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IL-16 Promotes Leukotriene C₄ and IL-4 Release from Human Eosinophils via CD4- and Autocrine CCR3-Chemokine-Mediated Signaling

Christianne Bandeira-Melo,* Kumiya Sugiyama,* Lesley J. Woods,* Mojabeng Phoofolo,* David M. Center,† William W. Cruikshank,† and Peter F. Weller‡*‡

Human eosinophils are potential sources of inflammatory and immunomodulatory mediators, including cysteinyi leukotrienes, chemokines, and cytokines, which are pertinent to allergic inflammation. We evaluated the means by which IL-16, a recognized eosinophil chemoattractant, might act on eosinophils to affect their capacity to release leukotriene C₄ (LTC₄) or their preformed stores of chemokines (eotxin, RANTES) or Th1 (IL-12) or Th2 (IL-4) cytokines. IL-16 dose dependently (0.01–100 nM) elicited new lipid body formation, intracellular LTC₄ formation at lipid bodies, and priming for enhanced calcium ionophore-activated LTC₄ release. IL-16 also elicited brefeldin A-inhibitable, vesicular transport-mediated release of preformed IL-4, but not IL-12, from eosinophils. CD4 is a recognized IL-16R, and accordingly anti-CD4 Fab, soluble CD4, and a CD4 domain 4-based IL-16 blocking peptide inhibited the actions of IL-16 on eosinophils. Although CD4 is not G-protein coupled, pertussis toxin inhibited IL-16-induced eosinophil activation. IL-16 actions were found to be mediated by the autocrine activity, not of platelet-activating factor, but rather of endogenous CCR3-acting chemokines. IL-16 induced the rapid vesicular transport-mediated release of RANTES. The effects of IL-16 were blocked by CCR3 inhibitors (met-RANTES, anti-CCR3 mAb) and by neutralizing anti-eotxin and anti-RANTES mAbs, but not by platelet-activating factor receptor antagonists (CV6209, BN5201). RANTES and eotxin each enhanced LTC₄ and IL-4 (but not IL-12) release. Therefore, IL-16 activation of eosinophils is CD4-mediated to elicit the extracellular release of preformed RANTES and eotxin, which then in an autocrine fashion act on plasma membrane CCR3 receptors to stimulate both enhanced LTC₄ production and the preferential release of IL-4, but not IL-12, from within eosinophils. The Journal of Immunology, 2002, 168: 4756–4763.

IL-16 may participate in other forms of inflammation, including inflammatory bowel disease (13), in which eosinophils may be a source of IL-16 (14). The functions of IL-16 in these varied forms of immunologically mediated diseases may be complex. For instance, IL-16 can inhibit MLR- and anti-CD3-activated lymphocyte responses (15, 16), can participate in dendritic cell-T cell interactions (4, 17), can enhance pro-inflammatory cytokine release from monocytes (18), and can inhibit IL-5 production by Ag-stimulated T cells in atopic subjects (19).

We had previously shown that IL-16 was a potent (ED₉₀ ~ 10⁻¹² M) chemoattractant for human eosinophils and that this activity was dependent on CD4 expressed by eosinophils (2). IL-16, however, did not appear to enhance other responses of eosinophils, such as their capacity for enhanced leukotriene C₄ (LTC₄) formation or their capacity to “degranulate,” as assessed by fluid phase assays of granule-derived arylsulfatase B release (2). Just as there is an increasing recognition of the varied activities of IL-16, there has been an increased understanding of the complexities of eosinophil cell biology and functioning in varied immunologic responses. Eosinophils may have functions in immune responses extending beyond their conventional “degranulation”-based effector responses, and these may include functioning as APCs to promote Th2 CD4⁺ responses (20, 21). Eosinophils are now recognized to contain preformed stores of diverse cytokines and chemokines within their cytoplasmic granules (22). Thus, in addition to their distinctive cationic proteins, eosinophil granules contain chemokines (e.g., eotxin (23) and RANTES (24, 25)) and cytokines with

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3 Abbreviations used in this paper: LTC₄, leukotriene C₄; EIA, enzyme immunoassay; PTX, pertussis toxin; sCD4, soluble CD4; PAF, platelet-activating factor; BFA, brefeldin A; GPCR, G protein-coupled receptor.
disparate and potentially opposing activities, notably including the prototypical Th2 cytokine IL-4 (26–28) and the Th1 cytokine IL-12 (29–31). The regulated release of these preformed cytokines occurs not by exocytotic fusion of granules with the plasma membranes to extrude all granule contents, but rather by selective processes based on vesicular mobilization and transport of these granule-derived cytokines (32–34).

