

# Intracrine Cysteinyl Leukotriene Receptor-mediated Signaling of Eosinophil Vesicular Transport-mediated Interleukin-4 Secretion

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## Abstract

We investigated whether cysteinyl leukotrienes (cysLT) are intracrine signal transducers that regulate human eosinophil degranulation mechanisms. Interleukin (IL)-16, eotaxin, and RANTES stimulate vesicular transport-mediated release of preformed, granule-derived IL-4 and RANTES from eosinophils and the synthesis at intracellular lipid bodies of LTC<sub>4</sub>, the dominant 5-lipoxygenase-derived eicosanoid in eosinophils. 5-Lipoxygenase inhibitors blocked IL-16-, eotaxin-, and RANTES-induced IL-4 release; but neither exogenous LTC<sub>4</sub>, LTD<sub>4</sub>, nor LTE<sub>4</sub> elicited IL-4 release. Only after membrane permeabilization enabled cysLTs to enter eosinophils did LTC<sub>4</sub> and LTD<sub>4</sub> stimulate IL-4, but not RANTES, release. LTC<sub>4</sub>-elicited IL-4 release was pertussis toxin inhibitable, but inhibitors of the two known G protein-coupled cysLT receptors (cysLTRs) (CysLT1 and CysLT2) did not block LTC<sub>4</sub>-elicited IL-4 release. LTC<sub>4</sub> was 10-fold more potent than LTD<sub>4</sub> and at low concentrations (0.3–3 nM) elicited, and at higher concentrations (>3 nM) inhibited, IL-4 release from permeabilized eosinophils. Likewise with intact eosinophils, LTC<sub>4</sub> export inhibitors, which increased intracellular LTC<sub>4</sub>, inhibited eotaxin-elicited IL-4 release. Thus, LTC<sub>4</sub> acts, via an intracellular cysLTR distinct from CysLT1 or CysLT2, as a signal transducer to selectively regulate IL-4 release. These results demonstrate that LTC<sub>4</sub>, well recognized as a paracrine mediator, may also dynamically govern inflammatory and immune responses as an intracrine mediator of eosinophil cytokine secretion.

Key words: eotaxin • interleukin-16 • RANTES • leukotriene C<sub>4</sub> • 5-lipoxygenase

## Introduction

Eosinophils, one of the principal cell types recruited to and activated at sites of allergic inflammation, are major participants in the pathogenesis of asthma and other forms of allergic disorders (1, 2). Eosinophils have the potential to generate and release diverse lipid and protein mediators critical to the development and perpetuation of allergic inflammation. Eosinophils are major sources of cysteinyl leukotrienes (cysLTs),\* LTC<sub>4</sub> and its extracellular derivatives, LTD<sub>4</sub> and LTE<sub>4</sub> (3). These 5-lipoxygenase (LO) pathway-

derived eicosanoids have many well recognized actions as paracrine mediators, causing bronchoconstriction, mucous hypersecretion, increased microvascular permeability, bronchial hyperresponsiveness, and eosinophil infiltration (4–6). In addition, eosinophil-specific granules contain proteins that include not only their distinctive cationic granule proteins, but also over two dozen preformed cytokines (7). Amongst these eosinophil-derived cytokines are the CC chemokines, RANTES (8) and eotaxin (9), and the prototypical Th2 cytokine, IL-4 (10, 11). 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 (12). The actions of IL-4 are not limited to the initiation of Th2 responses, but may also stimulate other cellular responses that contribute to manifestations of allergic diseases (12, 13). Unlike CD4<sup>+</sup> T cells, eosinophils store IL-4 (11), RANTES (14, 15), and eotaxin (9), already preformed within eosinophil specific granules. Preformed IL-4 and RANTES may be rapidly re-

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\*Abbreviations used in this paper: BFA, brefeldin A; cysLT, cysteinyl leukotriene; cysLTR, cysteinyl leukotriene receptor; HETE, hydroxyeicosatetraenoic acid; LO, lipoxygenase; LT, leukotriene; MAP, mitogen-activated protein; PAF, platelet-activating factor; PI3K, phosphoinositide-3 kinase; PKC, protein kinase C; PTX, pertussis toxin; UDP, uridine diphosphate; UTP, uridine triphosphate.

leased within minutes from eosinophil granules by vesicular transport (14–16), but the physiological stimuli and intracellular signal transduction mechanisms that elicit their release are not yet fully defined.

For the generation of cysLTs in eosinophils, arachidonic acid liberated from membrane phospholipids is acted on by 5-LO in concert with the 5-LO-activating protein (FLAP) to form LTA<sub>4</sub> (17). LTC<sub>4</sub> synthase conjugates reduced glutathione to LTA<sub>4</sub> to form LTC<sub>4</sub> (18). Production of LTC<sub>4</sub> within eosinophils may occur at perinuclear membranes or cytoplasmic lipid bodies (17, 19, 20). LTC<sub>4</sub> is not permeable across the plasma membrane and its release from eosinophils is mediated by a distinct ATP-dependent export mechanism (21). Extracellularly, LTC<sub>4</sub> is further metabolized to LTD<sub>4</sub> by  $\gamma$ -glutamyl transpeptidase or  $\gamma$ -glutamyl leukotrienase (22, 23) and to LTE<sub>4</sub> by a dipeptidase (24). These cysLTs exert their actions by engaging specific receptors. To date, two cysLT receptors (cysLTRs) have been cloned and characterized, the CysLT1 and CysLT2 receptors (25–28). These receptors can be distinguished with pharmacologic inhibitors and by their differing ligand binding affinities, as studied in transfected cells. CysLT1, which is also activated by the pyrimidinergic ligand, uridine diphosphate (UDP) (29), binds LTD<sub>4</sub> with a 10-fold greater affinity than it binds LTC<sub>4</sub> (IC<sub>50</sub>'s of  $\sim$ 1 vs.  $\sim$ 350 nM, respectively) (25), whereas CysLT2 binds LTC<sub>4</sub> and LTD<sub>4</sub> with equal affinities (IC<sub>50</sub>'s of  $\sim$ 3–7 nM) (27, 28). In addition, various findings suggest the existence of other, not yet cloned, cysLTRs (30–32).

