Frequency and characterization of antigen-specific IL-4– and IL-13–producing basophils and T cells in peripheral blood of healthy and asthmatic subjects

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Background: Both basophils and T cells are known to secrete IL-4 and IL-13 after activation with either nonspecific stimuli or specific antigen, but the relative contribution of these 2 cell types to overall cytokine production is unclear.
Objectives: To further characterize basophil cytokine production and compare it with that of T cells, we examined the frequency of IL-4– and IL-13–producing basophils and T cells in human PBMCs by means of flow cytometry after activation in allergic asthmatic and normal subjects.
Methods: PBMCs obtained from whole blood after Percoll gradient were activated with specific antigen or ionomycin and fixed. PBMCs were made permeable; stained with antibodies to IgE, CD3, and either IL-4 or IL-13; and analyzed by means of flow cytometry.
Results: Preformed cytokines were not detected in unactivated basophils. After ionomycin activation, 60% to 90% of basophils from both control and allergic asthmatic subjects expressed IL-4 and IL-13. Specific antigen induced cytokine expression by 10% to 20% of basophils from the asthmatic group only. After specific antigen activation, basophils accounted for 4 times more IL-4–producing cells than did T cells. IL-4 and IL-13 production at 2 hours was exclusively from basophils. After allergen activation, CD40 ligand was upregulated on a subset of peripheral blood basophils.
Conclusions: These data demonstrate that basophils are the predominant peripheral blood cells that express IL-4 and IL-13 in the first 6 hours after antigen activation and strengthen the putative role of basophils both in IgE production and in the generation of allergic inflammation. (J Allergy Clin Immunol 1999;104:811-9.)

Key words: *Basophils, T cell, cytokines, flow cytometry, human, allergy, asthma*

Abbreviations used
CD40L: CD40 ligand
FcεRI: High-affinity IgE receptor
PBS-S: PBS-saponin
PE: Phycoerythrin
PE/Cy5: Phycoerythrin/Cyanin 5

Basophils have been implicated in acute allergic reactions, including asthma, by their release of immediate mediators, such as histamines, leukotrienes, and prostaglandins. Additionally, because basophils secrete both IL-4[1] and IL-13[2,3] after high-affinity IgE receptor (FcεRI) cross-linking, it has been suggested that basophils may contribute to CD40-dependent Ig class switching,[4,5] the upregulation of costimulatory molecules on B cells,[4,6,7] and the upregulation of vascular cell adhesion molecule-1 on endothelium.[8,9] IL-4 is also required for T-cell differentiation to the T_{H2} phenotype.[10] As such, basophils, in addition to T cells, may thus help to orchestrate allergic inflammation.

To further explore the hypothesis that basophils contribute to IL-4 and IL-13 production in allergic inflammation, we examined the frequency of basophils producing these cytokines after nonspecific stimuli and after antigen exposure and compared it with that of T cells producing the same cytokines. Because basophil cytokine expression has been examined on the protein and messenger (m)RNA levels by using bulk assays, such as ELISA[11] northern blot analysis,[12] and reverse transcriptase PCR,[13] and because the surface phenotype of basophils has been effectively determined,[14-18] we elected to examine basophil cytokine expression at the single cell level by using flow cytometry. To use this technology, we first identified basophils among the FcεRI-bearing cells in peripheral blood. We then compared cytokine expression by basophils from allergic asthmatic and control subjects and determined the relative contribution of basophil and T-cell fractions of PBMCs to total cytokine expression. Finally, because both CD40 ligation and IL-4 production are required for IgE synthesis, we investigated the modulation of CD40 ligand (CD40L) expression on T cells and basophils after in vitro allergen stimulation. As will be shown, the data clearly support the hypothesis that basophils are a major source of IL-4 and IL-13 when compared with the production of these cytokines by T cells and that strategies

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TABLE I. Baseline characteristics of allergic asthmatic patients

Patient	Age (y)	Antigens eliciting positive skin test responses*	Methacholine PC_{20} (mg/mL)	Corticosteroid treatment
1	25	Cat, rye, elm	0.25	Yes (inhaled)
2	42	Cat, *D pteronyssinus*, *D farinae*	25	No
3	25	Cat, rye, ragweed	0.25	No
4	28	Cat, elm, oak, *D pteronyssinus*, *D farinae*, rye	0.25	No
5	35	Cat, rye, ragweed	2.5	No
6	42	Cat, ragweed, rye, *D pteronyssinus*, *D farinae*	10	No
7	51	Cat, *D pteronyssinus*, *D farinae*	NP	Yes (inhaled)

NP, Not performed (FEV_1 less than 70% of the predicted value).
*A positive skin test response was defined as 5 mm or greater induration.

to interrupt basophil cytokine synthesis may be important in the successful treatment of allergic diseases.

METHODS

Reagents

Ionomycin and DNAse I (Calbiochem, La Jolla, Calif); brefeldin A, paraformaldehyde, Percoll, Ficoll-Diatrizoate 1.077 g/ml, and dimethyl sulfoxide (Sigma Chemical Co, St Louis, Mo); saponin (Fluka Biochemika, Ronkonkoma, NY); and standardized cat hair (10,000 AU/mL) and *Dermatophagoides pteronyssinus* (30,000 AU/mL) allergenic extracts (Bayer Co, Elkhart, Ind) were purchased. The recombinant major cat allergen of *Felis domesticus* (rFel d 1) was a gift of ImmuLogic Corporation (Waltham, Mass).

Monoclonal antibodies

FITC-conjugated anti-CD3 and anti-IL-2 (clone 5322.111; Becton Dickinson Immunocytochemistry Systems, San Jose, Calif); mouse anti-IgE FITC, anti-CD2, CD19 phycoerythrin/cyanin 5 (PE/Cy5), anti-HLA-DR PE/Cy5, anti-IL-3Rα PE (CD123), anti-CD40L PE, anti-IL-4 PE (clones MP4-25D2 and 8D4), IL-5 PE (clone 5Al0), and IL-13 PE (clone 5A2); allophycocyanin-conjugated streptavidin, PE- and FITC-conjugated mouse IgG1, PE-conjugated rat IgG1, biotinylated goat IgG, purified anti-IL-4, and anti-IL-13 (Pharmingen); PE/Cy5-conjugated mouse anti-human CD14 and CD16 (Caltag Laboratories, Burlingame, Calif); and biotin-conjugated goat anti-human IgE (Biosource, Camarillo, Calif) were obtained commercially. Anti-FcεRI (clone 15.1) was a gift of Dr J. P. Kinet and was FITC labeled.