We have now applied more sensitive, recently developed investigative methods (32, 33) to evaluate whether IL-16 can promote LTC4 formation by eosinophils and whether IL-16 can participate in the regulated and potentially differential release of chemokines or cytokines that are preformed within human eosinophils. Our data demonstrate that IL-16 can activate specific responses of human eosinophils and in a CD4-dependent manner elicit the release of RANTES and eotaxin from eosinophils, which then function as CCR3-mediated autocrine activators to enhance eosinophil eosinoid formation, and a selective release of specific cytokines from eosinophil granule stores.

Materials and Methods

Eosinophil purification

Peripheral blood was obtained with informed consent from 16 normal donors, and eosinophils were isolated as described (32). Briefly, after citrate-anticoagulated blood was mixed with 6% dextran-saline (MacGaw, Irvine, CA) and centrifuged and erythrocyte sedimentation, the leukocyte-enriched plasma was overlaid onto Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden) and centrifuged at 250 × g for 20 min. Granulocyte-enriched cell pellets were collected, washed at 4°C with calcium- and magnesium-free HBSS (HBSS−), and depleted of erythrocytes by hypotonic saline lysis. Eosinophils were negatively selected using the MACS system (Miltenyi Biotec, Auburn, CA) using the CytoAvid human eosinophil isolation kit (Miltenyi Biotec, Auburn, CA) with biotin-labeled anti-human eosinophil antibody and biotinylated magnetic beads (Miltenyi Biotec). Eosinophils were negatively selected using the MACS system (Miltenyi Biotec, Auburn, CA) using the CytoAvid human eosinophil isolation kit (Miltenyi Biotec, Auburn, CA) with biotin-labeled anti-human eosinophil antibody and biotinylated magnetic beads (Miltenyi Biotec). Eosinophil suspensions (10⁶/ml) were incubated (37°C, 5% CO2, 2 h) with IL-16 (0.01–100 nM; R&D Systems) or medium alone for 2 h. We have now applied more sensitive, recently developed investigative methods (32, 33) to evaluate whether IL-16 can promote LTC4 formation by eosinophils and whether IL-16 can participate in the regulated and potentially differential release of chemokines or cytokines that are preformed within human eosinophils. Our data demonstrate that IL-16 can activate specific responses of human eosinophils and in a CD4-dependent manner elicit the release of RANTES and eotaxin from eosinophils, which then function as CCR3-mediated autocrine activators to enhance eosinophil eosinoid formation, and a selective release of specific cytokines from eosinophil granule stores.

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Lipid body induction, staining, and enumeration

Eosinophils suspensions (10⁶/ml) were incubated (37°C) with IL-16 (0.01–100 nM; R&D Systems, Minneapolis, MN) or medium alone for 1 h and then centrifuged (350 rpm, 4 min) onto glass slides. Cytosin slides, while still moist, were fixed with 2% paraformaldehyde in HBSS−, rinsed in 0.1 M cacodylate buffer (pH 7.4), stained in 1.5% OsO4 (30 min), rinsed in dH2O, immersed in 1% thiocarbohydrazide (5 min), rinsed with 0.1 M cacodylate buffer, and stained with 1.5% OsO4 (3 min), rinsed, and then dehydrated and mounted (35). Lipid bodies were enumerated by light microscopy with a ×100 objective lens in 50 consecutively scanned cells. For each condition there was a unimodal distribution of lipid body numbers per eosinophils without wide variations.

