

A Comparison of Inflammatory Mediators Released by Basophils of Asthmatic and Control Subjects in Response to High-Affinity IgE Receptor Aggregation

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Key Words

Basophils • Cytokines • Chemokines • Leptin

Abstract

Background: In human blood basophils, cross-linking the high-affinity IgE receptor FcεRI with multivalent antigen activates a signaling pathway leading to secretion of inflammatory mediators and cytokine production. Basophils are known to play an important role in the pathogenesis of asthma but there has been no comprehensive examination of the effectors these cells produce. Here a study of the transcription and release of a selection of chemokines and cytokines from basophils was undertaken. **Methods:** A Cartesian antibody array provided an effective method of assaying for multiple cytokines and chemokines simultaneously. Results were verified by RT-PCR and ELISA assays. This allowed the comparison of freshly prepared peripheral blood basophil responses to cross-linking of the high-affinity IgE receptor, with and without preincubation with IL-3. **Results:** Evidence that human blood basophils produce the chemokines MIP-5, eotaxin and GM-CSF was provided by antibody array and RT-PCR analyses. Preincubation with IL-3 enhanced the expression and release of IL-13, IL-8 and mRNA transcripts encoding MIP-5 and GATA2 in basophils from both asthmatic and control subjects. Leptin mRNA transcription, storage and release in basophils are described for the first time. **Conclusions:**

Surveying cytokine and chemokines stored and released by peripheral blood basophils shows that asthmatic and control subjects share similar profiles even when their degranulation responses are distinct. Evidence is provided for the production of leptin, GM-CSF, eotaxin and MIP-5 by peripheral blood basophils. IL-3 preincubation enhances the production and release of IL-8 upon IgE receptor cross-linking.

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Introduction

In most human subjects fewer than 1% of peripheral blood leukocytes are basophils. Despite this low incidence, basophils have been identified as important effector cells in IgE-mediated inflammatory and allergic reactions. Basophil accumulation in the lungs has been shown to be a feature of fatal asthma in humans, while in mice the recruitment of basophils to the lung has been identified as an important step in establishing a primary type 2 helper T cell response [1, 2]. The high-affinity IgE receptor, FcεRI, is the principal immunoreceptor on the surface of human basophils. FcεRI bind circulating IgE with $K_d = 10^{-9}$ to 10^{-10} M. Basophils are activated when multivalent allergen cross-links these IgE-bound receptors. Cross-linking initiates a complex tyrosine kinase-dependent signaling pathway that results in calcium in-

flux, degranulation and synthesis of leukotrienes and platelet-activating factor [3, 4]. Basophil granules contain potent inflammatory mediators such as histamine, neutral proteases, proteoglycans and lysosomal enzymes [5, 6]. Although basophil-derived cytokines are proposed to play a key role in chronic inflammation, a profile of the mediators released by these activated cells has not been documented. With the development of multiplex assays, it is now possible to assay for multiple cytokines simultaneously with modest amounts of sample. Here we use an antibody array, combined with RT-PCR and ELISAs, to generate a profile of basal and stimulated cytokine production in human basophils isolated freshly from human blood.

Knowledge of basophil behavior and action *in vivo* is limited due to the paucity of cells. What information there is centers on the murine model but does lend some clues as to the possible influences of growth factors on these cells as they are activated and travel from the circulation to sites of inflammation [2, 7, 8]. These reports provide mounting evidence that IL-3 influences the basophil cytokine response. Here the effects of IL-3 preincubation on multiple basophil responses are measured simultaneously. Although IL-3 influences basophil responses, IL-3 concentrations in peripheral blood have eluded quantification. This study attempts to quantify IL-3 concentrations in serum and bronchial alveolar lavage fluid (BALF) to determine if there is any correlation between subjects' status as asthmatic or control or histamine release from basophils.

Materials and Methods

Percoll was from Pharmacia (Uppsala, Sweden); affinity-purified goat anti-human IgE (anti-IgE), Cartesian Array™ Human Cytokine Set 1 and IL-4 ultrasensitive ELISA were from Biosource (Camarillo, Calif., USA). MidiMacs separation columns and Midimacs magnetic columns were from Miltenyi Biotec (Auburn, Calif., USA). mRNA-Bee Tel-Test (Friendswood, Tex., USA) and RNA RNase ZAP were purchased from Ambion (Austin, Tex., USA). One-Step RT-PCR kit was from Qiagen (Valencia, Calif., USA), kb DNA Ladder from Stratagene (La Jolla, Calif., USA). Agarose, chloroform, bovine serum albumin and ethidium bromide were from Sigma (St. Louis, Mo., USA). Human recombinant IL-3 was from BD Pharmingen (San Diego, Calif., USA). 10× Hanks' balanced salt solution was from Invitrogen (Carlsbad, Calif., USA).

Primers were synthesized by the Center for Genetics in Medicine of the University of New Mexico's Department of Biochemistry and Molecular Biology. IL-3 ELISA was from R&D Systems (Minneapolis, Minn., USA). Leptin ELISAs were from Cayman Chemical Company (Ann Arbor, Mich., USA) and R&D Systems.

Table 1. Characteristics of the study population

Subject	Status	Maximum histamine release % of total	Basophils purified/ml of blood	Skin test	Figure
112	Con	7.94	9,615	neg	2a
174	Con	74.90	8,660	neg	2a
209	Con	99.35	7,327	neg	2a
220	Con	24.45	24,409	neg	2a
117	Con	84.72	16,667	neg	2b, 7b
209	Con	84.72	8,462	neg	2b
87	As	8.91	5,176	pos	2b
12	As	78.21	28,346	pos	2b, 2c
181	Con	71.89	15,403	neg	2c
130	Con	63.71	4,167	neg	2c
112	Con	3.95	9,918	neg	2c
212	As	63.71	12,479	pos	2c
87	As	0.03	6,066	pos	2c
241	As	4.11	28,306	pos	2c
112	Con	27.20	7,565	neg	3, 4
232	Con	67.44	29,580	neg	3, 4
241	As	5.48	39,013	pos	3, 4
234	As	13.93	7,647	pos	3, 4
231	Con	77.03	8,240	neg	5, 6, 7b
239	As	14.57	10,015	pos	5, 6
259	Con	35.77	24,596	neg	5, 6
112	Con	-1.27	11,122	neg	7a
234	As	9.37	10,244	pos	7a
232	Con	77.63	20,377	neg	7a
241	As	0.21	49,699	pos	7a
239	As	59.46	4,660	pos	7b
242	As	79.97	14,173	pos	7b
133	Con	44.70	18,740	neg	7b

16 controls (Con) and 12 asthmatics (As) were included in this study. Histamine release and basophil numbers/ml of blood are the numbers obtained at the first visit for each subject. Skin tests, reported as negative (neg) and positive (pos), showed no seasonal variation.