Subjects

After informed consent, peripheral blood was obtained from normal volunteers (n = 3) and from asthmatic subjects (n = 7) with cat or house dust mite allergy without pets in the home. Allergic asthmatic subjects had a greater than 1-year history of symptoms consistent with asthma, a methacholine PC_{20} of 25 mg/mL or less, and 3 or more positive skin test responses (≥5 mm induration) to a panel of 7 tested allergens (cat, elm, ragweed, Bermuda grass, *D farinae*, *D pteronyssinus*, and rye) (Table I). Nonatopic control subjects were defined by having a negative history of allergic disease, negative skin test responses, and a methacholine PC_{20} of greater than 25 mg/mL. The clinical protocol was approved by the National Institute of Allergy and Infectious Disease Institutional Review Board.

Culture conditions

For basophil stimulations, PBMCs were obtained from EDTA-anticoagulated whole blood after of Percoll gradient (Sigma Chemical Co) at a density of 1.08 g/mL.[19] PBMCs were suspended in RPMI-1640 complete media (10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, 25 μg/mL gentamicin, 50 μmol/L 2-mercaptoethanol, 1 mmol/L pyruvate, and nonessential amino acids [Biofluids, Rockville, Md]). Cells were incubated at 2×10^6 PBMCs/mL in 24-well plates (Costar, Cambridge, Mass) at 37°C. Basophil stimulations were performed by using either 1 μmol/L ionomycin or allergen extracts (1:1000 dilution). Brefeldin A (10 μg/ml) was added at the start of the culture to inhibit cytokine release. Cytokine staining was not detectable in basophils activated in the absence of brefeldin A (data not shown). After the noted time, 50 μg/mL DNAse I was added to each well, and cells were incubated for 5 minutes, washed in cold PBS, and fixed in 4% paraformaldehyde at room temperature for 5 minutes. Arrest of fixation was obtained by washing cells in ice-cold PBS 0.1% BSA, with centrifugation at 1500*g* for 10 minutes. The cell pellets were then resuspended in 1 mL of PBS with 10% dimethyl sulfoxide, placed in aliquots, and stored at –80°C for subsequent analysis.

Basophil-T cell comparative cultures were performed on Ficoll-Diatrizoate–isolated PBMCs. Cells were activated at 37°C with either Fel d 1 (2 μg/mL) or no antigen in RPMI-1640 complete media supplemented with anti-CD28 ascites 1:1000 (clone CK248, generously provided by L. Moretta, Genova, Italy) at a density of 4×10^6 cells/2 mL in 16 × 125-mm tissue culture–treated round-bottom tubes (Corning Glass, Corning, NY). After 2 hours, brefeldin A (10 μg/mL) was added, and the cells were incubated for an additional 4 hours and then fixed as above. Cultures examining CD40L were analyzed at 2 hours, without the addition of brefeldin A.

Flow cytometry

Cryopreserved fixed cells were thawed at 37°C, washed, and stained for cell surface markers and intracellular cytokines, as previously described.[20] Briefly, the cell pellets were resuspended in PBS with 0.1% saponin (PBS-S) and 5% nonfat dry milk for 1 hour to block nonspecific binding and then stained with mAbs at 4°C for 30 minutes. The cells were washed twice in PBS-S and stained with allophycocyanin-conjugated streptavidin for an additional 30 minutes at 4°C. Finally, the samples were washed in PBS-S and resuspended in 300 μL of PBS-BSA for analysis. Blocking with unlabeled anticytokine mAb was performed in the same manner, with the addition of 100 μg/mL of either unlabeled anticytokine mAb or isotype control, for 1 hour before the addition of labeled anticytokine mAb.

Acquisition and analysis

Data were acquired on a 2-laser FACSCALIBUR (Becton Dickinson Immunocytometry Systems) flow cytometer, which allowed the simultaneous detection of 4 fluorescent parameters. Typically, 100,000 to 400,000 total events were acquired, depending on the

