Cutting Edge: Eotaxin Elicits Rapid Vesicular Transport-Mediated Release of Preformed IL-4 from Human Eosinophils

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Eosinophils, prominent in Th2-driven immune responses, including asthma and allergic and parasitic diseases, may have multiple roles in these diseases. As effector cells eosinophils, based in part on their release of cationic granule proteins and lipid mediators, may contribute to the immunopathogenesis of allergic diseases (1). Additional functional roles for eosinophils are indicated by findings that eosinophils may exert immunomodulatory activities via interactions with T and B lymphocytes. For these potential interactions with lymphocytes, human eosinophils express costimulatory surface proteins, including CD40, CD28, CD86, and MHC class II Ags (2–4), and produce over two dozen cytokines, including the prototypical Th2 cytokine IL-4 (5).

Like eosinophils, IL-4 is a hallmark of allergic and parasitic disorders. IL-4 contributes to the polarization toward Th2 differentiation and promotes IgE class switching (6, 7). The actions of IL-4 are not limited to the initiation of Th2 responses, but also may stimulate other cellular responses that contribute to manifestations of allergic diseases (6). Potential cellular sources of IL-4 include CD4+ T cells (7), mast cells (8), basophils (9), NK1.1+ T cells (10), γδ T cells (11), and eosinophils (12–14). Within eosinophils, unlike CD4+ T cells, IL-4 is stored as a preformed pool within eosinophil-specific granules (13–17). A role for eosinophil-derived IL-4 has been demonstrated in a murine system in which the i.p. instillation of *Schistosoma mansoni* eggs led to the enhanced generation over 12 h of IL-4 derived from peritoneal exudate eosinophils (12). Because nothing is known about how IL-4 within human eosinophils might be mobilized, we have investigated stimuli and mechanisms that lead to the extracellular release of IL-4 stored within normal donor-derived eosinophils.

**Materials and Methods**

**Purification of human eosinophils**

Eosinophils were isolated from the blood of 18 healthy nonatopic donors by negative selection using the MACS anti-CD16 immunomagnetic bead procedure (Miltenyi Biotec, Auburn, CA) (18). Eosinophil purity and viability was >99% and >95%, respectively.

**Detection of intracellular IL-4**

Intracellular IL-4 was analyzed by 1) immunofluorescence microscopy of cytospin preparations of paraformaldehyde-fixed/saponin-permeabilized eosinophils stained with Alexa546-labeled (Molecular Probes, Eugene, OR) anti-IL-4 mAb (clone 3010.211) or isotype control mouse IgG1 (both obtained from R&D Systems, Minneapolis, MN) and viewed with a TE300 Nikon fluorescence microscope; 2) flow cytometry (FACScan, CellQuest software; BD Biosciences, Mountain View, CA) of paraformaldehyde-fixed/saponin-permeabilized eosinophils stained with PE-conjugated anti-IL-4 mAb (clone 3010.211) or isotype control mouse IgG1 (both obtained from BD PharMingen, San Diego, CA); and 3) ELISA (R&D kit) of post-nuclear supernatants (14,000 × g for 20 min) of eosinophil lysates (in 1 mM DTT, 1 mM EDTA, 0.1% SDS and 1% Nonidet P-40, pH 7.5, 150 mM NaCl, and 20 mM HEPES with protease inhibitors).
ELISA for detection of eosinophil-released IL-4

Eosinophils (2 × 10⁶ cells in 1 ml) were incubated for 1 h (37°C) with chemokines (R&D Systems) or A23187 (Sigma, St. Louis, MO) in RPMI 1640 medium containing 0.1% OVA. Eosinophil supernatant IL-4 was measured by ELISA (sensitivity 10 pg/ml; R&D Systems).

EliCell assay for the detection of eosinophil-released IL-4

The EliCell assay, a gel-phase dual Ab capture and detection assay based on microscopic observations of individual viable cells, was performed as detailed (18) to enumerate the proportion of eosinophils releasing IL-4 and to electronically quantitate the average relative amounts of IL-4 released extracellularly. A biotinylated goat polyclonal anti-IL-4 Ab (20 μg/ml; R&D Systems) was used as capturing Ab and an Alexa546-labeled anti-IL-4 mAb (R&D Systems) was used (400 μl of 10 μg/ml) to detect released IL-4. Alexa546-labeled mouse IgG1 was included as a nonimmune isotype control. An irrelevant biotinylated capture Ab was substituted in combination with the Alexa546-labeled anti-IL-4 detection Ab to ascertain that 1) cell permeabilization had not allowed detection of intracellular IL-4; and 2) retention of surface-released IL-4 was dependent on the immobilized anti-IL-4 capturing Ab.