IL-13 and IL-8 ELISA kits were from RayBio (Norcross, Ga., USA). Human histamine ELISA kit was from Beckman Coulter (Fullerton, Calif., USA).

Human Subjects

This study was approved by the Human Research Review Committee (HRRC) at the University of New Mexico. A total of 28 subjects participated in this part of the study, 12 asthmatics and 16 controls. Characteristics of the subjects are summarized in table 1. All gave informed consent, were identified by study numbers and were nonsmokers between 18 and 60 years old. Allergy skin testing, methacholine challenges, BALF collection and phlebotomy were performed in the UNM General Clinical Research Center (GCRC). Skin allergy tests were performed when

joining the study and every 2 years thereafter. Subjects were classified as either atopic asthmatic or nonatopic controls based on the results of allergy skin tests using aeroallergens common in the Albuquerque area (Fremont cottonwood, juniper-mountain cedar, Bermuda grass, bluegrass, fescue, Russian thistle, kochia, western ragweed, cat dander, and house dust mite). A subject was considered atopic if he or she had two or more positive skin prick tests (3 mm or greater wheal diameter and no wheal in response to normal saline). Methacholine challenges were performed under physician supervision. Asthma was defined as either a 15% or more increase of FEV₁ in response to 200 µg albuterol or increased sensitivity to inhaled methacholine. A positive methacholine test was defined as a decrease of the FEV₁ of at least 20% after inhalation of 16 mg/ml or less methacholine. Control subjects were all nonsmokers, nonasthmatic and also nonatopic based upon negative skin tests. Asthmatic subjects were all atopic and had mild asthma.

Basophil Purification

Basophils were purified from an average of 115 ml peripheral venous blood. Briefly, basophils were enriched by Percoll gradient centrifugation followed by negative selection [9]. A portion of these cells was used for histamine release; reactions were carried out as described previously [10]. All basophil preparations were assayed for histamine release at both 0.1 and 1 µg/ml anti-IgE concentrations. The percent histamine reported is the highest percentage recorded of these incubation conditions. The average purity of these preparations was 99%. Contaminating cells were primarily lymphocytes and neutrophils. Purification took an average of 5 h.

Basophil Culture

Basophils were suspended at a density of 200,000 to 1.5 million cells/ml in sterile Eppendorf tubes containing Hanks' medium supplemented with 0.1% BSA. This incubation medium was used to remove the possible confounding effects of additional factors found in serum-supplemented media. Cells in a final volume of 100 µl were incubated at 37°C in a CO₂ incubator. Cell activation was by cross-linking of IgE-occupied FcεRI receptors by supplementing with 1 µg/ml anti-human IgE. After incubation cells were centrifuged at 400 g for 3 min and the pellets stored at -70°C until RNA isolation. Supernatants were removed and stored at -70°C for cytokine and histamine analysis. In experiments where basophils were incubated with IL-3, purified basophils were incubated in the same manner as described above but 10 ng/ml of IL-3 was added to the incubation media. Prior to the completion of incubations, aliquots of cells were activated for either 2 or 4 h with 1 µg/ml anti-IgE. Cells were harvested by centrifugation.

Enzyme-Linked Immunosorbent Assays

ELISAs were performed according to the manufacturer's instructions. Histamine released was quantified after incubation with 0.1 and 1 µg/ml anti-IgE. Sensitivity for each assay was as follows: IL-3: 7.4 pg/ml, IL-4: 0.27 pg/ml, leptin: 7.8 pg/ml, IL-13: 25 pg/ml, IL-8: 3 pg/ml and histamine 0.5 nM.

PCR Analysis

Semiquantitative RT-PCR was performed as follows: total cellular RNA was isolated using RNA-Bee according to the manufacturer's instructions. 0.1 µg/ml of total RNA was then amplified

by One-Step RT-PCR using the appropriate primers under the following conditions: denaturation, 94°C for 30 s, annealing, 60°C for 30 s and extension for 1 min at 72°C in an Eppendorf Mastercycler Gradient instrument. The number of cycles employed was dependent on the target. GAPDH-specific primers were as follows: sense, 5'-TGGTATCGTGGGAAGGACTCATGAC-3'; antisense, 5'-ATGCCAGTGAGCTTCCCGTTCAGC-3'. IL-4-specific primers were as follows: sense, 5'-TGCCTCACATTGTCACTGCAA-3'; antisense, 5'-AGCCCTGCAGAAGTTTCTCT-3'. IL-13-specific primers were as follows: sense, 5'-GGAGCTGGTCAACATCACGC-3'; antisense, 5'-CGTTGATCAGGGATTCCAGG-3'. IL-6-specific primers were as follows: sense, 5'-CGGGAACGAAAGAGAAGCTCTA-3'; antisense, 5'-GGCGCTTGTGGAGAAGGAG-3'. Leptin-specific primers were as follows: sense, 5'-GTGCGGATTCTGTGGCTTT-3'; antisense, 5'-GGAATGAAGTCCAAACCGGTG-3'. GATA2-specific primers were as follows: sense, 5'-CAAGCCCAAGCGAAGACTGT-3'; antisense, 5'-GTGCCCGTTGGCGTTTC-3'. MIP-5-specific primers were as follows: sense, 5'-CTCCTGCCTCATGCTTGTTG-3'; antisense, 5'-CTGGATTTTCCAGTGGGAAGCTT-3'. IL-17-specific primers were as follows: sense, 5'-TCCTGGGAAGACCTCATTGG-3'; antisense, 5'-AATTTGGGCATCCTGGATTTC-3'. ML-1-specific primers were as follows: sense, 5'-ACCGGTACCCCTCGGAAGT-3'; antisense, 5'-CTGGATGGGAACGGAATTCA-3'. GATA3-specific primers were as follows: sense, 5'-GCGGGCTCTATCACAAAATGA-3'; antisense, 5'-GCTCTCCTGGCTGCAGACAGC-3'. Eotaxin-1-specific primers were as follows: sense, 5'-CATGAAGGTCTCCGCAGCACTTCT-3'; antisense, 5'-CCAGATACTTCATGGAATCCTGC-3'. GM-CSF-specific primers were as follows: sense, 5'-CTGCTGCTGAGATGAATGAAG-3'; antisense, 5'-GCACAGGAAGTTTCCGGGGT-3'. Ten microliters of the final reaction mixture was visualized on a 1% agarose gel containing 0.3 mg/ml ethidium bromide. Gel images were captured with a high-performance Cohu CCD camera (San Diego, Calif., USA). Densitometry image analysis was performed using Image J V1.33 (NIH). Cycle numbers varied depending on the transcript amplified. GAPDH acted as constitutive control.