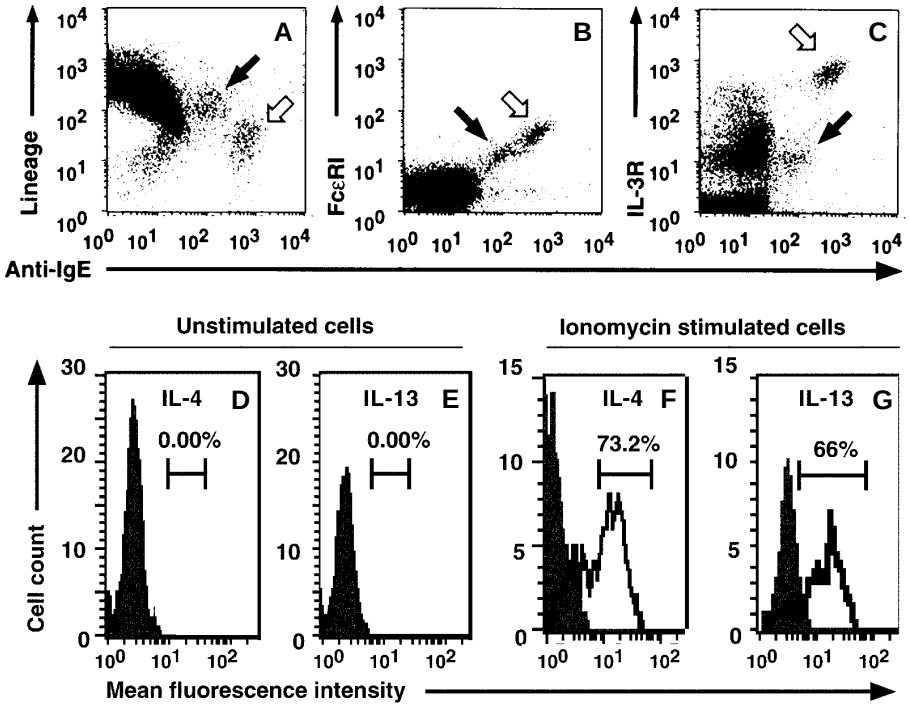


FIG 1. Identification of basophils and determination of their cytokine expression by flow cytometry. PBMCs were fixed, permeabilized, and stained with antibodies to IgE, lineage markers (CD2, CD14, CD16, and CD19), FcεRI, IL3Rα, and CD45. Dot plots of IgE versus lineage **(A)**, FcεRI **(B)**, and IL-3Rα **(C)** were generated. The IgE^{high}, lineage^{-} population (**A**, *open arrow*) defined a population of FcεRI^{high} and IL-3Rα^{high} cells (**B** and **C**), as noted by the *open arrow*. Additionally, an IgE^{int}, lineage^{int} monocyte population was noted (**A**, **B**, and **C**; *filled arrows*). After isolation, PBMCs were fixed either immediately (**D** and **E**) or 2 hours after activation with ionomycin (**F** and **G**). Cells were then permeabilized and stained for IgE and lineage and either IL-4 (**D** and **F**) or IL-13 (**E** and **G**). Cytokine staining is shown as a *bold line* and *open histogram*. Staining controls *(gray histograms)* consisted of an isotype-matched control mAb (**D** and **E**) or blocking of the mAb with an excess of unlabeled anticytokine (**F** and **G**). These data are representative of 3 independent experiments.

cytokine and the cell fraction being studied. List mode files were analyzed by using CELLQUEST (Becton Dickinson Immunocytometry Systems) software.

Statistical analysis

Final data were expressed as means ± SD. The paired *t* test was performed to compare intrasubject differences of cytokine and CD40L expression between T cells and basophils. A *P* value of less than .05 was considered as significant.

RESULTS

Identification of basophils

The determination of basophil cytokine expression by flow cytometry requires an unambiguous staining approach to identify and gate on basophils. To achieve this goal, we used a combination of inclusion and exclusion markers, anti-IgE, and a panel of lineage-specific mAbs (CD2, CD14, CD16, and CD19), as previously described,[14,15] which allowed the unambiguous identification of the basophil population (Fig 1, *A, open arrow*). To verify that this IgE^{high} lineage population consisted of basophils, we performed multiparameter flow cytometry with antibodies to IgE, the lineage panel, and either FcεRI, CD123/IL-3Rα, or CD45. As expected, this IgE^{high} population *(open arrows)* expressed high levels of IL-3Rα and FcεRI (Fig 1, *B* and *C*) and intermediate CD45 staining (data not shown), which is consistent with a basophil population (mean, 1.7% PBMCs; range, 1.4% to 3.7% PBMCs; n = 7). Some cells with larger scatter (data not shown) demonstrated intermediate levels of IgE, FcεRI, and lineages but expressed low levels of IL-3Rα, which is consistent with a monocyte population *(filled arrows)*. Additionally, small numbers of the IgE^{high} lineage cells expressed high levels of HLA-DR, which is consistent with a dendritic cell lineage.[21,22] These cells accounted for a mean of 0.3% (range, 0.08%-0.6%; n = 6) of the IgE^{high} lineage population. For subsequent experiments, PBMCs were simultaneously stained with a battery of lineage markers (anti-CD2, ani-CD14, anti-CD16, and anti-CD19) and anti-IgE. Cytokine-producing basophils were assessed with PE-conjugated anticytokine mAbs by gating on IgE^{high} lineage cells.

Quantitative expression of IL-4 and IL-13 by basophils

Preformed IL-4 has been reported in basophils.[23,24] We then first examined unstimulated basophils from nonatopic subjects (n = 3) for the presence of IL-4. To

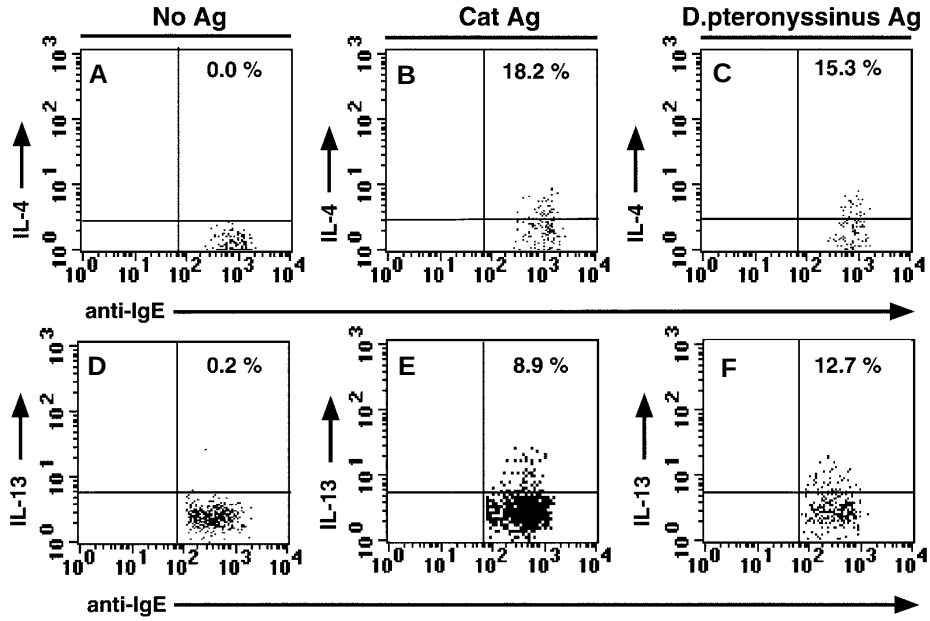


FIG 2. Basophil cytokine expression after antigen *(Ag)* activation. PBMCs were activated in vitro with no antigen (**A** and **D)**, cat antigen (**B** and **E**), or *D pteronyssinus* antigen (**C** and **F**) for 2 hours **(A-C)** or 6 hours **(D-F)**. Cells were then fixed, permeabilized, and stained for IgE, lineage, and either IL-4 **(A-C)** or IL-13 **(D-F)**. After gating on IgE^{high} $lineage^{-}$ basophils, a cytokine versus IgE dot plot was generated. Statistical markers were placed on the basis of isotype-matched control staining. Similar data were obtained in 3 independent experiments. The percentages given in each panel represent the percentage of basophils producing cytokines.

address the possibility of unequal recognition of preformed IL-4 by individual anti-IL-4 clones,[24] we used 2 anti-IL-4 mAbs (25D2 and 8D4) known to recognize different epitopes on the IL-4 molecule. Clone 25D2 clearly showed an absence of IL-4 staining (Fig 1, *D*), whereas clone 8D4 demonstrated a small but distinct number of IL-4–positive basophils (0.03%; data not shown). In parallel experiments IL-13 was not detected in unstimulated basophils (Fig 1, *E*). Similar results were obtained for unstimulated basophils from atopic patients (data not shown).

