Eotaxin Potentiates Antigen-Dependent Basophil IL-4 Production

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Basophils are a major source of IL-4, which is a critical factor in the generation of allergic inflammation. Eotaxin induces chemotaxis mediated through the CC chemokine receptor 3 (CCR3) present on basophils as well as eosinophils and Th2 cells, thereby promoting cell recruitment. To determine whether eotaxin has other proinflammatory activity, we examined the effect of eotaxin on basophil IL-4 expression by flow cytometry. Eotaxin alone had no effect on basophil IL-4 production, but further increased allergen-stimulated IL-4 expression. Eotaxin also enhanced IL-4 release from purified basophils 2- to 4-fold, as determined by ELISA (p < 0.01). Addition of eotaxin to cultures resulted in a 40-fold left shift in the dose response to Ag. This effect was obtained with physiologic concentrations of eotaxin (10 ng/ml), was abrogated by an Ab to the CCR3 receptor, and was noted with other chemokine ligands of CCR3. Additionally, eotaxin augmented IL-3 priming of basophil IL-4 production in a synergistic manner (p < 0.01). In contrast, no priming was observed with either IL-5 or GM-CSF. These results establish a novel function for eotaxin and other chemokine ligands of CCR3: the potentiating effect of Ag-mediated IL-4 production in basophils, and suggest a potential nonchemotactic role for CC chemokines in the pathogenesis and amplification of inflammation. The Journal of Immunology, 1999, 163: 2877–2882.

Materials and Methods

Reagents

DNase I (Calbiochem, La Jolla, CA); brefeldin A, paraformaldehyde, and DMSO (Sigma, St. Louis, MO); saponin (Fluka Biochemika, Ronkonkoma, NY); standardized cat hair (10,000 biological allergy units (BAU)/ml) allergic extracts (Bayer, Elkhart, IN); and recombinant human IL-3, IL-5, GM-CSF, eotaxin, eotaxin-2, RANTES, and MCP-2, -3, and -4 (Peprotech, Rocky Hill, NJ) were purchased.

Monoclonal Abs

Anti-CD2, CD19 PE/cyanin 5, anti-IL-4 PE (clone MP4-25D2), allopseudocytin-conjugated streptavidin, rat IgG1 PE, mouse IgG2a (PharMingen, San Diego, CA); anti-CD14 and CD16 PE/cyanin 5 (Caltag Laboratories, Burlingame, CA); anti-CXCR3 (R&D Systems, Minneapolis, MN); and goat anti-human IgE biotin (Biosource, Camarillo, CA) were obtained commercially. Anti-CCR3 (clone 7B11) was a gift of Dr. Paul Ponath (Leukosite, Cambridge, MA).

Subjects

Allergic asthmatic subjects had a greater than 1-yr history of symptoms consistent with asthma, a methacholine PC20 <25 mg/ml, and three or more positive aeroallergen skin tests (>5 mm of induration), including cat, of a panel of nine tested. The clinical protocol was approved by the National Institute of Allergy and Infectious Diseases Institutional Review Board. All subjects signed informed consent.

Cell culture

PBMC were obtained from EDTA-anticoagulated whole blood processed over a 1.080 g/cm3 Percoll gradient. PBMC were suspended in RPMI 1640 (Life Technologies, Grand Island, NY), supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 25 µg/ml gentamicin, 50 µM 2-ME, 1 mM pyruvate, and nonessential amino acids (Biofluids, Rockville, MD). Cells were incubated at 2 x 10^6 PBMC/ml in 24-well plates (Costar, Cambridge, MA), in the presence of 10 µg/ml brefeldin A. Basophil stimulation was performed using cat allergen (10 BAU/ml, except where noted), with or without eotaxin (10 ng/ml). Other chemokines (eotaxin-2, RANTES, MCP-3, 3, 4) were used with identical concentration. Where indicated, PBMC were preincubated with anti-CCR3, anti-CXCR3 mAbs (10 µg/ml), or rIL-3, rIL-5, rGM-CSF (50 ng/ml), for 20 min at 37°C. After 4 h, 50 µg/ml DNase I was added to each well, cells were incubated for 5 min, washed, fixed in 4% paraformaldehyde at room temperature for 5 min, and washed (16).
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Basophil purification

When indicated, peripheral leukocytes were separated over a discontinuous Percoll gradient (1.080 and 1.069 g/cm³), at 400 × g for 25 min at room temperature (17). The basophil-enriched interphase was harvested, washed twice, and further purified by negative selection using Ab-coated paramagnetic beads (anti-CD2, CD7, CD14, CD15, CD16, CD36, CD45RO, and anti-HLA-DR) (Miltenyi Biotec, Bergisch Gladbach, Germany) (18). The final cell suspensions consisted of 30–50% basophils.