Understanding the mechanisms governing the synthesis of eosinophil cysLTs and their potential actions on eosinophils themselves are important because it is increasingly likely that eicosanoids synthesized within cells, including eosinophils, may have other roles in regulating cell functions, in addition to the more recognized paracrine activities in inflammation. Indeed, eicosanoids and related lipids are increasingly being recognized to have autocrine effects. For instance, eosinophil cysLTs exert autocrine effects to enhance eosinophil survival (33). The enhanced plasma membrane expression of activation-related CD69 on human eosinophils induced by platelet-activating factor (PAF) and IL-5 is dependent on endogenous eosinophil-derived 5-LO metabolites (34).

Although eosinophils express the two known, seven transmembrane spanning, G protein-coupled cysLTRs, CysLT1 and CysLT2 (28, 35), little is known about the plasma membrane or intracellular distribution of these or other eicosanoid receptors in eosinophils. On other cells, expression of specific G protein-coupled prostaglandin receptors is beginning to be localized at intracellular sites, including the nuclear envelope (36). The localization of cyclooxygenase and LO pathway enzymes and their intracellular eicosanoid-specific receptors at the nuclear envelope and the nucleoplasm may relate less to paracrine mediator formation and more to intracrine signal-transducing activities pertinent to more local transcriptional or other cellular functions (37, 38).

We have evaluated whether eosinophil-derived cysLTs function as autocrine or even intracrine mediators involved in the stimulated release of IL-4 from eosinophils. We find that CCR3 chemokine receptor-initiated signaling, which elicits the vesicular transport-mediated release of preformed IL-4 from eosinophils (16, 39), is dependent on the formation of new 5-LO pathway-derived eicosanoids. Endogenous LTC<sub>4</sub> formed at eosinophil lipid bodies, and not extracellular LTC<sub>4</sub>, acting via distinct intracellular cysLTRs, is an intracrine downstream signaling mediator of CCR3-elicited IL-4 release from eosinophils.

## Materials and Methods

**Eosinophil Purification.** Peripheral blood was obtained with informed consent from 16 normal donors, and eosinophils were isolated with minor modifications of procedures described previously (15). In brief, after citrate-anticoagulated blood was mixed with 6% dextran saline (McGaw) to facilitate erythrocyte sedimentation, the leukocyte-enriched plasma was overlaid onto Ficoll-Paque Plus (Amersham Biosciences) 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<sup>-/-</sup>), and depleted of erythrocytes by hypotonic saline lysis. Eosinophils were negatively selected using the StemSep™ system (StemCell Technologies Inc.) with an eosinophil enrichment mixture of antibodies against CD16, CD2, CD14, CD19 and CD56 plus magnetic colloid. The viability of freshly isolated cells was >95% (by trypan blue exclusion) and eosinophil purity was >99% (by HEMA3® staining; Fisher Scientific). Purified cell suspensions were adjusted to 10<sup>6</sup> or 15 × 10<sup>6</sup> cells/ml in RPMI 1640 medium containing 0.1% endotoxin-free ovalbumin (Sigma-Aldrich) for use in fluid- or gel-phase assays, respectively.

**LTC<sub>4</sub> Assays.** Eosinophil suspensions (10<sup>6</sup> cells/ml) were washed in HBSS<sup>-/-</sup>, resuspended in 1 ml of HBSS containing calcium and magnesium, and stimulated with 0.1  $\mu$ M A23187 (Sigma-Aldrich) for 15 min (37°C). Reactions were stopped on ice, and cell suspensions were centrifuged (500 *g* for 10 min; 4°C). Cell pellets were extracted for 30 min with methanol and centrifuged. Methanol extracts were dried under nitrogen and resuspended in HBSS<sup>-/-</sup> to volumes equivalent to 10<sup>6</sup> eosinophils/ml. Cell supernatants and pellet extracts were assayed for LTC<sub>4</sub> by enzyme immunoassay (sensitivity <7.8 pg/ml) (Cayman Chemical) for detection of released and intracellular levels of LTC<sub>4</sub>, respectively. Intracellular formation of LTC<sub>4</sub> within eosinophils embedded in an agarose matrix was evaluated as described previously using carbodiimide fixation of newly formed LTC<sub>4</sub> before its immunofluorescent localization with an Alexa-488-labeled (Molecular Probes) rat anti-LTC<sub>4</sub>/LTD<sub>4</sub>/LTE<sub>4</sub> mAb (clone 6E7; Sigma-Aldrich) (20).

**EliCell Assays for Detecting IL-4 and RANTES Secretion.** The EliCell assay, a gel-phase dual antibody capture and detection assay based on microscopic observations of individual viable cells, was performed as detailed (15, 16) to enumerate the proportions of eosinophils secreting preformed IL-4 or RANTES and to electronically quantitate (in arbitrary units × 10<sup>6</sup>) the average relative amounts of each cytokine secreted. Biotinylated goat polyclonal antibodies against IL-4 and RANTES (each at 20  $\mu$ g/ml; R&D Systems) were used as capturing antibodies and paired with Alexa546-labeled anti-IL-4 and anti-RANTES mAb (400  $\mu$ l of 10  $\mu$ g/ml; R&D Systems) to detect released IL-4 and RANTES, respectively. Alexa546 labeling was performed as per a protocol from

Molecular Probes. Controls to ascertain the specificity of extracellular immunodetection of these two cytokines and to confirm that the detected cytokines were not from the intracellular pool were performed. No IL-4 or RANTES staining was found either when Alexa546-labeled mouse IgG<sub>1</sub> was used as a nonimmune isotype control or when the biotinylated capture antibodies (necessary to immobilize cytokines at their extracellular sites of release) was substituted with a biotinylated irrelevant control antibody.

**Stimuli and Treatments.** Eosinophils were stimulated with IL-16 (100 nM; R&D Systems), RANTES (6 nM; R&D Systems), eotaxin (6 nM; R&D Systems), the cysLTs, LTC<sub>4</sub>, LTD<sub>4</sub>, or LTE<sub>4</sub>, (0.03–3,000 nM; Cayman Chemical), PAF (1 μM), 5(S)-hydroxyeicosatetraenoic acid (HETE) (1, 10, and 100 nM; Cayman Chemical), uridine triphosphate (UTP) or UDP (each 3 and 3,000 nM; Sigma-Aldrich), or calcium ionophore (0.5 μM; Sigma-Aldrich) for time periods ranging from 5 min to 3 h.