To examine maximal expression of IL-4 and IL-13, PBMCs from nonatopic patients (n = 3) were stimulated with ionomycin (1 μmol/L). We analyzed IL-4 expression by using both the 25D2 and 8D4 clones, which yielded an average of 71.7% ± 1.5% (Fig 1, *F*) and 91.5% ± 2.1% positive cells, respectively (data not shown). In parallel experiments IL-13 was expressed by 63.1% ± 2.8% of cells (Fig 1, *G*). Basophils from atopic asthmatic subjects (n = 3) showed a similar result (data not shown). To provide a more rigorous proof of the specificity of antibody staining, cells were incubated with an excess of unconjugated anticytokine mAb before staining with PE-labeled anticytokine mAb.[20,25] By using such an approach, cytokine staining was blocked to baseline levels (Fig 1, *F* and *G*, *shaded histograms*).

Effects of in vitro antigen stimulation on cytokine expression

Experiments were next performed to examine the effects of specific allergen (cat or house dust mite antigen extract) on cytokine expression by basophils from asthmatic (n = 3) and control subjects (n = 3). To this end, we analyzed in vitro allergen-stimulated PBMCs from allergic asthmatic patients for basophil IL-4 and IL-13 expression. When basophils were cultured without antigen, no cytokine expression was seen, regardless of the allergic status of these patients (Fig 2, *A* and *D*). In contrast, in the atopic asthmatic donors, the addition of allergen induced the expression of both IL-4 and IL-13. Similar frequencies of IL-4– and IL-13–positive cells were found, ranging from 10% to 20% of basophils, in response to either cat or mite antigen (Fig 2, *B*, *C*, *E*, and *F*).

Kinetic profiles of cytokine expression

The kinetics of antigen-induced IL-4 and IL-13 expression by basophils were examined by incubating PBMCs with or without cat antigen extract for 0 to 8 hours. Antigen-dependent IL-4 expression from asthmatic patients peaked at 2 hours and declined significantly thereafter ($P < .01$; Fig 3, *A*). Additional experiments showed that little if any IL-4 was expressed 1 hour after activation (data not shown). In contrast, IL-13 showed delayed kinetics, although significant IL-13 expression was observed after 2 hours of incubation ($P < .05$; Fig 3, *B*). In contrast, antigen-activated basophils from nonatopic control subjects did not express either cytokine. Consistently, basophils from both asthmatic and control subjects cultured without antigen failed to express IL-4 and IL-13.

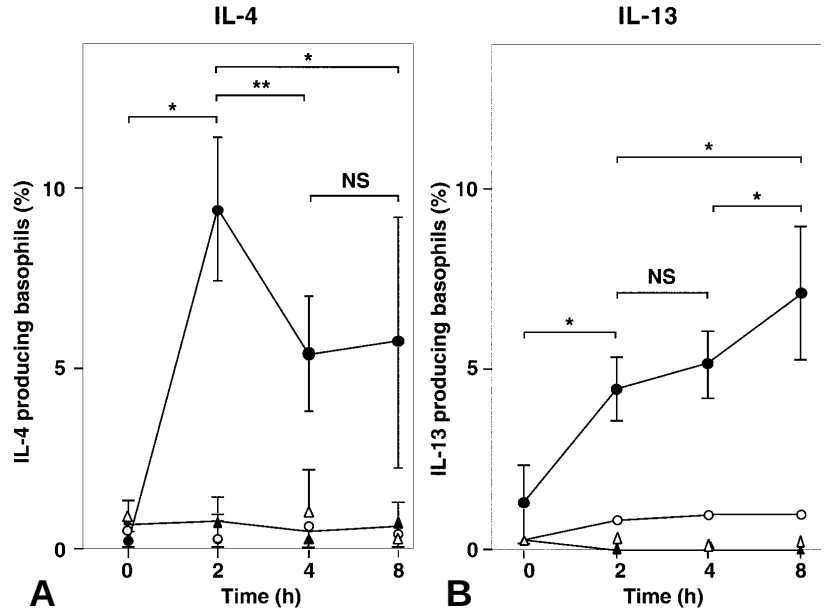


FIG 3. Kinetics of cytokine expression by antigen-stimulated basophils. PBMCs from asthmatic subjects with cat allergy (*circles*, n = 3) or nonatopic control subjects (*triangle*, n = 3) were stimulated in vitro with either cat antigen *(closed symbols)* or no antigen *(open symbols)*. Cells were then stained for IgE, lineage, and either IL-4 **(A)** or IL-13 **(B)**. After gating on IgE^{high}, $lineage^{-}$ cells, the frequency of cytokine-producing basophils was determined. The percentage of cytokine-producing basophils is shown, and values represent the mean ± SD of 3 independent experiments. Statistically significant differences are noted by a single asterisk ($P < .05$) and double asterisks ($P < .01$). *NS,* Not significant.