In some experiments, eosinophils were pretreated (37°C) for 30 min with specific inhibitors: 1) a neutralizing anti-CCR3 mAb (clone 61828.111) or isotype-matched control rat IgG (both 10 μg/ml; R&D Systems); 2) pertussis toxin (10 or 100 ng/ml; Calbiochem, La Jolla, CA); 3) actinomycin D and cycloheximide (both at 1 and 10 μM; Calbiochem); or 4) brefeldin A (0.1 and 1 μg/ml; Biomol, Plymouth Meeting, PA). Statistical comparisons were made by ANOVA followed by Student Newman-Keuls 1 test with differences considered significant when p < 0.05.

Results and Discussion

IL-4, a major mediator in the induction and regulation of allergic and parasitic immune responses, is not produced exclusively by activated Th2 lymphocytes. Earlier studies established that human eosinophils synthesize and store IL-4 within their specific granules and suggested, based on immunocytochemistry, that there might be subpopulations of IL-4-positive and -negative eosinophils (13, 14). Our analyses of populations of eosinophils from normal donors extend these findings by demonstrating that all circulating eosinophils contained preformed IL-4, as evidenced by a uniformly positive, unimodal pattern of intracellular IL-4 immunoreactivity by flow cytometry (Fig. 1A, top) and by high level, granule-associated eosinophil anti-IL-4 immunostaining (94.3 ± 2.5% positive cells; mean ± SD, n = 4) by fluorescence microscopy (images not shown). By ELISA of cell lysates, eosinophils contained more IL-4 (32.3 ± 6.2 pg/2 × 10⁶ cells) than did PBMCs (2.3 ± 0.9 pg/2 × 10⁶ cells, means ± SEM, n = 7) from the same donors (Fig. 1A, bottom). Thus, in addition to the presence of IL-4 in tissue eosinophils in bronchial biopsies of asthmatic patients and

FIGURE 1. IL-5 enhances eotaxin-induced release of preformed IL-4 from eosinophils. A, Eosinophils from nonatopic donors contain preformed IL-4. Top, Flow cytometry histogram of intracellular IL-4 in saponin-permeabilized eosinophils, with Alexa546-labeled anti-IL-4 mAb (dashed line) and nonimmune IgG1 (solid line). No anti-IL-4 staining was detected with nonpermeabilized eosinophils (data not shown). Result is representative of findings from four donors. Bottom, ELISA-assayed quantities of preformed IL-4 in lysates of eosinophils and PBMCs from seven normal donors. Bars denote the ELISA-assayed quantities of preformed IL-4 in lysates of eosinophils and PBMCs from seven normal donors. Bars denote the mean ± SD from five donors. ∗ and ∗∗, p < 0.05 and p < 0.01, respectively, compared with unstimulated eosinophils.

B, EliCell assays of IL-4 released extracellularly from eosinophils (captured with a biotinylated anti-IL-4 Ab and detected with Alexa546-labeled anti-IL-4 mAb). Dose-responses of eotaxin-induced IL-4 release at 1 h with and without concomitant 2 nM IL-5 were expressed both as the average fluorescence intensities for immunoreactive IL-4 around 50 individual eosinophils (images not shown). In some experiments, eosinophils were pretreated (37°C) for 30 min with specific inhibitors: 1) a neutralizing anti-CCR3 mAb (clone 61828.111) or isotype-matched control rat IgG (both 10 μg/ml; R&D Systems); 2) pertussis toxin (10 or 100 ng/ml; Calbiochem, La Jolla, CA); 3) actinomycin D and cycloheximide (both at 1 and 10 μM; Calbiochem); or 4) brefeldin A (0.1 and 1 μg/ml; Biomol, Plymouth Meeting, PA). Statistical comparisons were made by ANOVA followed by Student Newman-Keuls 1 test with differences considered significant when p < 0.05.
in allergen-induced cutaneous late-phase reactions in atopic subjects (15, 19), human blood-derived eosinophils are a major potential source of IL-4.