LTC4 measurements

After samples were taken for lipid body enumeration, cell suspensions (10⁶/ml) were washed in HBSS−, suspended in calcium- and magnesium-containing calcium- and magnesium-free HBSS (HBSS−; Sigma-Aldrich) for 15 min (37°C). Reactions were stopped on ice, cell suspensions were centrifuged (500 × g for 10 min; 4°C), and supernatants were assayed for LTC4 by enzyme immunoassay (EIA) (2) sensitivity,<7.8 pg/ml (Cayman Chemicals, Ann Arbor, MI). Intracellular formation of LTC4 within eosinophils embedded in an agarose matrix was evaluated as described previously using carbodiimide fixation of newly formed LTC4 before its immunofluorescent localization with an Alexa488-labeled (Molecular Probes, Eugene, OR) rat anti-LTC4/LTD4/LTE4 mAb (clone 6E7; Sigma-Aldrich) (35).

EliCell assay for detection of released IL-4, IL-12, and RANTES

The EliCell assay, a gel-phase dual-Ab capture and detection assay based on microscopic observations of individual viable cells, was performed as described (32) to enumerate the proportions of eosinophils releasing preformed cytokines or chemokines (IL-4, IL-12, or RANTES) and to electronically quantify (in arbitrary units × 10⁷) the average relative amounts of each cytokine released extracellularly. Biotinylated goat polyclonal Abs against IL-4, IL-12, and RANTES (each at 20 μg/ml; R&D Systems) were used as capturing Abs and paired with Alexa546-labeled mouse anti-IL-4, anti-IL-12, and anti-RANTES mAb (400 μl of 10 μg/ml; R&D Systems) to detect released IL-4, IL-12, and RANTES, respectively. Alexa486 labeling was performed per a protocol from Molecular Probes. Controls to ascertain the specificity of extracellular immunodetection of these three cytokines and to confirm that the detected cytokines were not from the intracellular pool were performed. No IL-4, IL-12, or RANTES staining were found either when Alexa486-labeled mouse IgG1 was used as a non-immune isotype control or when the biotinylated capture Abs (necessary to co-incubate with a biotinylated irrelevant control Ab. As evaluated by LIVE/DEAD fluorescent assay (Molecular Probes), eosinophil viability at the end of assays was >80%.

Stimuli and treatments

Eosinophils were stimulated with IL-16 (0.01–100 nM; R&D Systems), RANTES, or eotaxin (6 nM; R&D Systems) for time periods ranging from 5 min to 3 h. For inhibitor studies, cells were pretreated for 30 min with pertussis toxin (PTX) (20 ng/ml; Calbiochem, La Jolla, CA), recombinant soluble CD4 (sCD4) (50 ng/ml; R&D Systems), Fab of anti-CD4 (clone OKT4; American Type Culture Collection, Manassas, VA) and anti-HLA class I (clone W6/32; Sigma-Aldrich) mAbs (1 μg/ml), anti-CCR3 mAb (clone 61828.111; R&D Systems), or isotype control rat IgG2a at 10 μg/ml (BD PharMingen, San Diego, CA), met-RANTES (60 nM; R&D Systems), the platelet-activating factor (PAF) receptor antagonists CV6209 and BNS0201 (10 μM; Bionol, Plymouth Meeting, PA), brefeldin A (BFA) (0.1 and 1 μg/ml; Bionol), or their vehicles, as indicated. Alternatively, cells were co-incubated with 10 μg/ml of anti-eotaxin (clone 43911.11) and anti-RANTES (clone 21445.1) mAb (each from R&D Systems) or with their matching nonimmune mouse IgG1. Inhibitors were prepared in RPMI 1640 containing 0.1% of endotoxin-free OVA. PTX was substituted in DMSO, and the final DMSO concentration was <0.01% and had no effect on eosinophils. A peptide from domain 4 of human CD4, peptide 2H-QLCLSLDSDLG(135-135)-amide, and a scrambled control peptide 3H-DLQQLSLSC-amide, were purchased from Research Genetics (Huntsville, AL) (36).