Relative frequency of cytokine-positive basophils and T cells

Both IL-4 and IL-13 influence IgE production and the generation of late-phase allergic inflammation.[26-29] In addition to T cells, basophils may be found within sites of allergic inflammation,[30,31] raising the possibility that basophil secretion of IL-4 and IL-13 may play a role in the generation or maintenance of the inflammatory response. To determine the relative contribution of basophils and T cells to antigen-induced cytokine production, PBMCs from allergic asthmatic patients were cultured with or without the recombinant major cat antigen Fel d 1 for 6 hours. Basophils and T cells were defined as being IgE^{+}, $lineage^{-}$ and $CD3^{+}$ cells, respectively, and the frequency of each cell type was calculated as a function of the total number of PBMCs analyzed (Fig 4, *A*). Both basophils and lymphocytes cultured without antigen failed to express IL-4 (Fig 4, *B*) or IL-13 (data not shown). After antigen stimulation, basophils accounted for a much larger number of IL-4–producing cells than did T cells (Fig 4, *C*). When the frequency of cytokine-producing cells was calculated out of the total number of basophils, rather than PBMCs, similar results were obtained to those noted in Fig 2. Similar results were seen for IL-13 (Fig 4, *D*). Only T cells were found to express IL-2 and IL-5 (Fig 4, *E* and *F*), which is consistent with the know spectrum of basophil cytokine production.[1,28]

Contribution of basophils and T cells to cytokine expression

To quantify the relative contributions of the 2 cell types, rFel d 1–stimulated PBMCs were similarly analyzed for IL-4 (7 donors) and IL-13 (5 donors). IL-4–producing basophils comprised 0.2% ± 0.1% (mean ± SD) of PBMCs, whereas T cells accounted for 0.05% ± 0.03% of PBMCs ($P < .001$). The frequency of IL-13–positive basophils (0.09% ± 0.05%) was greater than that of T cells (0.04% ± 0.03%), but the difference was not statistically significant ($P = .12$; Fig 5, *A*).

Contribution of basophils and T cells to CD40L expression

The interaction between CD40 and its ligand (CD40L) is an additional requisite for IgE switching.[5] Previous reports using immunohistochemistry have shown that CD40L is constitutively expressed on purified peripheral blood basophils[32,33] and is weakly upregulated after phorbol myristate acetate-ionomycin activation.[33] To determine whether basophil CD40L is upregulated under physiologic conditions, we examined basophil CD40L expression after antigen stimulation. CD40L was constitutively and similarly expressed at low levels on both resting basophils and T cells. In contrast, we found that 2 hours after antigen exposure, CD40L was upregulated on basophils ($P < .05$) but not on T cells (Fig 5, *B*).

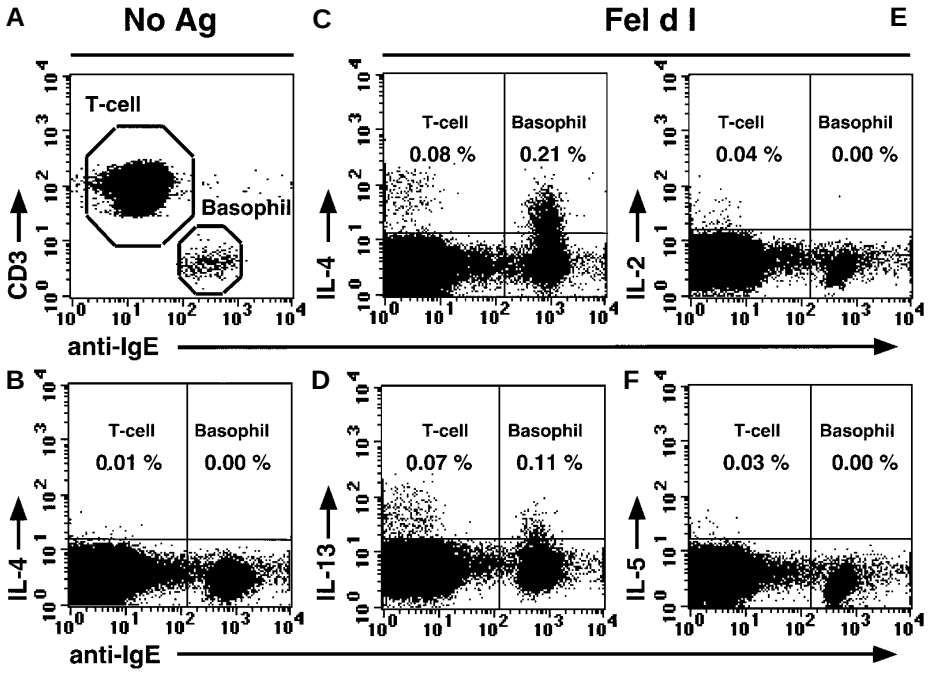


FIG 4. Simultaneous identification of basophil and T cell cytokine production. PBMCs from an asthmatic subject with cat allergy were stimulated in vitro with either no antigen *(Ag)* (**A** and **B**) or rFel d 1 **(C-F)** for 6 hours and fixed. Cells were then stained for IgE, lineage, CD3, and either IL-4 (**B** and **C**), IL-13 **(D)**, IL-2 **(E)**, or IL-5 **(F)**. **A,** IgE^{high}, $lineage^{-}$ basophils and $CD3^{+}$ T cells were combined within a single gate. **B-F,** This additive gate was then used to determine the frequency of cytokine-expressing PBMCs that could be attributed to basophils (IgE^{high}) or T cells (IgE^{low}). This figure is representative of 5 independent experiments. The frequencies given in each panel represent the cytokine-producing T cells or basophils as a percentage of the total number of PBMCs.

