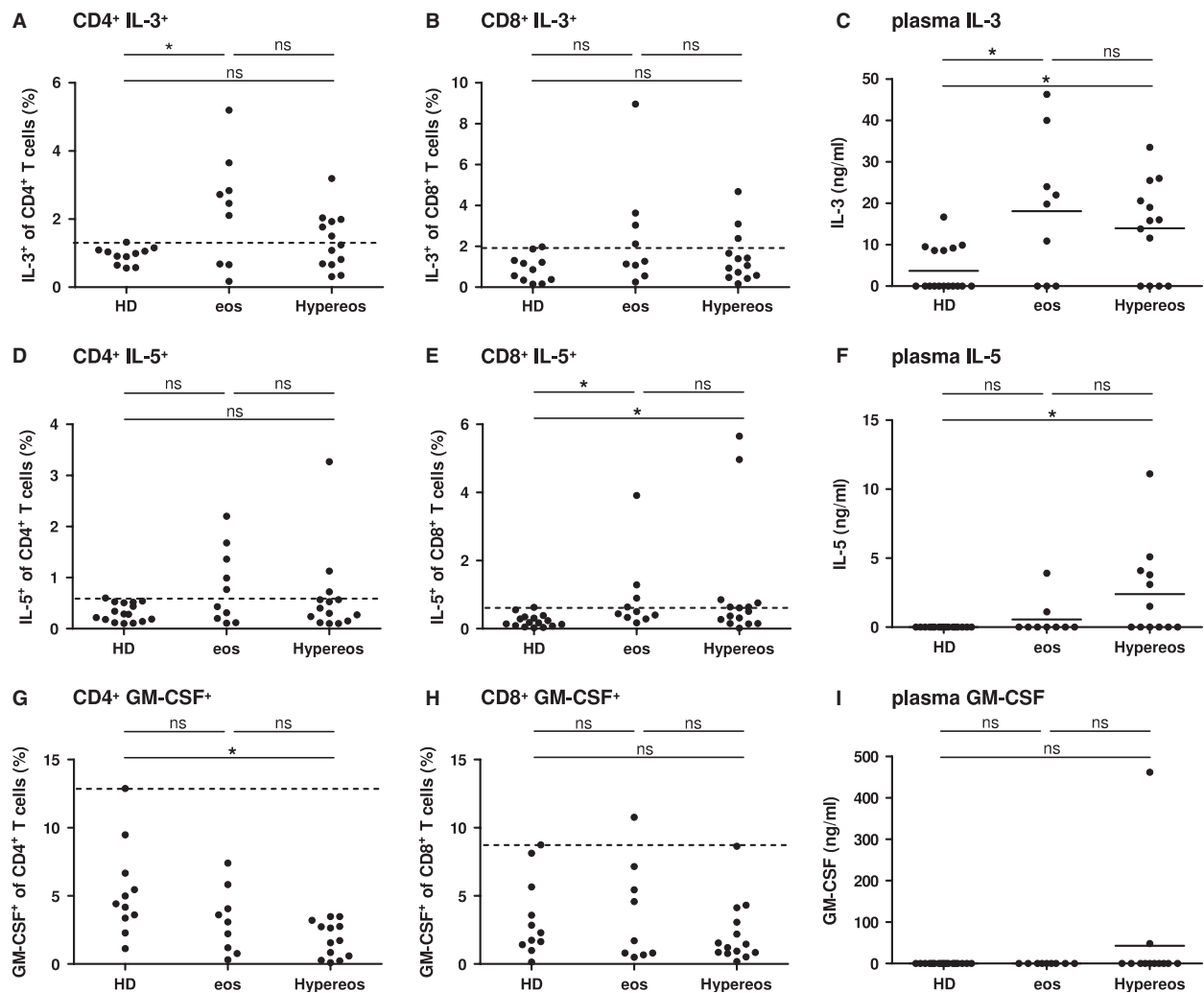


Figure 1 Cytokine expression in T cells and plasma cytokines. Cytokine expression was determined by flow cytometry of peripheral blood CD4⁺ and CD8⁺ T cells following stimulation with PMA/ionomycin. Healthy donors had <500 eosinophils/ μ l of blood. Patients were designated as eosinophilic (500–1500 eosinophils/ μ l) or hypereosinophilic (>1500 eosinophils/ μ l). Each dot represents results from a single donor and is shown as percentage of (A) IL-3⁺