FIGURE 1. Dose responses of IL-16-induced lipid body formation (A), priming for enhanced LTC4 production (B), and IL-4 release (C and D) from human eosinophils at 1 h. Lipid bodies are expressed as the mean number enumerated in 50 consecutive eosinophils. LTC4 quantities released by 10⁶ eosinophils in 1 ml after 15-min activation with 0.1 μM A23187 were assayed by EIA in cell supernatants. IL-4 release, detected in RPMI 1640 containing 0.1% of endotoxin-free OVA, PTX was substituted in DMSO, and the final DMSO concentration was <0.01% and had no effect on eosinophils. A peptide from domain 4 of human CD4, peptide 2H-QLCLSLDSDLG(135-135)-amide, and a scrambled control peptide 3H-DLQQLSLSC-amide, were purchased from Research Genetics (Huntsville, AL) (36).
**Statistical analysis**

Data were expressed as means ± SD. Percentage inhibition with antagonists was calculated in comparison with stimulated increases in lipid body numbers, LTC₄ production, or IL-4 release above baselines. Statistical comparisons were done by ANOVA followed by Newman-Keuls Student’s test. Differences were considered significant when p < 0.05. Correlation coefficients evaluating lipid body numbers vs quantities of LTC₄ released were determined from the means of four different experiments by linear regression with significance (F test) at p < 0.05.

**Results**

**IL-16 primes for enhanced LTC₄ production and elicits lipid body formation in eosinophils**

IL-16 very effectively primed eosinophils for increased LTC₄ release in response to a submaximal 0.1 μM concentration of calcium ionophore A23187. Prestimulation of eosinophils for 1 h with IL-16 dose dependently elicited increases in A23187-induced LTC₄ production (Fig. 1B). At 100 nM, IL-16-primed eosinophils released ~6-fold as much LTC₄ as did eosinophils challenged with A23187 alone. Because the formation of lipid bodies and LTC₄ synthesis at these organelles provides a basis for this enhanced capacity for LTC₄ release (35), we analyzed the effect of IL-16 on the number of cytoplasmic lipid bodies. Resting eosinophils contained ~9.4 ± 2.3 lipid bodies (mean ± SD, n = 10). IL-16 dose dependently induced the formation of new lipid bodies within eosinophils, doubling their initial numbers (Fig. 1A). The increased quantities of LTC₄ generated by eosinophils primed with increasing concentrations of IL-16 correlated highly with the increased numbers of lipid bodies (r = 0.89; p < 0.05; n = 4), in accord with similar correlations observed previously with other specific chemoattractants (i.e., PAF, eotaxin, eotaxin-2, eotaxin-3, and RANTES) (35, 37–39). In the absence of A23187 activation, eosinophils stimulated for 1 h with IL-16 only (100 nM) did not release extracellular quantities of LTC₄ sufficient to be detectable by EIA (data not shown), but they did synthesize new LTC₄ immunodetectable at lipid bodies within 33 ± 7% (mean ± SD, n = 3) of IL-16-stimulated eosinophils (Fig. 2D).

**IL-16 elicits IL-4, and not IL-12, release from eosinophils**

Eosinophils contain preformed stores of almost two dozen cytokines resident largely within eosinophil-specific granules (22), and these include IL-4 and IL-12, two cytokines with potentially disparate and opposing activities. As detected with a sensitive solid-phase dual-Ab capture and detection assay developed to detect the extracellular release of eosinophil chemokines and cytokines (32), IL-16 stimulated the release of IL-4 from eosinophils. IL-16-stimulated eosinophils showed a punctate pattern of immunoreactive IL-4 released at discrete loci proximate to the cell surface, compatible with a vesicular transport-mediated process of IL-4 release (Fig. 2A). No IL-4 staining was found with nonstimulated eosinophils (Fig. 1, C and D), when the Alexa546-labeled anti-IL-4 detection Ab was replaced by an Alexa546-labeled isotype non-immune control (data not shown), or when biotinylated anti-IL-4 capture Ab (which immobilizes IL-4 at its extracellular sites of release) was substituted with a biotinylated irrelevant control (data not shown). The last condition assured that neither intracellular nor membrane-bound IL-4 was being detected in the nonpermeabilized eosinophils. In contrast, IL-16 did not stimulate the release of...
CD4 receptors mediate the effects of IL-16 on eosinophils