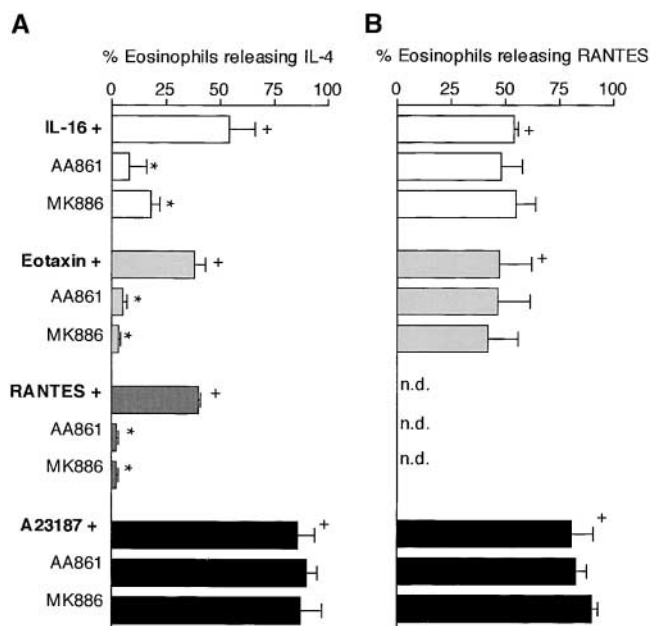
For inhibition studies, cells were pretreated for 30 min with 20 ng/ml pertussis toxin (PTX; Calbiochem); 5-LO pathway inhibitors, MK886 (a FLAP and LTC<sub>4</sub> synthase inhibitor) or AA861 (5-LO inhibitor) (10 μM; Biomol); brefeldin A (BFA) (0.1 and 1 μg/ml; Biomol); the protein kinase C (PKC) inhibitors, chelerythrine (10 μM) and staurosporin and calphostin C (1 μM; Biomol); the phosphoinositide-3 kinase (PI3K) inhibitors, wortmannin (1 μM) and LY294002 (10 μM; Biomol); the tyrosine kinase inhibitors, 10 μM herbimycin and 10 μM genistein (Biomol); the mitogen-activated protein (MAP) kinase inhibitors, 10 μM PD98059, 10 μM U0126, 10 μM and SB203580 (Biomol); or their vehicles, as indicated. Alternatively, cells were coincubated with cysLTR antagonists, MK571, LY171833, or BAYu9773 (10 μM; Biomol); eotaxin (6 nM); inhibitors of LTC<sub>4</sub> carrier-proteins, probenecid (0.5–2 mM; Sigma-Aldrich); MK571 (10 μM) and cyclosporin A (1 μg/ml; Sigma-Aldrich); or their vehicles, as indicated. Stock solutions of stimuli and inhibitors were prepared in HBSS<sup>-/-</sup> containing 0.1% of endotoxin-free ovalbumin, aliquoted and stored at -20°C. AA861, MK886, LY294002, calphostin C, herbimycin, genistein, PD98059, U0126, SB203580, LY171833, BAYu9773, and A23187 were diluted in DMSO. The final DMSO concentration was <0.01% and had no effect on eosinophils.

**Permeabilization of Viable Eosinophils.** To enable the entrance of cysLTs into eosinophils, freshly purified eosinophils (at 2 × 10<sup>6</sup> cells/ml) were semi-permeabilized using a protocol published elsewhere (40) with minor modifications. In brief, cells were incubated for 1 h at room temperature in a semi-permeant solution (1% methanol and 1% DMSO in HBSS<sup>-/-</sup>). Cells were washed in RPMI, mixed with agarose matrix, and used in EliCell assay. Alternatively, after 1 h of semi-permeabilization, eosinophils were washed and either stained with osmium for analysis of lipid body content (41) or checked by flow cytometry, as described earlier (16), to verify that the normal content of preformed IL-4 was unchanged. The success of the permeabilization procedure was confirmed by the ability of eosinophils to rapidly incorporate 10 μM pyrenedodecanoic acid (p96; Molecular Probes), a blue fluorescent fatty acid, in their lipid bodies within 15 min, whereas control nonpermeabilized cells showed no p96 staining (not depicted).

**Statistical Analysis.** Data were expressed as means ± SD. Percent inhibition with antagonists was calculated in comparison to stimulated increases in LTC<sub>4</sub> 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.

## Results

**Endogenous 5-LO Pathway-derived Eicosanoids Regulate Eosinophil IL-4 Secretion.** We have shown previously that preformed eosinophil granule stores of IL-4 may be rapidly and selectively released extracellularly by vesicular transport-mediated mechanisms activated via CCR3 either by exogenous eotaxin and RANTES or by these chemokines released from IL-16-stimulated eosinophils (16, 39). These CCR3-acting chemokines also prime calcium ionophore (A23187)-activated eosinophils for greater extracellular release of the dominant 5-LO eicosanoid of eosinophils, LTC<sub>4</sub>, and directly elicit 5-LO activation and low level formation of LTC<sub>4</sub> detectable intracellularly at lipid body domains (20). Therefore, we investigated whether 5-LO-derived eicosanoids might have signal-transducing roles in regulating cytokine secretion from human eosinophils. Pretreatment with two mechanistically distinct inhibitors of 5-LO (AA861 and MK886) blocked IL-16-, eotaxin-, and RANTES-induced IL-4 release from eosinophils (Fig. 1 A). In contrast, pretreatment of eosinophils with either AA861 or MK886 did not inhibit IL-16- or eotaxin-induced release of RANTES (Fig. 1 B). The 5-LO inhibitors did not inhibit the cytolytic and exocytotic release of either IL-4 or RANTES elicited by A23187 (Fig. 1). Thus, eosinophil-derived 5-LO metabolites participate in CCR3