cells of CD4⁺ T cells, (B) IL-3⁺ cells of CD8⁺ T cells, (D) IL-5⁺ cells of CD4⁺ T cells, (E) IL-5⁺ cells of CD8⁺ T cells, (G) GM-CSF⁺ cells of CD4⁺ T cells, and (H) GM-CSF⁺ cells of CD8⁺ T cells. The dashed line corresponds to the maximum percentage detected in healthy donors. Plasma levels of (C) IL-3, (F) IL-5, and (I) GM-CSF were determined by ELISA. Lines depict the mean values. ns = not significant, * = $P < 0.05$.

GM-CSF in CD4⁺ and CD8⁺ T cells in isolated PBMCs of patients and healthy donors following stimulation with PMA/ionomycin (Table S1 and Fig. 1). Plasma cytokine measurements as well as viability assays were performed in parallel to ICC (Table S1 and Fig. 1). While IL-5 was undetectable and IL-3 was low or undetectable in the plasma of healthy donors, most patients had detectable and in some cases increased levels of plasma IL-5 and/or IL-3 (Fig. 1C, F), and their eosinophils often showed delayed apoptosis *in vitro* owing to *in vivo* cytokine exposure (Table S1), as previously observed (9). GM-CSF, however, was only detectable in two patients (Table S1, Fig. 1I). No correlations were found between absolute eosinophil numbers and levels of plasma IL-3 or IL-5 in patients (IL-3: $R^2 = 0.0108$, $P = 0.6539$; IL-5: $R^2 = 0.043691$, $P = 0.3505$).

Intracellular cytokine staining of patient-derived T cells revealed some surprising results. The vast majority of eosinophilic patients had an increased percentage of IL-3-producing CD4⁺ T cells (Fig. 1A), and 8 of 25 patients (32%) had increased IL-5⁺CD4⁺ T cells (Fig. 1D) as expected, supporting the idea of increased T-helper-mediated cytokine production as a potential cause for reactive eosinophilia. However, a third of the patients also showed drastically increased IL-3 or IL-5 (Fig. 1B,E) production in the CD8⁺ T-cell compartment (IL-3: 32%, IL-5: 36%), suggesting a role of CD8⁺ T cells and the small Tc2 subset of CD8⁺ T cells in (hyper) eosinophilia. Interestingly, IL-5-producing CD8⁺ T cells have been reported in asthma and atopic dermatitis patients and may correlate with disease severity in asthma (12, 13). While the increased IL-3-/IL-5-producing CD4⁺ T cells that we found in eosinophilic patients were expected, the finding that many hyper eosinophilic patients had approximately baseline levels of IL-3-producing CD4⁺ T cells was not. However, these patients did have detectable and, in some cases, high plasma levels of IL-3, suggesting a non-T-cell source, such as IgE-activated mast cells (14, 15), basophils (16), or T cells that have emigrated into the inflamed tissue (s). Similarly, in patients in whom plasma IL-5 was detected in the absence of increased IL-5-producing T cells, other cellular sources must be considered, such as the recently described type 2 innate lymphoid cells (ILC2) (17, 18). It should be noted that eosinophils themselves are also known to express and release cytokines under inflammatory conditions (19). Owing to the heterogeneity of the responses, there were no significant correlations between absolute eosinophil numbers and the percentage of a specific subset of IL-5- or IL-3-producing T cells (CD4⁺IL-5⁺: $R^2 = 0.0694$, $P = 0.2245$, CD4⁺IL-3⁺: $R^2 = 0.0308$, $P = 0.4347$, CD8⁺IL-5⁺: $R^2 = 0.1008$, $P = 0.1306$, CD8⁺IL-3⁺: $R^2 = 0.0047$, $P = 0.7618$).

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In contrast to what has been described for bronchoalveolar-lavage-derived T cells in asthmatics (20), our data do not support a role of T-cell-derived GM-CSF in non-IgE-mediated (hyper)eosinophilia. GM-CSF-producing CD4⁺ T cells were diminished (Fig. 1G), and GM-CSF⁺CD8⁺ T cells remained largely unchanged (Fig. 1H) rather than increasing in patients compared with controls. In agreement with this finding, only two patients exhibited detectable plasma levels of GM-CSF, and these patients had exceedingly low fractions of GM-CSF-producing T cells. In these patients, we could demonstrate that GM-CSF was derived from large-cell carcinomas of the lung. One of these cases has been reported elsewhere (21).