DISCUSSION

By using flow cytometry, we were able to unambiguously define the basophil population and to demonstrate coexpression of high levels of FcεRI and IL-3Rα (Fig 1). However, FcεRI is not specific for basophils but is also expressed by monocytes,[34] dendritic cells,[22] and eosinophils.[35] We noted an IgE^{int}, $FcεRI^{int}$, $lineage^{int}$ population consistent with a monocyte lineage as further demonstrated by their characteristic scatter and low IL-3Rα expression. Neither the monocytic nor dendritic cell (IgE^{high} and $HLA\text{-}DR^{high}$) populations expressed cytokines after allergen activation (data not shown). Eosinophils were excluded by the use of 1.080 Percoll gradient, characteristic scatter,[36] and the expression of cytoplasmic CD16.[37]

We examined IL-4 in unstimulated basophils by using 2 noncompeting anti-IL-4 mAbs (clones 25D2 and 8D4), which demonstrated little if any preformed cytokine (Figs 1, 2, and 3). It is unclear whether the minimal IL-4 that was detected with the 8D4 clone is significant because our results in T cells suggest this mAb has lower specificity than the 25D2 clone (data not shown). Furthermore, the frequency of cells detected containing preformed IL-4 is very low and close to the limits of detection of the technique. This amount was equivalent to 1% to 2% of that detected in subsequent experiments examining antigen-activated basophils. Preformed IL-13 was not found within unstimulated basophils, which is in agreement with previous reports.[23,32]

Two previous studies have demonstrated IL-4 immunoreactivity in unstimulated basophils by using immunocytochemistry[24] and flow cytometry.[38] The former study examined basophils after 18 hours of priming with IL-3, which may have activated the basophils and produced the low level of IL-4 expression observed. The latter study examined basophils immediately after isolation and detected IL-4 in 40% to 70% of basophils. These results, obtained by using one of the same clones we have used in our study, are difficult to reconcile with our own findings. Singularly, we have rigorously demonstrated the specificity of our antibody by blocking IL-4 staining with unlabeled anti-IL-4 mAb (Fig 1, *F* and *G*).[20]

Previously, it has been shown by immunocytochemistry that 10% of basophils express IL-4 after ionomycin stimulation,[24] suggesting a restricted expression of IL-4 within a subset of basophils, which is reminiscent of the situation found in T cells[20,39] and mast cells.[40] In contrast, we found that the vast majority of basophils were capable of IL-4 and IL-13 expression (Fig 1). This leads us to conclude that, unlike T cells, all or nearly all basophils have the capacity for IL-4 and IL-13 expression. The similari-

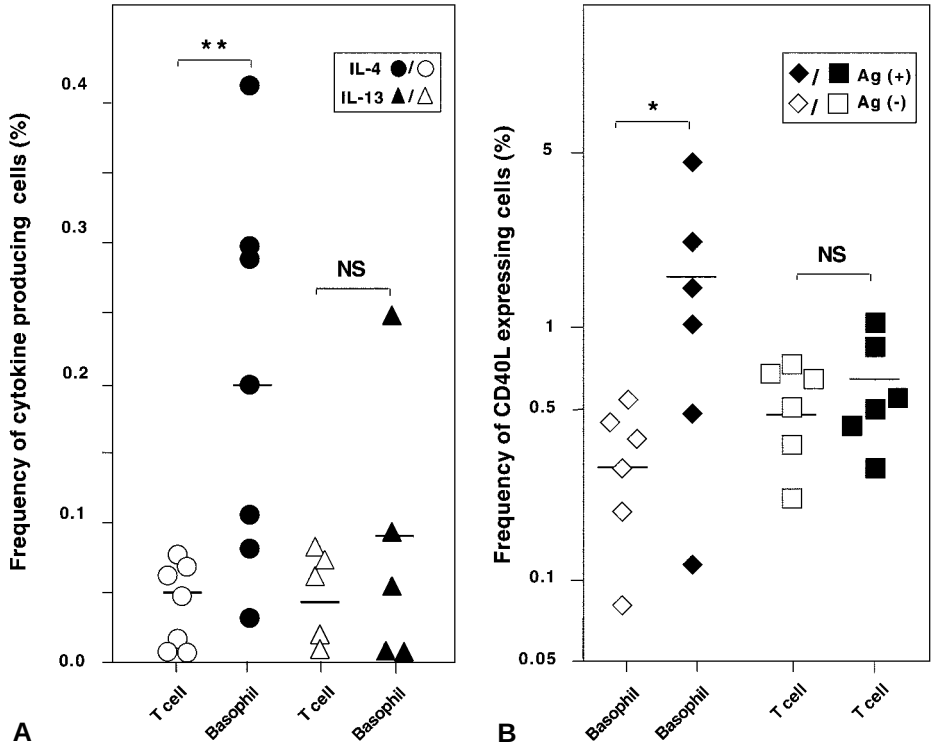


FIG 5. Cytokine and CD40L expression by basophils and T cells after Fel d 1 activation. PBMCs from donors with cat allergy were activated with Fel d 1 and analyzed as per Fig 4. **A,** The frequencies of IL-4 (*circles*, 7 donors) and IL-13 (*triangles*, 5 donors) expressing basophils *(filled symbols)* and T cells *(open symbols)* were determined as a function of the total number of PBMCs. **B,** The frequency of basophils *(diamonds)* and T cells *(squares)* expressing CD40L after media *(open symbols)* or Fel d 1 *(filled symbols)* activation were determined as a function of the total number of basophils and T cells, respectively (n = 6). Statistically significant differences are noted by a single asterisk ($P < .05$) and double asterisks ($P < .01$).

ty between atopic and control basophil responses reflects the universal capacity of basophils to produce both IL-4 and IL- 13 independent of disease status.

In sensitive individuals antigen activation yielded 10% to 20% of basophils immunostaining for cytokine, despite careful titrations of antigen dose and duration of activation (Fig 2). This may represent a kinetic effect in which antigen activation yields more transient IL-4 expression, resulting in only a fraction of cytokine-expressing cells staining at any one time point. Although brefeldin A has been demonstrated to have no effect on cytokine mRNA expression or overall protein synthesis in basophilic cell lines,[41] its effect on fresh ex vivo basophils has not been studied.

We have shown that after antigen activation, IL-4 and IL-13 expression by basophils have different kinetics (Fig 3). This rapid de novo expression of IL-4, as detected by intracellular staining, is earlier than previous reports that used ELISA[3,23,42] and suggests that this unique feature of basophil cytokine release may contribute to the initiation and amplification of allergic inflammation. Conversely, the delayed expression of IL-13 by basophils may suggest a role in the maintenance of allergic inflammation. In contrast to these results, after antigen stimulation we have not been able to detect T cell IL-4 expression until 6 hours (data not shown).