Because the chemoattractant activity of IL-16 for human eosinophils is mediated by IL-16 engagement of CD4 (2), we evaluated whether IL-16 promotion of LTC₄ and IL-4 release was likewise mediated through CD4 expressed on eosinophils. Treatment of eosinophils with either soluble CD4 or neutralizing Fab of OKT4 anti-CD4 mAb (but not with a control mAb to surface-expressed class I MHC protein) blocked lipid body formation, priming for enhanced LTC₄ production and IL-4 release from eosinophils stimulated with IL-16 (Fig. 3, left panels). Of note, Fab were used because the binding of CD4 with whole OKT4 mAb mimics IL-16 stimulation of eosinophils (2). Thus, IL-16-elicited eosinophil activation was mediated via CD4 expressed by eosinophils.

The amino acid sequence of the extracellular IgG-like region of CD4 contains four domains designated D1 through D4. Recent studies reported that the binding sites for IL-16 within the CD4 molecule are within the D4 residues (36, 40). To further investigate the specific CD4 domain required for IL-16 binding and eosinophil activation, eosinophils were pretreated with an octapeptide (peptide 2) derived from the D4 region of CD4 molecule. As shown in Table I, peptide 2 but not peptide 3 (a control peptide with a random scrambled amino acid sequence) abolished IL-16-induced lipid body formation, enhanced LTC₄ production, and IL-4 release. It is of note that neither these peptides nor the Fab of anti-CD4 affected the resting status of nonstimulated eosinophils or the lipid body formation and LTC₄ and IL-4 release from RANTES-stimulated eosinophils (data not shown). These data indicate that the effects of IL-16 on eosinophils depend on IL-16 binding to the D4 domain of CD4 expressed by eosinophils.

Involvement of G protein-coupled CCR3 activation in IL-16-initiated eosinophil activation

Although membrane-bound CD4 molecules are not G protein-coupled receptors (GPCRs), we evaluated whether endogenously generated ligands that signal via GPCR might be downstream mediators of responses initiated by IL-16 engagement of CD4. Inhibition of G protein activation by PTX pretreatment blocked IL-16-elicited lipid body formation, priming for enhanced LTC₄ production and IL-4 release from eosinophils (Fig. 3, left panels). PTX-sensitive GPCRs known to elicit lipid body formation and enhanced eicosanoid synthesis include the PAF receptor (35, 37). Pretreatment with two specific PAF receptor antagonists (CV6209 and BN52021) did not inhibit IL-16-elicited lipid body formation, priming for enhanced LTC₄ production, or IL-4 release from eosinophils (Fig. 3, right panels). In contrast, IL-16-mediated activation of eosinophils was blocked by

**Table I. Inhibitory effect of peptide 2 derived from the D4 domain of CD4 molecule on IL-16-induced eosinophil activation**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Lipid Bodies/ Eosinophil</th>
<th>LTC₄ (ng/ml)</th>
<th>% Eosinophils Releasing IL-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium control</td>
<td>8.0 ± 1.4</td>
<td>0.2 ± 0.2</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>IL-16 (100 nM)</td>
<td>15.0 ± 1.4</td>
<td>2.4 ± 1.3</td>
<td>62 ± 2</td>
</tr>
<tr>
<td>+ Peptide 2 (10 μg/ml)</td>
<td>15.5 ± 0.7</td>
<td>1.9 ± 0.8</td>
<td>51 ± 4</td>
</tr>
<tr>
<td>+ Peptide 2 (100 μg/ml)</td>
<td>8.0 ± 2.6*</td>
<td>0.4 ± 0.2*</td>
<td>31 ± 5*</td>
</tr>
<tr>
<td>+ Peptide 3 (10 μg/ml)</td>
<td>17.5 ± 2.1</td>
<td>2.5 ± 0.1</td>
<td>68 ± 6</td>
</tr>
<tr>
<td>+ Peptide 3 (100 μg/ml)</td>
<td>17.0 ± 1.4</td>
<td>2.8 ± 1.1</td>
<td>58 ± 1</td>
</tr>
</tbody>
</table>