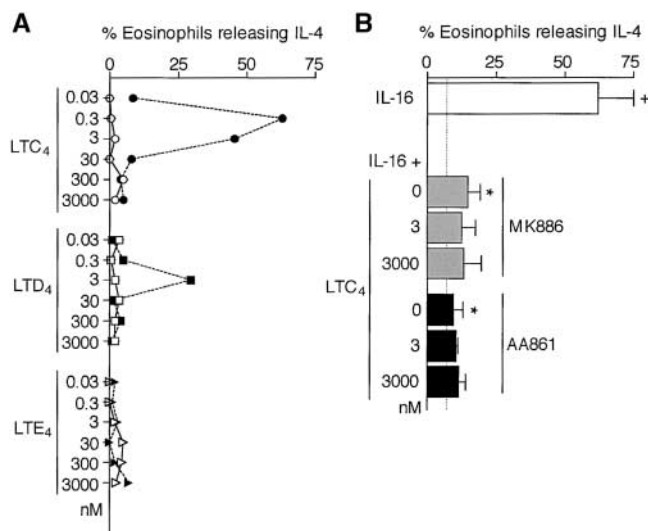


**Figure 1.** Effects of 5-LO pathway inhibitors on IL-16-induced, eotaxin-, RANTES-, and calcium ionophore (A23187)-induced IL-4 (A) or RANTES (B) release. Human eosinophils were pretreated for 30 min with 10 μM AA861 or MK886, as indicated. Cells were mixed in EliCell gel-matrix and stimulated for 1 h with IL-16 (100 nM), eotaxin (6 nM), RANTES (6 nM), or A23187 (0.5 μM). Results were expressed as the percentages of eosinophils releasing IL-4 or RANTES extracellularly. Values are means ± SD from three independent assays. + and \* represent P < 0.01 compared with nonstimulated and cytokine/chemokine-stimulated eosinophils, respectively. Nonstimulated eosinophils exhibited 8 ± 4% positivity for IL-4 (n = 7) and 5 ± 3% positivity for RANTES release (n = 5).

chemokine-initiated signaling to release IL-4, but not RANTES, from eosinophils.

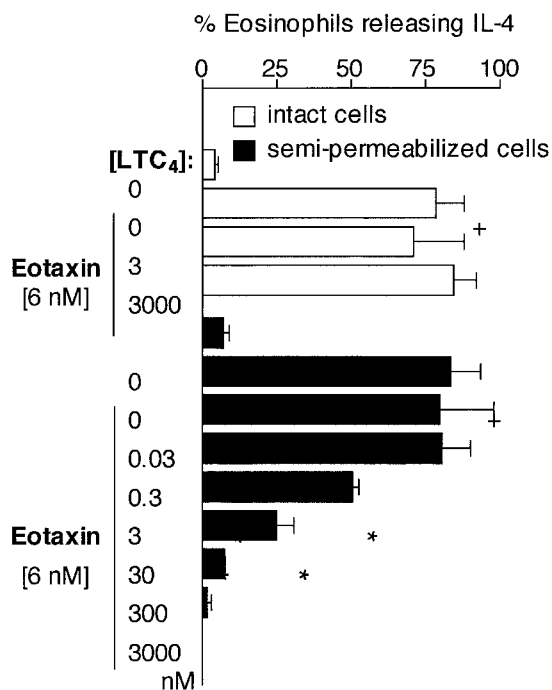
**Cysteinyl LTs Are Intracrine Signals Regulating Eosinophil IL-4 Secretion.** We evaluated which 5-LO pathway-derived eicosanoids, generated within and potentially released from eosinophils in response to CCR3 activation, were involved in IL-4 secretion from eosinophils. We first investigated LTC<sub>4</sub> and its extracellular derivatives, LTD<sub>4</sub> and LTE<sub>4</sub>. As shown in Fig. 2 A (open symbols), none of the three cysLTs at concentrations ranging from 0.03 to 3,000 nM were able to induce IL-4 or RANTES (not depicted) release from intact eosinophils. Likewise, exogenous 5-HETE (1, 10, and 100 nM), another potential eosinophil 5-LO-derived eicosanoid, failed to trigger IL-4 release from intact eosinophils (not depicted). 1 μM PAF, which can activate the 5-LO pathway in eosinophils (41, 42), also did not elicit IL-4 release (not depicted).

To ascertain whether the 5-LO product LTC<sub>4</sub> was necessary but not sufficient to induce IL-4 secretion from eosinophils, eosinophils were pretreated with 5-LO pathway inhibitors (AA861 or MK886) and stimulated with IL-16 with or without exogenous LTC<sub>4</sub> (3 or 3,000 nM). As shown in Fig. 2 B, the co-stimulation of eosinophils with exogenous LTC<sub>4</sub> was unable to restore the ability of IL-16 to induce IL-4 release that was blocked by the 5-LO inhibitors. These data indicate that extracellular stimulation with exogenous LTC<sub>4</sub> is ineffective in regulating IL-4 release from intact eosinophils.



**Figure 2.** Exogenous LTC<sub>4</sub> and LTD<sub>4</sub>, but not LTE<sub>4</sub>, stimulate IL-4 secretion only from plasma membrane-permeabilized eosinophils. (A) Intact (open symbols) or permeabilized (closed symbols) viable eosinophils were stimulated for 3 h with a range of cysLT concentrations (0.03–3,000 nM), as indicated. Results were expressed as the percentages of eosinophils releasing IL-4 extracellularly. Values are representative from three independent assays. (B) Exogenous LTC<sub>4</sub> was not able to restore IL-16-induced IL-4 release from intact eosinophils blocked by pretreatments (30 min) with 5-LO pathway inhibitors (AA861 and MK886 at 10 μM). Values are means ± SD from three independent assays. + and \* represent P < 0.01 compared with nonstimulated (7 ± 2% positive, dashed line) and IL-16-stimulated eosinophils, respectively.