Measurement of IL-4 release

After purification, Ag-activated basophils were cultured in 96-well plates (Costar), at a density of 500,000 cells/well, with or without eotaxin (10 ng/ml), in absence of brefeldin A. After 4-h incubation, the cultures were harvested and centrifuged, and the supernatant was removed. Cell-free supernatants (stored at −80°C) were then assayed for IL-4 by ELISA (R&D Systems). The assay has a sensitivity of 10 pg/ml.

Flow cytometry

Cells were stained for surface cell markers and intracellular cytokines, as described (16). Briefly, the cell pellets were resuspended in PBS with 0.1% saponin and 5% nonfat dry milk (PBS-S-milk) for 1 h to block nonspecific binding, and stained with mAbs at 4°C for 30 min. The cells were washed twice and stained with streptavidin allophycocyanin for an additional 30 min, washed, and analyzed.

Acquisition and analysis

Data were acquired with a two-laser FACSCALIBUR flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA), which allowed the simultaneous detection of four fluorescent parameters. Typically, 300,000 total events were acquired. List mode files were analyzed using Cellquest software (Becton Dickinson Immunocytometry Systems).

Statistical analysis

Final data were expressed as the mean ± SD. The paired t test was performed to compare differences of cytokine expression. A p value of <0.05 was considered as significant.

Results

Eotaxin augments Ag-dependent basophil IL-4 production

Prior studies have reported that eotaxin is chemotactic for basophils, and at high concentrations (1 µg/ml) releases histamine and generates leukotrienes in these cells (11, 19). We have recently shown that basophils are the predominant cellular source of IL-4 in PBMC following allergen activation in vitro (2). We thus sought to determine whether eotaxin modulates this basophil IL-4 production. PBMC were activated with cat Ag extract and eotaxin (10 ng/ml), and basophil IL-4 production was detected using flow cytometry by gating on IgEhigh cells that were negative for a panel of markers. The assay has a sensitivity of 10 pg/ml.

FIGURE 1. Eotaxin augments Ag-specific basophil IL-4 production. A, PBMC were incubated in the absence (a and c) or presence (b and d) of eotaxin (10 ng/ml) and in the absence (a and b) or presence (c and d) of cat Ag for 4 h, then stained for IgE, lineage markers (CD2, -14, -16, -19), and IL-4, and analyzed by flow cytometry. The frequency of IL-4-producing basophils was determined by gating on the IgEhigh , lineage− population. B, Basophil IL-4 production, as determined by flow cytometry. Mean ± SD of 12 donors is shown, and results are expressed as the percentage of maximal IL-4 expression. The mean maximal IL-4 production (100%) was 21.8%. C, Purified basophils were incubated with or without eotaxin (10 ng/ml) and in the absence or presence of cat Ag, for 4 h. The frequency of IL-4-producing basophils was determined by flow cytometry. Mean ± SD of five donors is shown, and results are expressed as the percentage of maximal IL-4 expression. The mean maximal IL-4 production was 17%. D, Basophil IL-4 release into the supernatant was analyzed by ELISA. Mean ± SD of three independent experiments using three donors is shown. Each experiment was performed in triplicate. Statistically significant differences are presented in each panel.