The production of IL-4 after primary antigen stimulation of PBMCs has generally been taken as a measure of T-cell activation.[43] Our results graphically demonstrate the flaw in attributing PBMC IL-4 production solely to T_{H2} cells (Figs 3 to 5). The intracellular cytokine detection by our flow cytometric method provides a direct and specific assessment of cytokine expression from basophils, in which the relative proportions of T cells and basophils remain intact. Despite the fact that T cells outnumber basophils 30:1 in PBMCs, basophils were consistently responsible for 80% or more of the IL-4–producing cells. This divergent result is due to the fact that 10% to 20% of basophils express IL-4 after antigen activation, whereas only 1:1000 to 5000 T cells are antigen specific. These results underscore a fundamental difference between activation by the clonally distributed T-cell receptor and that of FcεRI. Cells activated by means of the latter mechanism, such as mast cells and basophils, despite their numerical scarcity may contribute significantly to local cytokine production. IL-13 expression was 2-fold more common in basophils than in T cells, but this difference was not statistically significant.

The basophil-T cell comparative cultures quantitate cytokine production almost directly ex vivo, thereby bypassing artifacts of in vitro clonal expansion and T_{H2} cell differentiation that may be a consequence of longer term cell cultures. Such short-term T-cell stimulations have proven to be a sensitive method to measure antigen-specific memory T-cell cytokine production[43,44] and compare favorably with tetramer staining.[45] A limitation of these comparative cultures is that the optimal conditions for activation of basophil cytokine production is different from that of T cells. In specific, basophil IL-4 production was optimal at lower Fel d 1 concentrations (≤0.1 μg/mL vs ≥2 μg/mL for T cells) and earlier time points (2 hours vs 6 hours for T cells; data not shown). Despite the fact that conditions optimal for T cell, but not basophil, activation were used in these comparative cultures, basophils still accounted for 4 times as many IL-4–producing cells, suggesting that these results may underestimate the contribution of basophils.

CD40L has been shown to be constitutively expressed by both the KU812 basophilic cell line and by freshly isolated blood basophils.[46] However, the techniques used did not allow for quantitation of CD40L expression on the latter population. Our results, obtained by using flow cytometry, allow the quantitative assessment of CD40L expression and confirm the constitutive low level of CD40L expression on unstimulated blood basophils. CD40L expression is upregulated on KU812 cells after activation with phorbol myristate acetate-ionomycin.[32] Our results (Fig 5, *B*) extend these findings by demonstrating a uniquely rapid upregulation of basophil CD40L detectable 2 hours after antigen activation. These results strengthen the putative role of basophils in Ig switching and IgE synthesis.

In summary, the data presented in this article demonstrate that most, if not all, basophils are capable of producing IL-4 and IL-13. This production is observed as early as 2 hours and can be induced by antigen. Basophils were responsible for the overwhelming majority of early IL-4–producing cells in PBMCs. These data combined with the demonstration of CD40L expression by basophils add weight to the conclusion that human basophils play a major role in the regulation of allergic inflammation.

REFERENCES

1. Brunner T, Heusser CH, Dahinden CA. Human peripheral blood basophils primed by interleukin-3 (IL-3) produce IL-4 in response to immunoglobulin E receptor stimulation. J Exp Med 1993;177:605-11.
2. Ochensberger B, Daepp GC, Rihs S, Dahinden CA. Human blood basophils produce interleukin-13 in response to IgE-receptor-dependent and -independent activation. Blood 1996;88:3028-37.
3. Li H, Sim TC, Alam R. IL-13 released by and localized in human basophils. J Immunol 1996;156:4833-8.
4. Gauchat JF, Aversa G, Gascan H, De Vries JE. Modulation of IL-4 induced germline epsilon RNA synthesis in human B cells by tumor necrosis factor-alpha, anti-CD40 monoclonal antibodies or transforming growth factor-beta correlates with levels of IgE production. Int Immunol 1992;4:397-406.
5. Jabara H, Fu SM, Geha S, Vercelli D. CD40 and IgE: synergism between anti-CD40 monoclonal antibody and interleukin-4 in the induction of IgE synthesis by highly purified human B cells. J Exp Med 1990;172:1861-4.
6. Punnonen J, Aversa G, Cocks BG, et al. Interleukin-13 induces interleukin-4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. Proc Natl Acad Sci USA 1993;90:3730-4.
7. Pene J, Rousset F, Briere F, et al. IgE production by normal human lymphocytes is induced by interleukin-4 and suppressed by interferons gamma and alpha and prostaglandin E2. Proc Natl Acad Sci USA 1988;85:6880-4.
8. Bochner BS, Klunk DA, Sterbinsky SA, Coffman RL, Schleimer RP. IL-13 selectively induces vascular cell adhesion molecule-1 expression in human endothelial cells. J Immunol 1995;154:799-803.
9. Schleimer RP, Sterbinsky SA, Kaiser J, et al. IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM- 1. J Immunol 1992;148:1086-92.
10. Abehsira-Amar O, Gibert M, Joly M, Theze J, Jankovic DJ. IL-4 plays a dominant role in the differential development of ThO into Th 1 and Th2 cells. J Immunol 1992;148:3820-9.
11. Schroeder JT, MacGlashan DW Jr, Kagey-Sobotka A, White JM, Lichtenstein LM. IgE-dependent IL-4 secretion by human basophils. The relationship between cytokine production and histamine release in mixed leukocyte cultures. J Immunol 1994;153:1808-17.
12. Arock M, Merle-Beral H, Dugas B, et al. IL-4 release by human leukemic and activated normal basophils. J Immunol 1993;151:1441-7.
13. Redrup AC, Howard BP, MacGlashan DW, Kagey-Sobotka A, Lichtenstein LM, Schroeder JT. Differential regulation of IL-4 and IL- 13 secretion by human basophils: their relationship to histamine release in mixed leukocyte cultures. J Immunol 1998;160:1957-64.
14. Takahashi K, Takata M, Suwaki T, et al. New flow cytometry method for surface phenotyping basophils from peripheral blood. J Immunol Methods 1993;162:17-21.
15. Bochner BS, McKelvey AA, Schleimer RP, Hildreth JE, MacGlashan DW. Flow cytometric methods for the analysis of human basophil surface antigens and viability. J Immunol Methods 1989;125:265-71.
16. Grattan CE, Walpole D, Francis DM, et al. Flow cytometric analysis of basophil numbers in chronic urticaria: basopenia is related to serum histamine releasing activity. Clin Exp Allergy 1997;27:1417-24.
17. Andersson U, Hallden G, Persson U, Hed J, Moller G, DeLey M. Enumeration of IFN-gamma-producing cells by flow cytometry. Comparison with fluorescence microscopy. J Immunol Methods 1988;112:139-42.
18. Jung T, Schauer U, Heusser C, Neumann C, Rieger C. Detection of intracellular cytokines by flow cytometry. J Immunol Methods 1993;159:197-207.
19. Warner JA, Reshef A, Macglashan DW. A rapid Percoll technique for the purification of human basophils. J Immunol Methods 1987;105:107-10.
20. Prussin C, Metcalfe DD. Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. J Immunol Methods 1995;188:117-28.
21. Olweus J, BitMansour A, Warnke R, et al. Dendritic cell ontogeny: a human dendritic cell lineage of myeloid origin. Proc Natl Acad Sci USA 1997;94:12551-6.
22. Maurer D, Fiebiger E, Ebner C, et al. Peripheral blood dendritic cells express FceRI as a complex composed of FceRIa- and FcRIg-chains and can use this receptor for IgE-mediated allergen presentation. J Immunol 1996;157:607-16.
23. Gibbs BF, Haas H, Falcone FH, et al. Purified human peripheral blood basophils release interleukin-13 and preformed interleukin-4 following immunological activation. Eur J Immunol 1996;26:2493-8.
24. Mueller RC, Heusser H, Rihs S, Brunner T, Bullock GR, Dahinden CA. Immunolocalization of intracellular interleukin-4 in normal human peripheral blood basophils. Eur J Immunol 1994;24:2935-40.
25. Prussin C. Cytokine flow cytometry: understanding cytokine biology at the single cell level. J Clin Immunol 1997;17:195-204.
26. Bradding P, Feather IH, Howarth PH, et al. Interleukin 4 is localized to and released by human mast cells. J Exp Med 1992;176:1381-6.
27. Nonanka M, Nonaka R, Woolley K, et al. Distinct immunohistochemical localization of IL-4 in human inflamed airway tissues. IL-4 is localized to eosinophils *in vivo* and is released by peripheral blood eosinophils. J Immunol 1995;155:3234-44.
28. Falcone FH, Dahinden CA, Gibbs BF, et al. Human basophils release interleukin-4 after stimulation with *Schistosoma mansoni* egg antigen. Eur J Immunol 1996;26:1147-55.
29. Paul WE. Cytokines. Poking holes in the network. Nature 1992;357:16-7.