Because cysLTs are not permeable across the plasma membrane and we have shown previously that LTC<sub>4</sub> is synthesized intracellularly at cytoplasmic lipid bodies within IL-16-, eotaxin-, or RANTES-stimulated eosinophils (20, 39), we investigated whether cysLTs acting intracellularly could trigger IL-4 release. To investigate such potential intracrine signaling, we permeabilized eosinophils in order to allow impermeable cysLTs to enter eosinophils. We established that permeabilized eosinophils in comparison with intact (nonpermeabilized) eosinophils retained their basal content of IL-4 (as assessed by flow cytometry) and their normal resting numbers of cytoplasmic lipid bodies, and were normally responsive to eotaxin in forming new lipid bodies (not depicted) or releasing IL-4 (Fig. 3). In contrast to intact eosinophils, both LTC<sub>4</sub> and LTD<sub>4</sub> stimulated IL-4 release (Fig. 2 A, closed symbols) but not RANTES release (not depicted) from permeabilized eosinophils. LTC<sub>4</sub> was more active than LTD<sub>4</sub>, with LTC<sub>4</sub> stimulating greater percentages of eosinophils to release IL-4 extracellularly and with peak activity (0.3 nM) at a 10-fold lower concentration than with LTD<sub>4</sub>. Notably, higher concentrations (≥30 nM) of either LTC<sub>4</sub> or LTD<sub>4</sub> did not elicit IL-4 release (Fig. 2 A). Neither LTE<sub>4</sub> (Fig. 2 B, closed symbols), 5-HETE (not depicted), nor PAF (not depicted) stimulated IL-4 release from permeabilized eosinophils. Therefore, intracellular LTC<sub>4</sub> formed after 5-LO activation

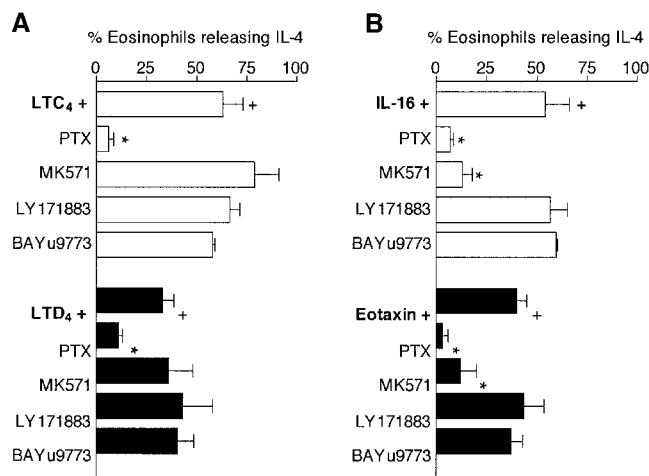


**Figure 3.** High concentrations of intracellular LTC<sub>4</sub> block eotaxin-induced IL-4 secretion from plasma membrane-permeabilized eosinophils. Intact (open bars) and permeabilized (closed bars) eosinophils were co-stimulated with 6 nM eotaxin plus different concentrations (0.03–3,000 nM) of LTC<sub>4</sub> (as indicated) for 3 h. Results were expressed as the percentages of eosinophils exhibiting secreted extracellular IL-4. Values are means ± SD from three independent assays. + and \* represent P < 0.01 compared with nonstimulated and eotaxin-stimulated eosinophils, respectively.

functions even at low concentrations as an intracrine, and not extracellular autocrine, mediator to regulate the differential secretion of IL-4 (vs. RANTES) induced by IL-16, eotaxin, or RANTES.

**Activation of a Novel Intracellular CysLTR Elicits IL-4 Secretion.** The characteristics of the receptor-mediated signaling initiated by intracrine LTC<sub>4</sub> were evaluated. Pretreatment of eosinophils with pertussis toxin (Fig. 4 A) significantly inhibited LTC<sub>4</sub>- and LTD<sub>4</sub>-elicited IL-4 secretion from permeabilized eosinophils, suggesting the involvement of a G<sub>i/o</sub>-like protein-coupled cysLTR. The two G protein-coupled cysLTRs that have been cloned and characterized are CysLT1 and CysLT2 (25–28). Human eosinophils contain mRNA transcripts for both CysLT1 and CysLT2 (28, 35). These receptors can be distinguished, in part, by their ligand binding affinities as studied in transfected cells. CysLT1, which is also activated by the pyriminidergic ligand, UDP (29), binds LTD<sub>4</sub> with a 10-fold greater affinity than it binds LTC<sub>4</sub>, whereas CysLT2 binds LTC<sub>4</sub> and LTD<sub>4</sub> with equal affinities. In contrast, the intracellular cysLTR mediating signaling for IL-4 release from permeabilized eosinophils was activated by LTC<sub>4</sub> at 10-fold lower concentrations than LTD<sub>4</sub> (Fig. 2 A; 0.3 vs. 3 nM, respectively). Moreover, the pyriminidergic ligands UTP and UDP (each at 3 and 3,000 nM), the latter an agonist for CysLT1, did not elicit IL-4 release from permeabilized eosinophils (not depicted).

We also used specific receptor antagonists of CysLT1 (MK571 and LY171883) that do not inhibit CysLT2, and BAYu9773, a receptor antagonist for CysLT1 that also de-



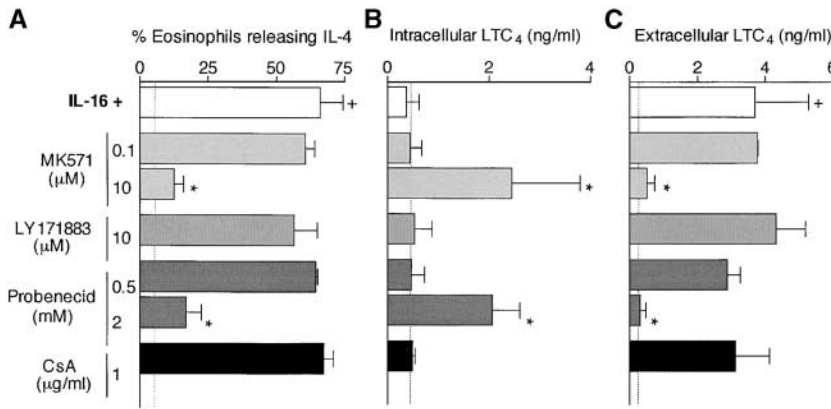
**Figure 4.** Effects of PTX and cysLTR antagonists on LTC<sub>4</sub>- and LTD<sub>4</sub>-elicited IL-4 secretion from plasma membrane-permeabilized eosinophils. (A) Permeabilized eosinophils were pretreated for 30 min with PTX (20 ng/ml), MK571 (10 μM), LY171883 (10 μM), or BAYu9773 (10 μM), and then stimulated for 3 h with LTC<sub>4</sub> (3 nM) or LTD<sub>4</sub> (3 nM). (B) Intact eosinophils were pretreated as described in A, and then stimulated for 1 h with 100 nM IL-16 or 6 nM eotaxin. Results were expressed as the percentages of eosinophils exhibiting secreted extracellular IL-4. Values are means ± SD from three independent assays. + and \* represent P < 0.01 compared with nonstimulated (6 ± 3% positive) and stimulated eosinophils, respectively.