Measurement of Cytokine and Chemokine Release Using Antibody Arrays

Purified basophils (average cell number 750,000) were lysed by 2 freeze-thaw cycles in 100 µl of H₂O either immediately or after incubation with 1 µg/ml anti-IgE for 24 h in medium. One hundred microliters of lysates or 50 µl of medium were incubated with a Cartesian Array Human Cytokine Set 1 membrane at 4°C overnight. The protocol was carried out according to the manufacturer's instructions. A control incubation with medium alone did not result in reactivity with any of the antibodies (data not shown). A map of antibody positions on the membrane is presented in table 2. Cytokine expression was imaged on a flat bed scanner. The relative levels of each cytokine was determined by averaging net light intensity of each pair of cytokine spots detected on the basis of gray-scale levels and then normalizing with positive controls set at 1.

BALF Collection and Concentration

Bronchoalveolar lavage (BAL) was performed in the UNM General Clinical Research Center (GCRC). Lidocaine was used for topical anesthesia of the upper airways. BAL was performed

Table 2. Human Cytokine Set 1 Cartesian Array map

Eotaxin	GM-CSF	IP-10	TNF- α	RANTES	Leptin	positive
Eotaxin	GM-CSF	IP-10	TNF- α	RANTES	Leptin	positive
IL-1 α	IL-1 β	IL-3	IL-4	IL-6	IL-8	negative
IL-1 α	IL-1 β	IL-3	IL-4	IL-6	IL-8	negative
IL-10	IL-12	IL-17	MIP-1 α	MIP-1 β	MIP-5	positive
IL-10	IL-12	IL-17	MIP-1 α	MIP-1 β	MIP-5	positive

18 antibodies are arranged in duplicate on the membranes.

in the right middle lobe of the right lung by infusing 300 ml of sterile saline solution in 60-ml aliquots. Saline was retrieved by suction. BALF was separated into acellular and cellular components by centrifugation (400 g for 10 min at 4°C). Supernatants were stored at -70°C until concentrated. Four-milliliter serum aliquots were concentrated to 800 μ l using Ultrafree-MC Centrifugal Filter Units, with a molecular weight cutoff of 10 kDa, in a microfuge. BALF was concentrated using Ultra-15 centrifugal filter devices (Amicon, Beverly, Mass., USA), with a molecular weight cutoff of 10 kDa, according to the manufacturer's recommendations. One hundred and twenty milliliters of BALF in 40-ml aliquots were centrifuged at 2,000 g using GS-6R centrifuge (Beckman Coulter, Fullerton, Calif., USA). Several centrifuge cycles were performed. Final sample volumes ranged between 400 and 800 μ l. Supernatants were stored at -70°C until analysis. The IL-3 concentration in BALF was determined in the concentrated sample. The results are given as picograms per milliliter in the unconcentrated BALF.

Results

Characteristics of the Study Population

Table 1 summarizes key characteristics of the subjects and their basophils, with linkage to relevant figures. All asthmatics were positive for at least 2 allergens in the panel. Most responded to nonseasonal allergens (dust mite and cat hair). Numbers of basophils per milliliter of blood were estimated by quantifying the basophils recovered after Percoll centrifugation and back-calculating to basophils per milliliter of blood. There was a trend towards a higher recovery of basophils from asthmatics than controls (17,990 basophils/ml in the 12 asthmatics vs. 14,050 basophils/ml in the 16 controls) but the difference was not statistically significant ($p=0.36$). As this study formed part of a larger 4-year study it was possible to examine this parameter in the much larger sample set that was available. The statistical analysis of numbers of basophils purified per milliliter of blood of 380 subjects is presented in figure 1. A statistically significant difference was found to exist with a p value of 0.0176. Although it is formally possible that the different yields of basophils from

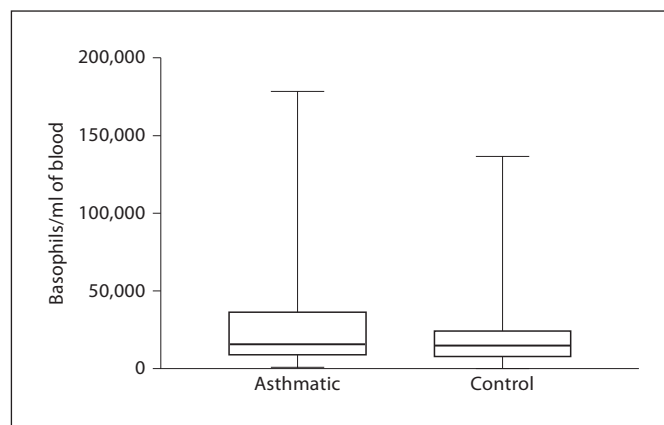


Fig. 1. Comparison of the numbers of basophils recovered after Percoll centrifugation from the blood of asthmatics and controls. Basophils were partially purified by Percoll gradient centrifugation from the blood of 150 asthmatic and 230 control subjects over a 4-year period. Basophils were counted and results used to back-calculate to the numbers of basophils/ml of blood. Data are from the first visit for each subject. The increased number of basophils in Percoll layers from the blood of asthmatics is statistically significant ($p=0.0176$).