Conclusion

While increased IL-5 and/or IL-3 plasma levels and their production by peripheral blood T cells is observed in many (hyper)eosinophilic patients, T-cell-derived GM-CSF does not appear to play a role in non-IgE-mediated eosinophilic disorders. In addition to CD4⁺ T helper cells, CD8⁺ T cells must also be considered as a potential eosinophil hematopoietin source in these patients.

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Author contributions

C.S. performed experiments and analyzed data. C.S. and H.U.S. designed the study and wrote the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Supporting Information

Additional Supporting Information, including supplementary methods, may be found in the online version of this article:

Table S1. Blood eosinophil counts, eosinophil haematopoietin-expressing T subpopulations, eosinophil apoptosis, and final clinical diagnosis of the patients included in this study.

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Online Supplement:

CD8⁺ T cells producing IL-3 and IL-5 in non-IgE-mediated eosinophilic diseases

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Materials and Methods

Patients

Patients included in the study did not show any evidence of atopic diseases or helminth infections. Li-heparin- and EDTA-anti-coagulated blood was drawn from subjects after obtaining their written informed consent to be enrolled in the study. The study was approved by the Ethics Committee of the Canton of Bern. In cases where an eosinophilic leukaemia was suspected, polymerase chain reaction (PCR) was performed for the FIP1-like 1/platelet-derived growth factor receptor- α (FIP1L1-PDGFR α) fusion mutation as described (11) and was negative for all hypereosinophilic patients included in this study. The kinetics of apoptosis in isolated peripheral blood eosinophils were determined as described previously (10). Patient characteristics are summarised in Table S1.

Intracellular cytokine staining

Peripheral blood mononuclear cells (PBMC) from peripheral Li-heparin-anti-coagulated blood were isolated by gradient centrifugation (Biocoll; Biochrome, Berlin, Germany) and cultured at 2×10^6 cells/ml in RPMI 1640 with 10% FCS for 6 h in the absence or presence of 0.5 μ g/ml phorbol-12-myristate-13-acetate (PMA) and 1 μ M ionomycin (both from Sigma-Aldrich, Buchs, Switzerland). Brefeldin A (Sigma-Aldrich) was added for the last 5 h of incubation. Cells were fixed and permeabilised (Perm2, BD Biosciences, San Jose, CA, USA), stained with antibodies against CD4 (FITC, BD Biosciences), CD8 (APC, BD Biosciences), and IL-3, IL-5, GM-CSF or isotype control (PE, all BioLegend, San Diego, CA, USA). For each, $\geq 50\,000$ events were acquired on a FACS Calibur (BD Biosciences) and data analysed using FlowJo software. CD4⁺ and CD8^{high} cells within the lymphocyte gate were analysed for cytokine expression. Numbers in Table S1 refer to cytokine positive cells within the respective CD4⁺ or CD8^{high} gate.

ELISA

Plasma was obtained from EDTA-anti-coagulated blood by centrifugation and cytokine levels determined by ELISA according to the manufacturer's instructions (IL-5, IL-3, GM-CSF; all R&D Systems, Minneapolis, MN, USA).

Table S1: Blood eosinophil counts, eosinophil haematopoietin-expressing T subpopulations, eosinophil apoptosis, and final clinical diagnosis of the patients included in this study