Because eosinophils contain preformed IL-4, the mechanisms to mobilize IL-4 from specific granule storage sites for its extracellular release need to be defined. Prior studies established the reusability of IL-4 from eosinophils activated with either serum-coated beads, IgA immune complexes, or calcium ionophore A23187 (13, 14, 20), but these nonphysiological stimuli are not selective and may be uniformly exocytotic and/or cytolytic (21, 22). Indeed with our methods, A23187 activation of eosinophils led to the cytoplasmic release of IL-4. Not only were the amounts of immunoreactive IL-4 released in eosinophil supernatants following A23187 stimulation (Table I) equivalent to the total preformed IL-4 content of eosinophils (Fig. 1A, bottom), but microscopy of eosinophils in EliCell assays demonstrated extensive IL-4 immunostaining of eosinophils (Table I) that exhibited morphological signs of cell damage (data not shown, but as previously illustrated for RANTES staining in A23187 stimulated eosinophils; Ref. 18).

An alternative mechanism to either exocytosis or cytolysis for the release of eosinophil granule-derived proteins has been indicated by ultrastructural observations of tissue and blood eosinophils activated in vivo. At least for the major cationic protein components of eosinophil-specific granules, selective losses of the core or matrix components of the granules and other findings suggest that eosinophil-specific granule contents may be mobilized by selective incorporation into small vesicles that traffic to the cell surface and release these granule contents by a process of "piecemeal" degranulation based on vesicular transport (23, 24). Recently, Lacy and coworkers identified IFN-γ as a physiological stimulus that in vitro induces piecemeal release of RANTES, a chemokine also stored preformed in eosinophil-specific granules (25); we have established a microscopic assay, the EliCell assay, to study the piecemeal degranulation process in eosinophils (18).

We evaluated the capacity of several eosinophil agonists to elicit IL-4 release from eosinophils. The C-C chemokines, eotaxin and RANTES, did not elicit IL-4 release at levels detectable by ELISA of supernatant fluids (Table I), consistent with a recent report that these cytokines elicited ELISA-detectable "degranulation" only if eosinophils were pretreated with cytochalasin B (26). In contrast with the EliCell assay, both eotaxin and RANTES stimulated release of IL-4 detectable extracellularly (Table I). No IL-4 staining was found with unstimulated eosinophils or when the Alexa546-labeled anti-IL-4 detection Ab was replaced by an Alexa546-labeled isotype IgG1 nonimmune control. Moreover, no IL-4 was detectable when the biotinylated anti-IL-4 capture Ab (which was necessary to immobilize IL-4 at its extracellular sites of release; Ref. 18) was substituted with a biotinylated irrelevant control Ab (data not shown). The latter condition assured that neither intracellular nor membrane-bound IL-4 was being detected in the non-permeabilized eosinophils, and the punctate pattern of immunoreactive IL-4 released at discrete loci proximate to the cell surface (Fig. 1C) (as confirmed by confocal microscopy; data not shown) was compatible with a vesicular transport-mediated process of IL-4 release.

IL-8, which does not stimulate normal eosinophils (27), did not elicit IL-4 release (Table I). Of greater interest, IFN-γ, at a concentration that effectively elicited vesicular transport-mediated release of RANTES from eosinophils (18, 25), failed to elicit IL-4 release (Table I), suggesting that differential signaling may function to selectively mobilize at least the cytokine proteins stored preformed in eosinophil granules.

IL-5 by itself did not elicit detectable IL-4 release from eosinophils (Fig. 1B), but IL-5 did enhance eotaxin-stimulated mobilization of IL-4. The simultaneous addition of IL-5 with even low concentrations of eotaxin (0.06 and 0.6 nM) evoked release of IL-4 from significant proportions of eosinophils, whereas these eotaxin concentrations alone did not stimulate detectable IL-4 release (Fig. 1B). IL-5 in concert with higher concentrations of eotaxin (6 and 60 nM) doubled the number of eosinophils releasing IL-4 and the amounts of IL-4 released (Fig. 1B). IL-5 also accelerated eotaxin-induced IL-4 release from eosinophils. Even as early as 5 min, IL-5 in concert with eotaxin enhanced both the percentages of eosinophils releasing IL-4 and the quantities of IL-4 being released (Fig. 2A).