Percoll could result from a difference in density between the basophils of asthmatics and controls, basophils were not detectable in other gradient fractions. The increased numbers of basophils in blood from asthmatics complement a previous analysis of the larger data set showing that maximum percentage histamine released from asthmatics is also higher than control [11].

Cytokine Complement of Basophils Isolated from Multiple Subjects Is Similar

To obtain a cytokine profile from unstimulated cells, freshly prepared peripheral blood basophils were lysed and the stored cytokines were surveyed using Human Cytokine Set 1 Cartesian Arrays that have an array sensitivity of less than 4 pg/ml for 18 different cytokines.

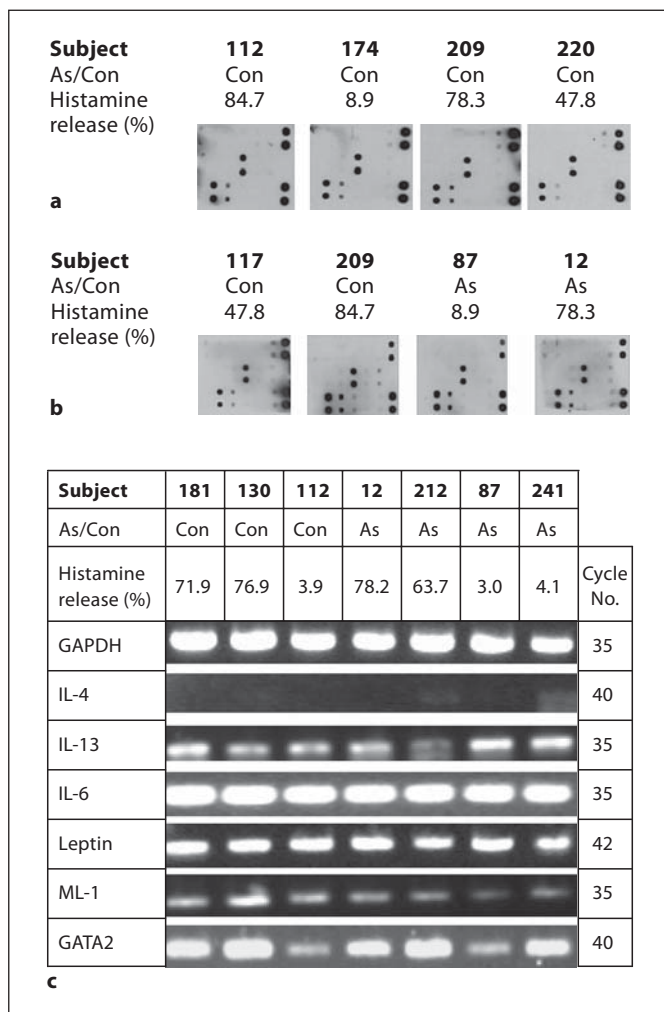
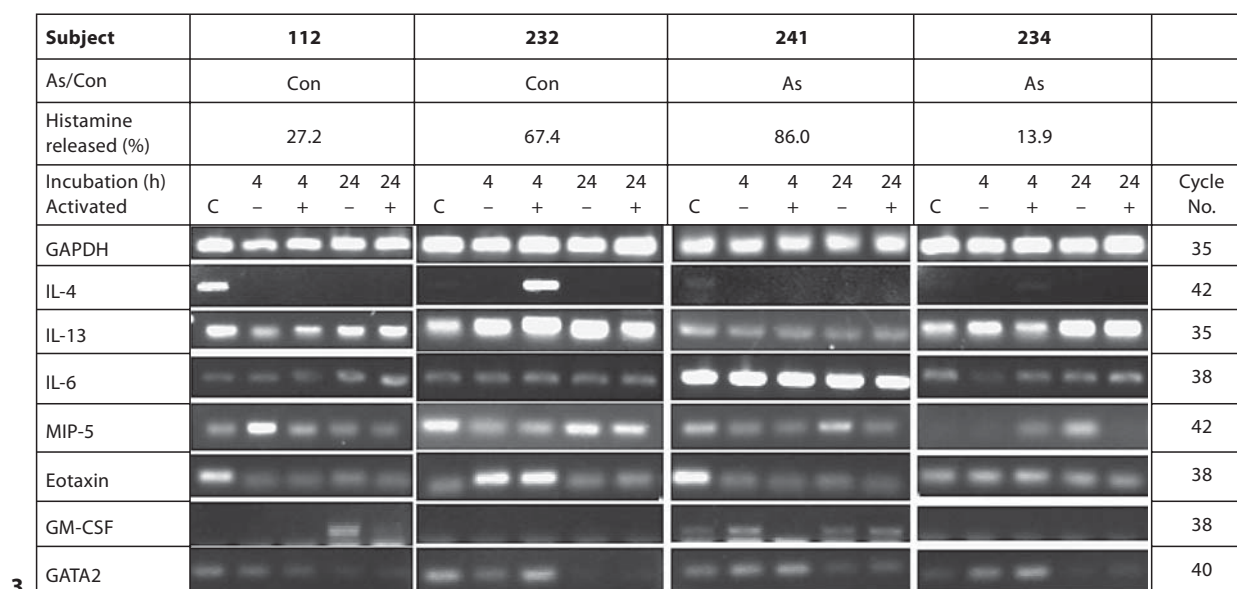


Figure 2a shows that IL-4 was the most consistently expressed cytokine in resting cells. Additionally leptin was detected in most samples. Cytokines resembling IL-12 and IL-17 were also present (but see below for lack of independent verification for these cytokines). The cytokines detected in resting cells were consistent, irrespective of percent histamine release or whether the subjects were asthmatic or controls. The same analysis was repeated using Hanks' BSA incubation media after receptor cross-linking (fig. 2b). Among the positive signals detected in the media were the presynthesized and stored cyto-

Fig. 2. Isolated basophils of control and asthmatic subjects share a similar cytokine transcript and protein profile. **a** Lysates of purified basophils from asthmatics (As) and controls (Con) were incubated with cytokine array membranes. In each case the percent histamine release was determined immediately after purification. **b** Purified basophils were incubated with 1 µg/ml anti-IgE for 24 h. The released proteins in the incubation media were detected by ELISA. **c** Comparison of mRNA transcripts in isolated basophils from different subjects.