sensitizes CysLT2 to cysLTs (27). As shown in Fig. 4 A, these antagonists of the two known cysLTRs failed to inhibit LTC<sub>4</sub>-elicited IL-4 secretion from permeabilized eosinophils, providing pharmacologic evidence that the intracrine signaling of LTC<sub>4</sub> was mediated by neither of the two known cysLTRs. In intact eosinophils stimulated to release IL-4 with either IL-16 or eotaxin, neither the CysLT1 antagonist LY171883 nor the dual receptor antagonist BAYu9773 inhibited IL-4 release (Fig. 4 B), again indicating in intact eosinophils that intracrine LTC<sub>4</sub> signaling was not acting via either CysLT1 or CysLT2 receptors.

Interestingly, pretreatment of eosinophils with MK571 did inhibit IL-4 secretion induced by both IL-16 and eotaxin (Fig. 4 B). MK571, in addition to being a CysLT1 antagonist, is also an inhibitor of multidrug resistance protein 1 (MRP1; references 43, 44), a protein carrier that mediates the extracellular export of impermeable LTC<sub>4</sub> from within cells (45, 46). To assess whether MK571's effect depended on its activity as an MRP1 inhibitor of LTC<sub>4</sub> export, we evaluated its effect on the intracellular levels of LTC<sub>4</sub> within IL-16-stimulated cells and compared its effect with another known inhibitor of LTC<sub>4</sub> export from eosinophils, probenecid (21). With intact IL-16-stimulated eosinophils, both inhibitors of LTC<sub>4</sub> transport, probenecid and MK571, blocked extracellular release of LTC<sub>4</sub> into eosinophil supernatants (see Fig. 5 C), increased intracellular LTC<sub>4</sub> levels (see Fig. 5 B), and inhibited IL-4 release (Fig. 5 A). Neither probenecid nor MK571 altered the intracellular content of IL-4, as assessed by flow cytometry in permeabilized eosinophils (16) (n = 3, not depicted). In contrast, control agents, LY171883, the CysLT1 inhibitor, and cyclosporin A, an inhibitor of another ATP-dependent, multidrug resistance P-glycoprotein transporter not involved in LTC<sub>4</sub> transport (47, 48), were without inhibitory effects on these three responses (Fig. 5). With inhibition of LTC<sub>4</sub> extracellular export in these IL-16-stimulated eosinophils, intracellular LTC<sub>4</sub> levels would be well in excess of ~5 nM LTC<sub>4</sub> concentrations measured in eosinophil extracts from 10<sup>6</sup> eosinophils/ml. The inhibition of IL-4 release by these elevated intracellular levels of LTC<sub>4</sub> would accord with the absence of IL-4 release with permeabilized eosinophils exposed to >30 nM LTC<sub>4</sub> concentrations either alone (Fig. 2 A) or together with eotaxin stimulation (Fig. 3).

We investigated the sites of LTC<sub>4</sub> formation using immunofluorescent localization of newly formed and carbodiimide-immobilized LTC<sub>4</sub> (20). As reported previously (20, 39), in IL-16- and RANTES-stimulated eosinophils (Fig. 6 A), agonist-induced and newly synthesized LTC<sub>4</sub> was focally localized at cytoplasmic lipid bodies. With inhibition of LTC<sub>4</sub> export with either probenecid or MK571, both the percentages of eosinophils and the numbers of intracellular lipid bodies exhibiting immunodetectable LTC<sub>4</sub> increased (Fig. 6).

**Downstream Signaling of LTC<sub>4</sub>-induced Vesicular Transport-mediated Eosinophil IL-4 Secretion.** We have shown recently that LTC<sub>4</sub> production at cytoplasmic lipid bodies within CCR3 chemokine-stimulated eosinophils