Eotaxin shifts the dose response to Ag

We next examined the effect of eotaxin on Ag dose response (Fig. 3). In Ag-alone cultures, we determined the Ag concentration necessary to obtain 50% of the maximal IL-4 expression, and then calculated the Ag concentration required to produce the same level of IL-4 expression in presence of eotaxin. In the six donors examined, eotaxin caused an approximate 40-fold shift (range 5.2- to 466-fold, geometric mean 39, median 43) in dose response toward a lower Ag concentration (p < 0.05). Additionally, for all donors,
Eotaxin augmentation of IL-4 production is mediated through CCR3

Eotaxin-induced chemotaxis, histamine release, and eicosanoid generation in basophils are mediated exclusively through CCR3 and are abrogated by the use of the 7B11 anti-CCR3 mAb (11). We performed blocking experiments using 7B11 to verify that the augmentation by eotaxin was specific and mediated via CCR3. The addition of anti-CCR3 mAb completely abrogated eotaxin augmentation of basophil IL-4 production (data not shown). Alternatively, the addition of either the irrelevant blocking mAb anti-CXCR3 or an isotype-matched control mAb was not associated with a significant inhibition of the eotaxin effect. These results demonstrate that the eotaxin augmentation of IL-4 expression is specific and exclusively mediated through the CCR3 receptor.

In addition to eotaxin, a number of CC chemokines (MCP-2, 3, 4, RANTES, and eotaxin-2) act on basophils through CCR-3, promoting both chemotaxis and inflammatory mediator release (11, 21, 22). We hypothesized that these additional CC chemokines, acting via CCR3, would similarly augment basophil IL-4 production. Ag-activated PBMC were thus incubated with the above chemokines, and the frequencies of IL-4-producing basophils were determined. All of the chemokines tested significantly increased Ag-specific IL-4 expression when compared with Ag activation alone (p < 0.01) (Fig. 4B). When examined in the absence of Ag activation, none of the CC chemokines tested had any effect on basophil IL-4 production (data not shown).

FIGURE 2. Concentration dependence of eotaxin activity. PBMC were incubated with (■) or without (□) Ag, and a range of eotaxin concentrations (0–50 μg/ml), and IL-4 production was assessed by flow cytometry. Mean ± SD of three independent experiments using three donors are shown, and results are expressed as the percentage of maximal IL-4 expression. The mean maximal IL-4 production was 16%. Statistically significant differences are noted: *, p < 0.05.

FIGURE 3. Eotaxin shifts the Ag dose response of basophils. PBMC from six allergic asthmatic subjects were incubated with (■) or without (○) eotaxin, and the noted concentrations of Ag. IL-4 production was analyzed by flow cytometry, and the results were expressed as the percentage of maximal IL-4 response. The number shown in the parenthesis is the calculated fold shift in the Ag dose response. This was determined by taking the ratio of the Ag concentration required to produce a 50% maximal IL-4 response in the noneotaxin-treated cultures (right arrowhead) over the Ag concentration required to generate that same absolute IL-4 response in the eotaxin-treated cultures (left arrowhead). The maximal response was 11.2%, 8.1%, 19.4%, 28.1%, 22.5%, and 13.1% for patients 1–6, respectively.
Eotaxin synergizes with IL-3 to augment Ag-dependent IL-4 production

IL-3, IL-5, and GM-CSF all prime basophils for histamine release and leukotriene production (23, 24), whereas only IL-3 has a clear role in priming basophils for IL-4 production (17, 20). Additionally, high concentrations of eotaxin (1 μg/ml) synergize with IL-3 to augment basophil histamine release in a non-Ag-dependent manner (11). To determine whether eotaxin also enhances cytokine priming of basophil IL-4 production, PBMC from three allergic asthmatic subjects were preincubated with IL-3, IL-5, or GM-CSF, and activated with cat Ag and eotaxin. Eotaxin (10 ng/ml) augmented Ag-mediated basophil IL-4 production by 3-fold \( (p < 0.05) \), whereas the addition of IL-3 alone increased Ag-mediated basophil IL-4 production by 1.5-fold (Fig. 5). The simultaneous addition of both eotaxin and IL-3 resulted in a 5-fold increase in IL-4 production \( (p < 0.01) \). In contrast, neither IL-5 nor GM-CSF, either alone or in combination with eotaxin, had any effect on IL-4 expression. Thus, the combination of eotaxin and IL-3 uniquely increased Ag-driven basophil IL-4 production in a synergistic manner. This synergy was Ag dependent and significant at higher Ag concentrations.