* Eosinophils were preincubated for 30 min with peptide 2 or 3 (irrelevant control), as indicated, and then stimulated with IL-16 (100 nM) for 1 h for induction of lipid bodies, priming for enhanced LTC₄ production, and IL-4 release. Results are means ± SD from three donors. *p < 0.05 compared with IL-16-stimulated eosinophils.
pretreatment with either the CCR3 antagonist met-RANTES (42) or a neutralizing anti-CCR3 receptor mAb (but not by an isotype control Ab) (Fig. 3, right panels). These findings suggested that actions of IL-16 to enhance LTC4 and IL-4 release from eosinophils might be mediated by endogenous CCR3-acting chemokines.

Eosinophils contain two chemokines, eotaxin and RANTES, that act via CCR3 and are stored preformed within eosinophil granules (23–25). We have shown that both RANTES and eotaxin induce lipid body formation, prime for enhanced LTC4 production (35), and elicit vesicular transport-mediated IL-4 release (43) from eosinophils via CCR3 activation. With the Elicell assay, we evaluated whether IL-16 elicited release of endogenous, preformed RANTES from eosinophils. Indeed, IL-16 stimulated the rapid release of RANTES that temporally preceded IL-16-induced IL-4 release (Fig. 4). Consistently, in kinetic studies with three different donors, IL-16 induced RANTES release as early as 5 min, whereas at least 30 min were needed for detectable IL-4 release, a time course consistent with autocrine intermediary roles for endogenously derived CCR3 agonists. With eosinophils incubated for 1 h with 100 nM IL-16 (n = 3), 57.6% exhibited extracellular RANTES release, and addition of sCD4 or OKT4 Fab anti-CD4 mAb during incubation with IL-16 inhibited the numbers of eosinophils releasing RANTES by 91.8 and 87.6%, respectively (Table II). In a punctate pattern similar to that for IL-4 release, released RANTES also appeared as extracellular focal spots close to eosinophil membrane, again indicating a vesicular transport-dependent secretory process (Fig. 2C). Interestingly, exogenous eotaxin also induced the rapid release of endogenous RANTES (Fig. 4, left panel). The RANTES-releasing activity of eotaxin was fully inhibited by PTX and a CCR3-blocking mAb and was partially inhibited by met-RANTES (Table II). These inhibitors of RANTES release, however, did not inhibit IL-16-induced release of RANTES.

To confirm the involvement of endogenously eosinophil-derived CC chemokines (RANTES and eotaxin) on IL-16-mediated activation of eosinophils, neutralizing anti-RANTES and anti-eotaxin mAbs were co-incubated with IL-16-stimulated eosinophils. Both mAbs inhibited IL-16-elicited lipid body formation, enhanced LTC4 production, and IL-4 release from eosinophils (Fig. 5), indicating that released chemokines were acting as autocrine activators. In addition, IL-16-induced release of RANTES was inhibited 34.3% by exogenous anti-eotaxin mAb and 0% by a control mAb (data not shown). Thus, IL-16 was directly mobilizing both RANTES and eotaxin from eosinophils for their extracellular release and autocrine activities. That IL-16 was eliciting the release of these chemokines by vesicular transport-mediated secretion was supported by findings with BFA, a vesicle formation inhibitor. Pretreatment with BFA blocked IL-16-induced RANTES release and enhanced LTC4 production and IL-4 release (Fig. 6).