We next evaluated mechanisms underlying the IL-5/eotaxin-elicted release of IL-4 from eosinophils. The release of IL-4 from IL-5/eotaxin-stimulated eosinophils was mediated through the G protein-linked CCR3 chemokine receptor. Pretreatment of eosinophils with a neutralizing anti-CCR3 receptor mAb reduced the percentages of eosinophils releasing IL-4 (71 ± 5% inhibition vs 0% with a control Ab) and the amounts of IL-4 released (98 ± 2% inhibition vs 11% with a control Ab) (both n = 3; p < 0.05). Likewise, pertussis toxin pretreatment at 10 and 100 ng/ml inhibited eotaxin-elicted IL-4 release and reduced the percentages of eosinophils releasing IL-4 by 56 ± 10 and 89 ± 3%, respectively (both p < 0.05, April 13, 2017). Neither actinomycin D, an inhibitor of transcription, nor cycloheximide, a protein synthesis inhibitor, suppressed eosinophil IL-4 release induced by IL-5/eotaxin (Fig. 2B). Therefore, the mechanisms underlying IL-5/eotaxin-elicted IL-4 release are not likely to require either the new synthesis of IL-4 or of other proteins that contribute to the secretory process. In contrast, brefeldin A, an inhibitor of vesicle formation, substantially suppressed the quantities of IL-4 released extracellularly around eosinophils without diminishing the overall number of cells releasing IL-4 (Figs. 1C and 2B).

Table I. ELISA and EliCell assays of IL-4 released from human eosinophils

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Dose</th>
<th>n</th>
<th>EL-4 (pg/ml)</th>
<th>% Eosinophils releasing IL-4</th>
<th>IL-4 fluorescence intensity × 10^4/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>500 U/ml</td>
<td>4</td>
<td>1.6 ± 0.3</td>
<td>1 ± 1</td>
<td>0.0 ± 0</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>6.0 nM</td>
<td>4</td>
<td>0.9 ± 0.4</td>
<td>1 ± 1</td>
<td>0.0 ± 0</td>
</tr>
<tr>
<td>RANTES</td>
<td>6.2 nM</td>
<td>3</td>
<td>1.7 ± 0.1</td>
<td>0%</td>
<td>0.0 ± 0</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.5 μM</td>
<td>4</td>
<td>45.4 ± 12.5**</td>
<td>89 ± 5**</td>
<td>5.2 ± 0.7**</td>
</tr>
</tbody>
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* Eosinophils were incubated with stimuli for 1 h. ELISA assessed IL-4 in 1 ml supernatant from 2 × 10^6 eosinophils. EliCell assessed both the percentages of eosinophils exhibiting extracellular IL-4 and the average electronically measured immunofluorescent intensities of extracellular IL-4. Results are means ± SD. *, p < 0.01 and **, p < 0.001 compared with medium. n, Number of different donors.
Our findings indicate that chemokines acting via CCRI3-initiated signaling pathways can very rapidly mobilize preformed stores of IL-4 from within human eosinophils. The means of extracellular release was by noncytotoxic, vesicular transport as indicated by the microscopic patterns of focal cell surface IL-4 release, the absence of required new protein synthesis, and the inhibition by a vesicle formation inhibitor, brefeldin A. In support of this mechanism, we have localized the vesicle-associated membrane protein-2 by immunogold electron microscopy not only at vesicles within eosinophils but also at the outer membrane of eosinophil-specific granules (28), indicating the capacity of secretory vesicles to traffic from eosinophil granule membranes. Although released IL-4 concentrations in supernatant fluids were not sufficient to be detectable by conventional ELISA, local concentrations of released IL-4 may effectively stimulate responses in tissue sites of eosinophil localization. IL-4 released by eosinophils may augment effector actions of eosinophils, including enhancing the IL-4-dependent generation of airway mucous secretion (29) or even the elicitation of further eosinophilia generation (30). Moreover, because airway eosinophils can traffic back to regional lymph nodes and effectively present airway-derived Ags to elicit proliferation of CD4+ T cells (31), local IL-4 release by eosinophils within lymph nodes, the thymus (32), or other sites may also modulate the local responses of lymphocytes. Thus, in contrast to CD4+ T cells in which IL-4 synthesis needs to be transcriptionally induced, human eosinophils have the capacity by means of vesicular transport to physiologically and rapidly release their preformed stores of IL-4.

References