Fig. 3. Analysis of mRNA transcripts in basophils with and without FcεRI cross-linking. Histamine release assays were carried out immediately on purified basophil preparations from asthmatics (As) and controls (Con). A portion of the purified cells was frozen immediately at -70°C to serve as control (C). Other basophils were incubated with or without 1.0 µg/ml anti-IgE for 4 or 24 h. mRNA isolated from these cells (0.1 µg/ml) was analyzed by semiquantitative RT-PCR. IL-12, IL-17, GATA3 and IL-8 transcripts were not detected in any of these subjects after 40 cycles of amplification.



kines already identified. Additional chemokines and cytokines synthesized and released in response to receptor cross-linking included IL-8, MIP-5, GM-CSF, eotaxin and MIP-1 α . This analysis revealed no differences between the release profiles of basophils from asthmatic and control subjects.

Cytokine Transcripts in Resting and Activated Basophils

Employing an antibody array can lead to false positives due to the potential for cross-reactivity with similar proteins and possibly interference by heterophilic antibodies. Therefore results were verified and extended by RT-PCR.

Figure 2c shows the amplification of mRNA transcripts for a selection of proteins of unstimulated basophils isolated from asthmatic and control subjects. IL-13 and IL-6 transcripts were consistently detected. Leptin transcripts were also found in all samples. IL-4 transcripts were expressed at variable, low levels despite the ready detection of IL-4 protein by array analysis.

Previous studies have sought to identify transcription factors in basophils [7, 12]. No GATA3 transcript was detected in any of the subjects of this study. In contrast, GATA2 transcripts were detected in each subject tested (fig. 2c) (interestingly at lower levels in two of three basophils characterized as low releasers of histamine).

As the resting cytokine transcripts detected were similar among basophils of different subjects, an activation time course was undertaken to determine if this would reveal any variations. The changes in mRNA expression (fig. 3) and release of proteins are reported (fig. 4). The greatest variation between subjects in expression (fig. 3) and release (fig. 4b) was recorded for IL-4. In contrast IL-13 expression was detected in each of the basophil controls and amplification of transcript did not alter significantly between resting and activated states (fig. 3). When released IL-13 protein was quantified it was only detected in subject 232 and then only in activated samples (fig. 4a).

IL-8 mRNA amplification was not detected in any of the subjects. ELISA analysis of the supernatants could only detect protein after 24 h incubation, and then only in 2 of the 4 subjects tested (fig. 4c). No change in IL-6 mRNA transcript amplification was detected after activation. Transcripts for the chemokines MIP-5, eotaxin and GM-CSF were detected in basophils of some subjects but there was no obvious upregulation upon activation (fig. 3).

To monitor the activation state of basophils during incubations the percent histamine released was determined after both 4 and 24 h of incubation with or without anti-

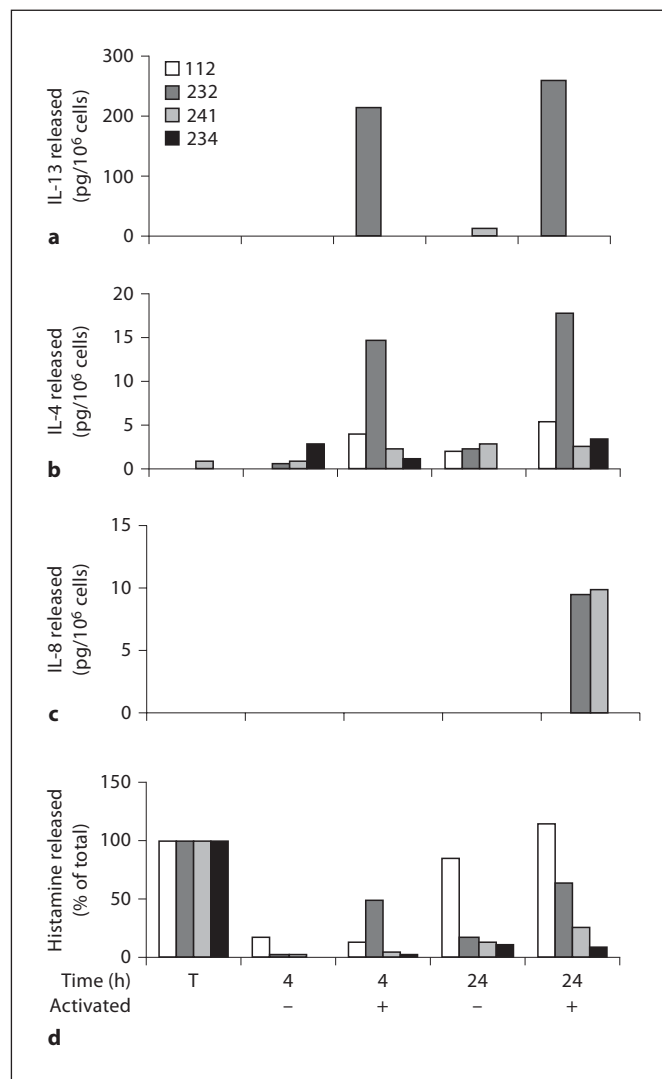


Fig. 4. Cytokine and histamine release from basophils with and without Fc ϵ RI cross-linking. Supernatants retrieved from the incubations described in figure 3 were analyzed by ELISA for IL-13 (a), IL-4 (b), IL-8/CXCL8 (c), and histamine (d). Total histamine (100%; T) was determined by assaying lysates from an aliquot of cells.

gen (fig. 4d). It is interesting that the data of patient 112 shows >100% release after 24 h. We presume this is because total histamine was measured without incubation and the cells synthesized a small amount of additional histamine in 24 h.