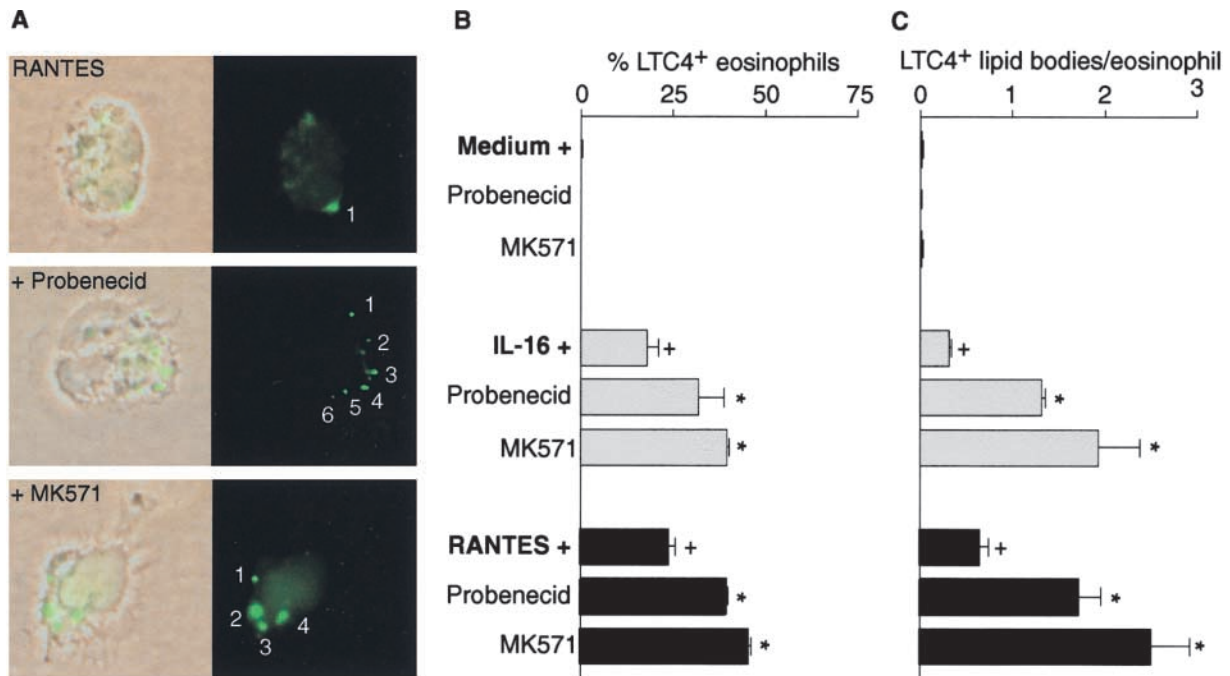


**Figure 5.** Blockade of the extracellular export of endogenously produced LTC<sub>4</sub> inhibits eosinophil IL-4 secretion. Intact eosinophils were pretreated for 30 min with MK571, LY171883, probenecid, or cyclosporin A (CsA), and then stimulated with IL-16 (100 nM) for 3 h. (A) The percentages of eosinophils exhibiting extracellularly secreted IL-4; (B) the intracellular concentration of LTC<sub>4</sub> within 10<sup>6</sup> eosinophils/ml; and (C) the extracellular concentration of LTC<sub>4</sub> released by 10<sup>6</sup> eosinophils/ml. Values are means ± SD from three independent assays. + and \* represent P < 0.01 compared with nonstimulated (dashed lines) and IL-16-stimulated eosinophils, respectively.

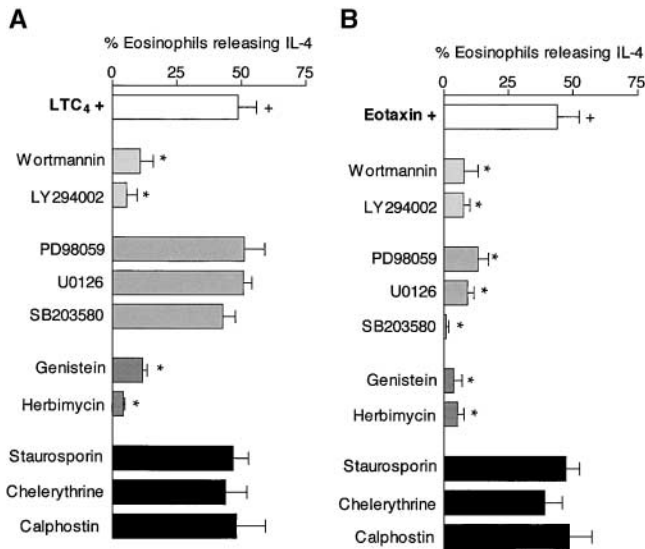
depended on activation of PI3K and MAP kinases (20). With specific kinase inhibitors, we investigated the downstream signaling of LTC<sub>4</sub>- and eotaxin-induced IL-4 release from eosinophils. Both LTC<sub>4</sub> and eotaxin-induced IL-4 release were blocked by inhibitors of PI3K (wortmannin, LY294002) and tyrosine kinases (genistein, herbimycin A), but not by PKC inhibitors (staurosporin, chelerythrine, and calphostin C; Fig. 7). In contrast, the MAP kinase inhibitors, PD98059 and U0126 (inhibitors of the extracellular signal-regulated kinases (ERK)1/2 activating kinase MEK [MAP ERK kinase]), and SB203586 (a p38 MAP kinase inhibitor), significantly inhibited eotaxin's actions

(Fig. 7 B) without affecting LTC<sub>4</sub>-induced IL-4 secretion (Fig. 7 A).

In IL-16-, eotaxin-, and RANTES-stimulated eosinophils, the mobilization and extracellular secretion of preformed IL-4 occurs rapidly by a selective, noncytotoxic, brefeldin A (BFA)-inhibitable, vesicular transport-mediated mechanism (20, 39). Similarly, LTC<sub>4</sub>-induced IL-4 release from permeabilized eosinophils was morphologically consistent with vesicular transport (not depicted) and was significantly inhibited by BFA (Table I), indicating that a vesicular transport-mediated process was also responsible for LTC<sub>4</sub>-elicited selective secretion of IL-4.



**Figure 6.** Effect of blockade of LTC<sub>4</sub> export on IL-16- and RANTES-elicited LTC<sub>4</sub> detection at lipid bodies within eosinophils. Intact eosinophils were pretreated for 30 min with 2 mM probenecid or 10 μM MK571, and then stimulated with medium alone, IL-16 (100 nM) or RANTES for 3 h. (A) Phase-contrast (left) and anti-LTC<sub>4</sub> immunofluorescent (right) microscopic images of identical fields of eosinophils within an agarose matrix are shown. Anti-LTC<sub>4</sub> immunofluorescent images (green) are overlaid on phase-contrast images to facilitate their intracellular LTC<sub>4</sub> localization within eosinophils. Inserted numbers indicate lipid bodies exhibiting positive anti-LTC<sub>4</sub> immunofluorescent staining. (B) The mean ± SD percentages of eosinophils exhibiting green immunofluorescent labeling for intracellular immunoreactive LTC<sub>4</sub>. Results in C are the mean ± SD numbers of lipid bodies exhibiting immunolabeling for LTC<sub>4</sub> within each scanned eosinophil (50 cells were scanned per slide).



**Figure 7.** Effect of kinase inhibitors on LTC<sub>4</sub>- and eotaxin-induced IL-4 secretion from eosinophils. (A) Plasma membrane-permeabilized eosinophils were pretreated for 30 min with wortmannin (1 μM), LY294002 (10 μM), PD98059 (10 μM), U0126 (10 μM), SB203580 (10 μM), genistein (10 μM), herbimycin (10 μM), staurosporin (1 μM), chelerythrine (10 μM), or calphostin C (1 μM), and then stimulated for 3 h with LTC<sub>4</sub> (3 nM). (B) Intact eosinophils were pretreated as described in A and then stimulated for 1 h with eotaxin (6 nM). Results were expressed as the percentages of eosinophils exhibiting extracellular IL-4. Values are means ± SD from three independent assays. + and \* represent P < 0.01 compared with nonstimulated (A, 6 ± 3% baseline positive; B, 5 ± 2% positive) and stimulated eosinophils, respectively.