**Discussion**

The capabilities of eosinophils to contribute to varied immunologic and inflammatory responses within tissues may be dependent on the activation of mechanisms that enhance their formation of eicosanoids, notably including LTC4, and their regulated release of various granule-stored proteins, which include not only their cardinal cationic proteins but also varied chemokines and cytokines with potentially diverse and disparate biologic activities. Because these responses may not be adequately studied in conventional fluid-phase assays, we have developed complementary techniques to evaluate the capacity of individual eosinophils to synthesize

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**Table II. Involvement of CD4 and CCR3 receptors on IL-16-induced RANTES release from eosinophils**

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Eosinophils Releasing RANTES</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-16 (100 nM)</td>
<td>57.6 ± 4.1</td>
<td>—</td>
</tr>
<tr>
<td>+ sCD4 (50 ng/ml)</td>
<td>5.0 ± 0.0*</td>
<td>91.8 ± 1.0*</td>
</tr>
<tr>
<td>+ OKT4 Fab anti-CD4 (1 μg/ml)</td>
<td>6.5 ± 2.1*</td>
<td>87.6 ± 4.4*</td>
</tr>
<tr>
<td>+ W6/32 Fab anti-HLA (1 μg/ml)</td>
<td>58.0 ± 0.7</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>+ PTX (20 ng/ml)</td>
<td>47.0 ± 9.8</td>
<td>18.1 ± 12.3</td>
</tr>
<tr>
<td>+ met-RANTES (60 nM)</td>
<td>61.5 ± 3.0</td>
<td>0.6 ± 1.1</td>
</tr>
<tr>
<td>+ Anti-CCR3 mAb (10 μg/ml)</td>
<td>58.5 ± 8.8</td>
<td>0.9 ± 0.8</td>
</tr>
<tr>
<td>+ rat IgG2a (10 μg/ml)</td>
<td>57.8 ± 2.0</td>
<td>2.7 ± 3.3</td>
</tr>
<tr>
<td>Eotaxin (6 nM)</td>
<td>49.5 ± 0.5</td>
<td>—</td>
</tr>
<tr>
<td>+ PTX (20 ng/ml)</td>
<td>1.4 ± 1.3*</td>
<td>99.6 ± 0.6*</td>
</tr>
<tr>
<td>+ Met-RANTES (60 nM)</td>
<td>27.0 ± 10.0*</td>
<td>49.9 ± 15.0*</td>
</tr>
<tr>
<td>+ Anti-CCR3 mAb (10 μg/ml)</td>
<td>5.0 ± 2.3*</td>
<td>95.5 ± 7.8*</td>
</tr>
<tr>
<td>+ rat IgG2a (10 μg/ml)</td>
<td>51.1 ± 3.5</td>
<td>1.2 ± 2.0</td>
</tr>
</tbody>
</table>

* Eosinophils were preincubated for 30 min with inhibitors of CD4/IL-16 binding and CCR3 activation, as indicated, and then stimulated with IL-16 (100 nM) or eotaxin (6 nM) for 1 h for induction of RANTES release. At 1 h, only 3.3 ± 2.8% (mean ± SD; n = 4) of unstimulated eosinophils showed basal release of RANTES. Results are means ± SD from at least three donors. *p < 0.05 compared with IL-16- or eotaxin-stimulated eosinophils.
LTC₄ and to release specific preformed chemokines and cytokines by a physiologic process of vesicular mobilization of granule-derived proteins for their transport and focal release outside of the plasma membrane of eosinophils. With techniques that can evaluate the functional responses of individual eosinophils, we have shown that both RANTES and eotaxin induce lipid body formation (35), prime for enhanced LTC₄ production (35), and elicit vesicular transport-mediated IL-4 release (43) from eosinophils via CCR3 activation. Our current results indicate that IL-16 can not only enhance specific responses of eosinophils, but that it can also identify major roles for eosinophil-derived eotaxin and RANTES in functioning as important autocrine intermediators of these IL-16-elicited responses.

IL-16 dose dependently elicited new lipid body formation, intracellular LTC₄ formation at lipid bodies, and priming for enhanced calcium ionophore-activated LTC₄ release. In our earlier studies with IL-16 (2), lesser concentrations of IL-16, a greater concentration of activating calcium ionophore, and a lack of methods to detect intracellular LTC₄ formation contributed to an inability to recognize that IL-16 stimulation of eosinophils could enhance the formation of the 5-lipoxygenase pathway-derived eicosanoid LTC₄. IL-16 also elicited BFA-inhibitable, vesicular transport-mediated release of preformed RANTES and IL-4, but not IL-12, from eosinophils. Although studies in CD4-knockout mice indicate that IL-16 may act on monocytes and dendritic cells via as yet unidentified CD4-independent mechanisms (44, 45), with human and mouse T cells CD4 is the recognized IL-16R (1).