GATA2 transcript amplification had a similar pattern in all samples tested. Amplified product was detected in the control and 4-hour incubation samples but decreased notably in the 24-hour incubation samples irrespective of activation state (fig. 3).

	231						239						259						
As/Con	Con						As						Con						
Histamine release (%)	77.0						14.6						35.7						
Incubation (h)	24	24	2	2	4	4	24	24	2	2	4	4	24	24	2	2	4	4	
Activated	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+	
+IL-3	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	Cycle No.
GAPDH																			33
IL-13																			42
IL-4																			35
IL-8																			35
Leptin																			35
ML-1																			35
IL-6																			33
GATA2																			35
MIP-5																			42

Fig. 5. Preincubation with IL-3 alters the transcription of a number of targets in basophils of asthmatic and control subjects. Histamine release assays were carried out immediately on purified basophils from asthmatic (As) and control subjects (Con). Basophils were incubated in the presence or absence of 10 ng/ml IL-3 alone. Cells were activated with 1 μ g/ml anti-IgE for 0, 2 or 4 h prior to the end of the 24-hour incubation. Isolated mRNA (0.1 μ g/ml) was analyzed by semiquantitative RT-PCR.

Although apparently detected by the Cartesian array, IL-12 and IL-17 transcripts were not amplified in any of the samples tested. Additionally, no evidence of IL-12 production was found using a combination of RT-PCR, ELISA and Luminex bead assays (results not shown). We suppose that the array data are false positives and emphasize the importance of independent analyses to confirm cytokine identities.

IL-3 Enhances Expression of a Number of Inflammatory Mediators in Basophils

The activation of purified basophils was repeated in the presence of IL-3 that enhances basophil survival and primes them for increased degranulation. Incubation with IL-3 alone caused increases in transcripts for IL-8, GATA2 and MIP-5 (fig. 5) and detectable IL-4 and IL-8 protein release (fig. 6b, c). Addition of antigen following priming with IL-3 caused a more robust increase in transcription, with significant increases in transcript levels for IL-4, IL-8 and GATA2 in all 3 subjects while MIP-5 and IL-13 transcript levels increased in 2 of the 3 subjects tested. IL-13 release was of the order of 10-fold greater when compared to the concentrations of ELISA detected protein in the absence of IL-3 (fig. 6a). Under the amplification conditions of this study, IL-8 mRNA was only detectable after preincubation with IL-3. IL-8 protein released increased from undetected to thousands of picograms per milliliter (fig. 6c). Neither IL-3 nor IL-3 com-

bined with antigen altered transcript levels for IL-6 or ML-1 (fig. 5).

There were two notable observations when histamine concentrations in the media of incubated basophils were determined. Preincubation of basophils with IL-3 did not result in spontaneous histamine release. After antigen stimulation in the presence of IL-3, however, histamine release was markedly higher than that seen in its absence (fig. 6d).

Estimations of IL-3 in sera and BALF samples were attempted. Even after concentrating serum samples 5-fold, IL-3 concentrations in some sera still fell below the stated sensitivity of the ELISA (7.4 pg/ml). Those samples were reported as 0. IL-3 concentrations, when corrected for concentration factors, were in the range of 2.84–40.77 pg/ml (table 3). Detected IL-3 concentrations in BALF (back-calculated to account for dilution during lavage) ranged from 0.1 to 0.46 pg/ml (table 3).

Leptin Is Produced in Basophils and Released in Response to Fc ϵ RI Receptor Cross-Linking

Cartesian array analysis suggested that leptin was synthesized, stored and released by basophils (fig. 2a, b). Confirming this, leptin mRNA was detected in each of the subjects tested (fig. 2c). The effects of IL-3 on leptin mRNA transcription upon activation were mixed, with the expression of subjects 231 and 239 decreasing after 2 h activation in the presence of IL-3 (fig. 6b). The amount