Discussion

We have demonstrated by flow cytometry (Fig. 1, A–C) that eotaxin enhances Ag-driven basophil IL-4 expression, and by ELISA (Fig. 1D) that it enhances IL-4 release. This effect is not influenced by the level of basophil purity (Fig. 1), leading us to conclude that basophils are the direct target of eotaxin. This effect is specifically mediated via the CCR3 receptor (Fig. 4A). Eotaxin both increases the frequency of IL-4–producing cells and shifts the Ag dose-response curve such that lower concentrations of Ag result in IL-4 production (Fig. 2). Recently, it has been demonstrated that IL-4 induces the expression of eotaxin in dermal and lung fibroblasts (25, 26). Taken together with our results, these findings suggest a positive feedback loop, in which both eotaxin and IL-4 act to enhance each other’s expression, thereby providing a mechanism to amplify Ag-initiated inflammation.

Although the best-characterized effect of chemokines is chemotaxis, there is precedent for chemokines acting as modulators of cytokine expression. Previous studies have demonstrated that after TCR activation, macrophage-inflammatory protein-1α and MCP-1 differentially augment either Th1 or Th2 cytokine production, respectively (27, 28). It is unclear whether these results truly represent a direct effect of chemokine upon T cell cytokine production or are simply the result of priming and differentiation of naive T cells during the 48-h culture. Our results, obtained after only 4 h of in vitro culture, suggest that chemokines can directly and rapidly augment Ag-induced basophil cytokine production. These findings suggest a critical interplay between chemokines and immunoregulatory cytokines, in which Ag-specific cytokine responses are highly dependent upon the surrounding chemokine milieu.

Consistent with previous studies (17, 20), IL-3 was found to prime Ag-induced basophil IL-4 production, whereas IL-5 and GM-CSF had no activity, either alone or in combination with eotaxin. Additionally, the magnitude of enhancement attributable to eotaxin was consistently of a greater magnitude than that of IL-3. These results further underscore the importance of eotaxin augmentation of basophil cytokine production and suggest that eotaxin and IL-3 may work in concert.

Four points underscore the relevance of these findings to clinical allergic diseases and asthma. First, the eotaxin concentrations required to obtain maximal cytokine augmentation (1–10 ng/ml) are similar to that encountered in vivo (15, 29) and notably are 1/100\(^{th}\)-1/500\(^{th}\) of that required for in vitro histamine release and leukotriene generation (11, 30). Second, basophil IL-4 augmentation by eotaxin uniquely requires FcεRI (high affinity IgE receptor)-mediated...
Eotaxin synergizes with IL-3 to augment Ag-dependent IL-4 production. PBMC were cultured with media (open symbols), or rhIL-3 (A), rhIL-5 (B), rhGM-CSF (C) (filled symbols) for 20 min, then activated with Ag (0–10 BAU/ml) in the presence (circles) or absence (squares) of eotaxin. IL-4 production was analyzed by flow cytometry, and the final results are expressed as the percentage of maximal IL-4 expression, in the eotaxin group (C). Mean ± SD from three independent experiments in three donors is shown. The mean maximal expression in the eotaxin group was 13.7%, 15.5%, and 16.7% for A, B, and C, respectively. Statistically significant differences are noted: *, p < 0.05; **, p < 0.01.

basophil activation and is not mediated by eotaxin alone. Thus, eotaxin induces IL-4 production only in the context of allergic disease and Ag activation. Third, eotaxin significantly lowers the threshold for basophil activation and IL-4 production by 40-fold, presumably resulting in the generation of allergic inflammation at lower levels of Ag exposure. This suggests that local eotaxin concentrations may have profound effects on IL-4 production and the consequent development of allergic inflammation. Fourth, IL-3, which is also present at inflammatory sites (31), synergistically augmented the eotaxin effect (Fig. 5).

These results establish a novel nonchemoattractant role for eotaxin: the potentiation of IL-4 expression, and suggest that eotaxin may generate and amplify allergic inflammation through multiple effector activities. The demonstration that other CC chemokines show a similar activity (Fig. 4B) reinforces the conclusion that chemokines play a significant role in the pathogenesis of allergic diseases. These results are also consistent with the assumption that chemokine antagonists may have therapeutic anti-inflammatory activity by decreasing cytokine expression in addition to their direct antichemoatctic effects.

Acknowledgments

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References