We previously established that IL-16-elicited chemotaxis of human eosinophils was CD4-dependent (2). In support of a central role for eosinophil-expressed CD4 in functioning as the signal-transducing receptor on eosinophils for IL-16, we demonstrated that anti-CD4 Fab, sCD4, and a CD4 domain 4-based IL-16 blocking peptide inhibited the actions of IL-16 on eosinophils. That IL-16 was not directly acting to enhance eosinophil LTC₄ formation and IL-4 release was indicated by the inhibition found with PTX, which catalyzes the ADP ribosylation of certain G protein subunits and uncouples PTX-sensitive G proteins from cell surface receptors (46). Potentially, CD4 on eosinophils might be interacting with the PTX-sensitive CXCR4, which is expressed on eosinophils (47). Alternatively, PTX-sensitive receptors for PAF or for chemokines acting via CCR3 might be involved. Bartemes et al. (41) recently documented a central role for endogenous PAF and its receptor in the augmented functional responses of eosinophils elicited by IL-5 or IgG, and these included enhanced LTC₄ formation and correlative increases in lipid body formation. Endogenous PAF generation and signaling through its receptor were not involved in IL-16-mediated signaling, because two PAF receptor antagonists (CV6209 and BN52021) were without effect. Instead, IL-16 actions were mediated by the autocrine activities of endogenous CCR3-acting chemokines. IL-16 induced the rapid vesicular transport-mediated release of RANTES, which temporally preceded later IL-4 release (Fig. 4). The effects of IL-16 were blocked by CCR3 inhibitors (met-RANTES and anti-CCR3 mAb) and by neutralizing anti-eotaxin and anti-RANTES mAbs. Both
RANTES and eotaxin each enhanced LTC4 and IL-4 (but not IL-12) release.

Thus, IL-16 acts to initiate the release of specific chemokines and cytokines that are stored preformed within eosinophils. Our findings provide insights into the mechanisms that regulate this mobilization and release process. First, as indicated by the finding that exogenous eotaxin also induced the rapid release of endogenous RANTES (Fig. 4, left panel; Table II), chemokines acting via CCR3 can enhance the release of additional CCR3-active chemokines from within eosinophils. This could provide a positive feedback loop amplifying the initial IL-16-elicted chemokine release and consequent eosinophil activation. Second, the vesicular transport-mediated process of eosinophil “degranulation” was highly selective. Notably, the IL-16-initiated transport and release of RANTES occurred in vesicles that were not also loaded with IL-4, whose release occurred only later in vesicles formed in response to the CCR3-active chemokines. Additional selectivity in the regulative release of eosinophil-derived cytokines was evident in the release of IL-4, but not of IL-12, by the IL-16- or CCR3 chemokine-activated eosinophils. Because eosinophils also contain preformed IL-16 (3), an ability for further autocrine activation of eosinophils exists, but at present the mechanisms that physiologically elicit IL-16 release from eosinophils have not been defined.

Thus, the capacity of IL-16 to activate specific functional responses of eosinophils has the potential to influence the nature of immunologic responses. IL-16 can directly eosinophils to make new LTC4, release the chemokines, RANTES, and eotaxin. These chemokines can act in an autocrine fashion on eosinophils via CCR3 and potentially on other cells that express CCR3, including basophils (48), mast cells (49), airway epithelial cells (50), and Th2 cells (51). Moreover, the differential release of IL-4, which can contribute to the polarization toward Th2 differentiation, promote IgE class switching, and stimulate other cellular responses in Th2 cells (51). In contrast, IL-16 can direct eosinophils to make new lymphocyte chemoattractants: IL-16 (lymphocyte chemotactic factor; LCF) and RANTES. J. Immunol. 156:2522.