Patient number	Blood eos/ mm ³	CD4 ⁺ IL-5 ⁺ (%)	CD8 ⁺ IL-5 ⁺ (%)	CD4 ⁺ IL-3 ⁺ (%)	CD8 ⁺ IL-3 ⁺ (%)	CD4 ⁺ GM-CSF ⁺ (%)	CD8 ⁺ GM-CSF ⁺ (%)	Plasma IL-5 (ng/ml)	Plasma IL-3 (ng/ml)	Plasma GM-CSF (ng/ml)	Impaired eosinophil apoptosis	Final clinical diagnosis
<i>Healthy donors (n = 17)</i>	< 500	0.10-0.60	0.04-0.63	0.56-1.32	0.15-1.98	1.13-12.88	0.14-8.74	<i>n.d.</i>	<i>n.d.-16.7</i>	<i>n.d.</i>	<i>no</i>	
1	1488	0.20	3.91	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	yes	Eosinophilic dermatitis
2	2047	0.40	0.02	n.a.	n.a.	n.a.	n.a.	11.1	33.5	n.d.	yes	HES
3	1522	0.30	0.27	1.50	0.73	3.21	3.07	n.a.	n.a.	n.a.	n.a.	Eosinophilic asthma
4	1296	0.99	0.17	2.72	3.63	5.83	7.16	1.1	19.8	n.d.	n.a.	Eosinophilic dermatitis
5	2016	0.10	0.76	1.93	n.a.	0.27	4.13	n.d.	13.8	n.d.	n.a.	Eosinophilic dermatitis
6	1650	0.53	0.15	0.82	0.48	3.48	1.55	3.1	25.5	n.d.	yes	Eosinophilic dermatitis
7	2140	0.72	5.65	1.99	3.10	2.64	4.32	1.5	n.d.	n.d.	yes	CLL
8	2160	n.a.	0.85	n.a.	0.43	n.a.	0.77	5.1	20.6	n.d.	yes	Eosinophilic dermatitis
9	1144	0.11	0.33	0.68	3.04	0.77	4.58	n.d.	46.5	n.d.	n.a.	Eosinophilic dermatitis
10	9976	0.57	0.37	1.08	2.39	1.56	0.85	n.d.	n.d.	462	no	NSCLC
11	3213	1.13	0.14	1.24	1.66	2.73	1.21	n.d.	26.0	n.d.	yes	HES
12	1430	0.12	0.29	0.66	0.55	1.19	0.67	3.9	40.0	n.d.	yes	Eosinophilic dermatitis
13	11296	0.15	0.51	0.66	1.40	0.59	0.95	4.1	16.0	48	yes	NSCLC
14	690	2.20	0.40	5.20	1.14	3.08	0.80	n.d.	24.0	n.d.	yes	Eosinophilic dermatitis
15	11154	3.27	4.97	2.04	0.58	2.76	2.19	3.8	19.0	n.d.	yes	Langerhans cell histiocytosis
16	1840	0.12	0.15	1.77	1.43	1.72	1.45	n.d.	n.d.	n.d.	no	Eosinophilic dermatitis

17	606	0.43	0.90	2.84	1.08	2.22	0.80	n.d.	22.0	n.d.	yes	Eosinophilic asthma
18	1924	0.27	0.64	0.35	0.94	0.84	0.84	n.a.	n.a.	n.a.	yes	Eosinophilic dermatitis
19	1599	0.57	0.61	0.69	1.06	0.23	0.52	n.d.	n.d.	n.d.	no	HES
20	1232	1.36	0.44	3.65	1.27	4.06	1.70	n.d.	n.d.	n.d.	n.a.	DRESS
21	1216	0.77	1.29	2.11	8.96	3.61	10.77	n.d.	n.d.	n.d.	n.a.	DRESS
22	1560	0.10	0.64	0.31	0.17	0.09	0.18	n.a.	11.6	n.a.	no	Eosinophilic dermatitis
23	1488	0.31	0.64	2.46	2.12	7.41	5.45	n.d.	n.d.	n.d.	n.a.	Eosinophilic dermatitis
24	1079	1.68	0.50	0.17	0.25	0.30	0.49	n.d.	10.9	n.d.	yes	Churg-Strauss Syndrome
25	3350	0.24	0.32	3.19	4.68	3.48	8.64	n.d.	15.8	n.d.	yes	HES

Numbers in bold indicate that levels are increased compared with normal donors.

n.a., data not available; n.d., not detectable; CLL, chronic lymphatic leukemia; DRESS, drug rash with eosinophilia and systemic symptoms; HES; hypereosinophilic syndrome; NSCLC, non-small cell lung cancer.

Detection limits: IL-5: 1.08 pg/ml; IL-3: 7.4 pg/ml; GM-CSF: 3 pg/ml

Impaired eosinophil apoptosis is defined by >50% viable eosinophils following a 24h culture period and/or resistance to Fas/ceramide-induced apoptosis (10).