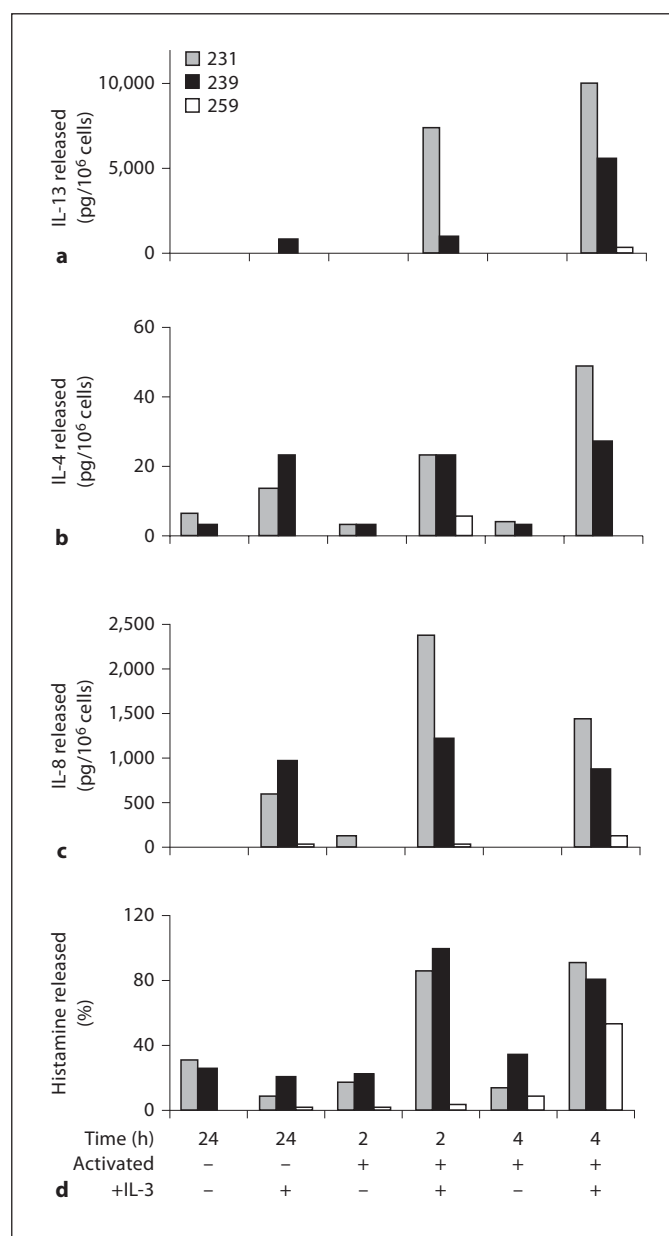


Fig. 6. Effect of IL-3 preincubation on cytokine and histamine release. Supernatants retrieved from the incubations in figure 5 were analyzed by ELISA. Detection of IL-13 (a), IL-4 (b), IL-8 (c) and histamine (d). Total histamine (100%) was determined by assaying lysates from an aliquot of cells.

of leptin stored in isolated cells from 4 subjects varied between 0.67 and 9.1 ng/10⁶ cells (fig. 7a, 0-hour data). Leptin release by some subjects' basophils was increased in response to FcεRI cross-linking (fig. 7a). Leptin release was not strongly altered when cells were preincubated for 24 h with IL-3 before the addition of antigen (fig. 7b).

Table 3. Analysis of IL-3 concentrations in serum and BALF of asthmatic and control subjects

a In serum

Subject		IL-3 pg/ml	Histamine released, %
87	As	0	10.3
10	As	0	10.9
212	As	0	62.0
241	As	0	0.0
239	As	0	44.7
247	As	0	77.5
42	As	5.5	88.0
234	As	7.7	9.4
239	As	7.8	10.8
14	Con	0	30.3
15	Con	0	44.7
133	Con	2.8	57.6
133	Con	5.9	60.0
253	Con	7.4	34.0
220	Con	40.8	17.4

b In BALF

Subject		IL-3 pg/ml
139	Con	0.01
163	Con	0.24
187	Con	0.46
173	As	0.11
172	As	0.12
171	As	0.13

Serum and BALF samples from asthmatic (As) and control (Con) subjects were concentrated as described in Materials and Methods. Percent histamine release from isolated basophils of these subjects was measured on the same day that serum samples were obtained. IL-3 concentrations in serum and BALF were determined by ELISA.

Discussion

We reported previously that basophils from asthmatics show a higher maximum percentage histamine than control basophils [11] and demonstrate here that more basophils are recovered after Percoll centrifugation from the blood of asthmatics than controls. Both characteristics may contribute to the onset or progression of disease. The present study was undertaken to determine if the complement of chemokines and cytokines stored or synthesized by basophils is also characteristically different

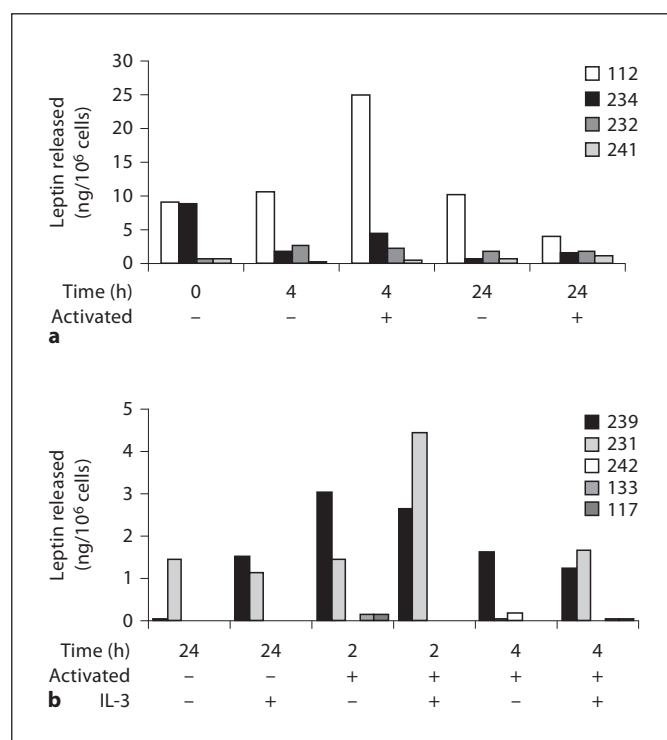


Fig. 7. Synthesis and release of leptin from basophils are inducible by cross-linking the FcεRI receptor. **a** Basophils from subjects described in figure 4 were lysed immediately (0 h) to measure stored leptin or incubated with or without 1 μg/ml anti-IgE for 4 and 24 h to measure leptin released into the incubation media. Lysates and media were analyzed by ELISA for leptin. **b** Purified basophils were incubated with or without 10 ng/ml IL-3 for 24 h. Prior to completion of this incubation period aliquots of cells were incubated with and without 1 μg/ml anti-IgE for 2 and 4 h. At the indicated time points the incubation media were retrieved by centrifugation and leptin released determined by ELISA.

between asthmatic and control subjects. In the past, investigations of inflammatory mediators synthesized, stored and released by basophils in response to FcεRI cross-linking have been difficult due to a combination of the low yields of high-purity cell preparations and the high costs of the assays. Here we used a multiplexed antibody array to reduce these problems and to evaluate the storage and release of multiple cytokines and chemokines from basophils simultaneously.

A total of 12 asthmatic and 16 control subjects were involved in the present study. Using an antibody array, we identified the same cytokines and chemokines stored in approximately the same proportions in basophils isolated from peripheral blood in this diverse subject panel. This result was confirmed by mRNA expression analysis and

in some cases by ELISA quantification. IL-4 was found to be the cytokine which varied most among subjects. The detection of IL-4 mRNA in the 4-hour resting sample in 3 out of the 4 subjects of this study combined with the detection of IL-4 protein in the media suggests that it may be released constitutively. This agrees with the conclusions of in vivo studies in mice using IL-4 reporters [2, 7]. In contrast, no constitutive release of IL-13 was detected.