30. Charlesworth EN, Hood AF, Soter NA, Kagey-Sobotka A, Norman PS, Lichtenstein LM. Cutaneous late-phase response to allergen. Mediator release and inflammatory cell infiltration. J Clin Invest 1989;83:1519-26.
31. Guo CB, Liu MC, Galli SJ, Bochner BS, Kagey-Sobotka A, Lichtenstein LM. Identification of IgE-bearing cells in the late-phase response to antigen in the lung as basophils. Am J Respir Cell Mol Biol 1994;10:384-90.
32. Gauchat JF, Henchoz S, Mazzei G, et al. Induction of human IgE synthesis in B cells by mast cells and basophils. Nature 1993;365:340-3.
33. Yanagihara Y, Kajiwara K, Basaki Y, Ikizawa K, Akiyama K, Saito H. Induction of human IgE synthesis in B cells by a basophilic cell line, KU812. Clin Exp Immunol 1997;108:295-301.
34. Maurer D, Fiebiger E, Ebner C, et al. Expression of functional high affinity IgE receptors (FcεRI) on monocytes of atopic individuals. J Exp Med 1994;179:745-50.
35. Gounni AS, Lamkhioued B, Ochiai K, et al. High affinity IgE receptor on eosinophils is involved in defense against parasites. Nature 1994;367:183-6.
36. Gopinath R, Nutman TB. Identification of eosinophils in lysed whole blood using side scatter and CD 16 negativity. Cytometry 1997;30:313-6.
37. Zhu X, Hamman KJ, Munoz NM, et al. Intracellular expression of FcεRI-II (CD16) and its mobilization by chemoattractants in human eosinophils. J Immunol 1998;161:2574-9.
38. Kon OM, Sihra BS, Till SJ, Corrigan CJ, Kay AB, Grant JA. Unstimulated basophils in atopic and nonatopic subjects express intracellular interleukin-4: detection by flow cytometry. Allergy 1998;53:891-6.
39. Elson LH, Nutman TB, Metcalfe DD, Prussin C. Flow cytometric analysis for cytokine production identifies T helper 1, T helper 2, and T helper 0 cells within the human CD4+CD27- lymphocyte subpopulation. J Immunol 1995;154:4294-301.
40. Bradding P, Okayama Y, Howarth PH, Church MK, Holgate ST. Heterogeneity of human mast cells based on cytokine content. J Immunol 1995;155:297-307.
41. Zhu FG, Gomi K, Marshall JS. Short-term and long-term cytokine release by mouse bone marrow mast cells and the differentiated KU-812 cell line are inhibited by brefeldin A. J Immunol 1998;161:2541-51.
42. De Vries JE, Zurawski G. Immunoregulatory properties of IL-13: its potential role in atopic disease. Int Arch Allergy Immunol 1995;106:175-9.
43. Imada M, Simons FE, Jay FT, Hayglass KT. Allergen-stimulated interleukin-4 and interferon-gamma production in primary culture: responses of subjects with allergic rhinitis and normal controls. Immunology 1995;85:373-80.
44. Waldrop SL, Pitcher CJ, Peterson DM, Maino VC, Picker LJ. Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: evidence for a novel antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. J Clin Invest 1997;99:1739-50.
45. Komanduri KV, Viswanathan MN, Wieder ED, et al. Restoration of cytomegalovirus-specific CD4+ T-lymphocyte responses after ganciclovir and highly active antiretroviral therapy in individuals infected with HIV-1. Nat Med 1998;4:953-6.
46. Murali-Krishna K, Altman JD, Suresh M, et al. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. Immunity 1998;8:177-87.

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