IL-17 protein was detected by the array, but no IL-17 RNA transcript was detected in any subject assayed. Kawaguchi et al. [13] had previously reported their failure to detect any IL-17 transcript in basophils but did identify a transcript with 70% homology to IL-17 which they called ML-1, also referred to as IL-17F. When ML-1 transcript was sought in basophils of this study it was found to be present in both asthmatic and control subjects. We hypothesize that there was cross-reactivity between ML-1 protein and the IL-17 antibody of the array. Currently we have no explanation for the other false positive on the array, IL-12.

Among the documented growth factors affecting basophils, IL-3 is the most potent, enhancing the release of cytokines, increasing cell viability and inhibiting basophil apoptosis [14–16]. Incubation with IL-3 did not cause direct histamine release and in the literature conflicting evidence has been presented on this point [17, 18]. In agreement with previously reported data [10], incubation over 24 h with IL-3 markedly enhanced antigen-stimulated histamine release. Interestingly GATA2 expression followed histamine release patterns. GATA2 transcripts levels decreased after 24 h incubation in the absence of IL-3 but were preserved in its presence (fig. 5b, 6b).

Preincubation with IL-3 also dramatically altered the expression and release of a number of cytokines. IL-3's enhancement of the release of both IL-4 and IL-13 from basophils upon receptor cross-linking has been extensively documented [14–17, 19]. We report for the first time that IL-3 also upregulates IL-8 transcript and protein levels in isolated peripheral blood basophils. The concentrations of IL-8 released after preincubation with IL-3 were significant when compared to those of IL-4 and IL-13.

The enhancement of transcript levels for chemokines and cytokines by IL-3 preincubation was not universal. GM-CSF, eotaxin and MIP-5 mRNAs were not detected as upregulated after preincubation or stimulation with IL-3, suggesting that these responses are under a different regulatory pathway. Recent studies indicate that IL-3 acts on downstream signaling events on two particular pathway locations [20]. Although the release of GM-CSF and

eotaxin were detected from KU812 cells, this is the first report of these proteins along with MIP-5 from isolated peripheral blood basophils [21]. Interestingly all three of these proteins and IL-8 function as chemokines [22–25]. This study therefore expands the number of chemokines that basophils could potentially release. These new findings suggest that basophils may have a greater role in late-phase asthmatic response, beyond the immediate release of potent proinflammatory mediators such as IL-4 and IL-13, by also recruiting neutrophils, eosinophils, T cells and monocytes to sites of inflammation.

Given the documented effects of IL-3 on basophils in vitro, there is still some question as to whether this cytokine could modulate basophil cytokine production in vivo [10, 14, 26]. Estimations of IL-3 concentrations in serum are sparse, but values in the range of 17–68 pg/ml for normal subjects have been reported [27]. The values recorded here fall slightly below this range (0–41 pg/ml). We could find few estimations of IL-3 concentrations in BALF in the literature but the concentrations recorded here (0.01–0.24 pg/ml) are in agreement with a report where concentrations of less than 1 pg/ml were typical [28]. Even accounting for our small sample set, it is obvious that the IL-3 concentrations used to supplement incubation media in this study and many others [14, 15] far exceed those detected in both serum and BALF. This does not negate the possibility that IL-3 exerts an effect on basophils. It has been reported that mast cells from lung can produce IL-3 at concentrations between 500 and 700 pg/10⁶ cells 8–24 h after activation [29]. Hence, it is possible that local IL-3 concentrations could be significantly higher than those detected here.

Basophil synthesis of leptin, suggested by the array results, was confirmed by mRNA detection and protein detection by ELISA. Leptin is a 16-kDa protein similar in structure to IL-2 and IL-12. It is principally synthesized and released by adipocytes. There have been reports of leptin modulating the functions of T cells, natural killer cells (NK) cells and monocyte-macrophages [30–32]. Reports of the effects of leptin on cells of the immune system include enhanced production of growth factors in macrophages and cytokines in dendritic cells, driving T cell polarization toward the Th1 phenotype and down-regulation of the apoptotic pathway in neutrophils [33, 34]. A few studies have examined leptin concentrations and correlations with asthma. Gurkan et al. [35] recorded higher leptin serum levels in asthmatic children over controls, while a broader study examined 5,876 subjects and reported that leptin is associated with asthma in women [36]. Basophils are unlikely to contribute sub-

stantially to circulating leptin, which has an arterial concentration of 7.3 ± 1.3 ng/ml [37]. However, basophil leptin may act in a paracrine rather than endocrine mode to modulate inflammatory events.

We conclude that isolated basophils with varying histamine release possess similar key cytokine and chemokine profiles, on isolation and after cross-linking of their Fc ϵ RI receptors. The findings that peripheral blood basophils release the chemokines GM-CSF and eotaxin along with MIP-5 indicate that basophils may play a greater role in late-phase asthmatic response, beyond the immediate release of potent proinflammatory mediators, by recruiting neutrophils, eosinophils, T cells and monocytes to sites of inflammation. Leptin production by basophils may indicate additional functions in inflammation. Importantly, the inclusion of IL-3 in incubations substantially alters the expression and release of both cytokines and chemokines. It is possible that activated basophils could sustain inflammation in asthmatic subjects through their interactions with IL-3 or other cytokines that may be present in the local environment of asthmatic but not control basophils.

Acknowledgements

The authors wish to thank the generous cooperation of volunteers who provided samples for this study. Tereassa Archibeque's dedicated effort to recruit, screen, retain and schedule volunteers is gratefully acknowledged. We also thank Biosource (Camarillo, Calif., USA) for performing Luminex bead assays for IL-12 and IL-17. This work was supported in part by grants P50 HL56384, RO1 GM49814 and M01RR000997 from the National Institutes of Health.

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