## Discussion

Amongst leukocytes, eosinophils are unique, in part, due to their complex content of proteins stored within their cytoplasmic granules. Mechanisms for differentially mobilizing these granule-stored proteins for their extracellular release may enable eosinophils to selectively and rapidly influence various immune, inflammatory, and other responses. In contrast to many leukocytes in which cytokines must be synthesized *de novo* before their release, eosinophils within their specific granules contain a diversity of already preformed chemokines and cytokines, including nominally Th1 and Th2 cytokines with diverse and potentially opposing functions (for review see reference 7). It is increasingly recognized that degranulation based on wholesale exocytosis that extrudes the full granule contents extracellularly does not provide for specificity of release of selected eosinophil granule contents. Instead, as yet poorly understood mechanisms can selectively load vesicles with specific granule proteins, and these vesicles may traffic to the cell surface to release these proteins at the cell surface (14, 16). Because the selective release of cytokines can provide a means for eosinophils to rapidly influence adjacent cells in normal or inflamed tissue sites, we investigated mechanisms involved in the selective mobilization and vesicle-mediated secretion of specific cytokines, including IL-4, a Th2-type cytokine with diverse actions pertinent to allergic and parasitic diseases (12, 13), and the chemokine RANTES. Our current

**Table I.** Vesicular Transport Mediates Intracellular LTC<sub>4</sub>-induced IL-4 Secretion from Eosinophils

Condition	Percent eosinophils releasing IL-4	IL-4 fluorescent intensity/cell
Medium	3.9 ± 5.1	4 ± 1
LTC <sub>4</sub>	37.8 ± 7.8 <sup>a</sup>	832 ± 196 <sup>a</sup>
+ BFA (0.1 μg/ml)	30.3 ± 8.8	472 ± 186 <sup>b</sup>
+ BFA (1 μg/ml)	19.3 ± 7.8 <sup>b</sup>	111 ± 70 <sup>b</sup>

Plasma membrane-permeabilized eosinophils were pretreated for 30 min with an inhibitor of vesicle formation, BFA, and then stimulated with 3 nM LTC<sub>4</sub>. After 3 h, IL-4 secretion was analyzed in EliCell assays. Results are either the percentages of eosinophils exhibiting extracellular IL-4 or the average of electronically measured immunofluorescent intensities of extracellular IL-4 (in arbitrary units). Values are means ± SD from three independent assays.

<sup>a</sup>P < 0.01 compared with nonstimulated eosinophils.

<sup>b</sup>P < 0.01 compared with LTC<sub>4</sub>-stimulated eosinophils.

studies provide several new insights into the signal transduction processes that contribute to the selective mobilization and release of specific granule-derived cytokines and chemokines from eosinophils.

Although eotaxin and RANTES each act via CCR3 to stimulate the secretion of both IL-4 and RANTES from eosinophils (15, 16, 39), only the release of IL-4 was dependent on the activation of 5-LO to form LTC<sub>4</sub> within eosinophils. Two 5-LO pathway inhibitors (MK886 and AA861) blocked eotaxin- and IL-16-stimulated IL-4 release but not RANTES release (Fig. 1); in permeabilized eosinophils, exogenous LTC<sub>4</sub> stimulated IL-4 (Fig. 2), but not RANTES, release. Thus, common activation via CCR3 can be differentially linked to downstream signaling mechanisms that for mobilization and release of IL-4, but not RANTES, are specifically dependent on 5-LO pathway activation to form LTC<sub>4</sub>.

Because cysLTs are not permeable across plasma membranes, it was possible to determine that LTC<sub>4</sub>, formed in response to CCR3-mediated activation, was functioning solely as an intracrine signal-transducing mediator. Exogenous LTC<sub>4</sub> and its derivative cysLTs did not stimulate IL-4 release from intact eosinophils, but LTC<sub>4</sub>, and to a lesser extent LTD<sub>4</sub>, did in eosinophils whose membranes were permeabilized to enable the intracellular entry of these cysLTs (Fig. 2). Thus, a novel role for endogenously formed LTC<sub>4</sub> within eosinophils as an intracellular signal-transducing mediator has been revealed.

Stimulation of eosinophils with CCR3-acting chemokines has been shown to lead to the formation of low levels of LTC<sub>4</sub> detectable by immunofluorescent microscopy at intracellular lipid bodies within eosinophils in the absence of detectable immunoassayed concentrations of extracellularly released LTC<sub>4</sub> (20). Several findings indicate that such low level intracellular LTC<sub>4</sub> may be functional in regulating IL-4 release. In permeabilized eosinophils, only low

levels of LTC<sub>4</sub> were active in stimulating IL-4 release, with maximal activity at 0.3 nM (Fig. 2). Greater LTC<sub>4</sub> concentrations (>3 nM) were inhibitory as indicated in permeabilized eosinophils (Fig. 2) and confirmed with the suppression of IL-4 release from eotaxin-stimulated, permeabilized eosinophils (Fig. 3). Similarly, the blockade of extracellular LTC<sub>4</sub> export with MK571 and probenecid raised intracellular LTC<sub>4</sub> levels in intact eosinophils and inhibited IL-16-stimulated IL-4 release. Whether the inhibition at higher LTC<sub>4</sub> concentrations represents high dose inhibition at a single type of cysLTR or engagement of another antagonistic receptor is unknown. Thus, LTC<sub>4</sub> has dose-dependent roles as an intracrine signal-transducing mediator. Notably, at low intracellular concentrations, newly synthesized LTC<sub>4</sub>, as detectable by immunofluorescent localization at lipid bodies in eotaxin- and RANTES-stimulated eosinophils (20), can signal to promote IL-4 secretion. At higher intracellular LTC<sub>4</sub> concentrations, LTC<sub>4</sub> inhibits this release of preformed IL-4. Agents such as MK571, which blocks the extracellular export of LTC<sub>4</sub> (in addition to inhibiting CysLT1; references 45, 46), may exert anti-inflammatory effects by increasing intracellular LTC<sub>4</sub> and thereby inhibiting the secretion of some eosinophil cytokines.

The IL-4-releasing activity of intracellular and not exogenous LTC<sub>4</sub> indicated that plasma membrane expressed cysLTRs, that for human eosinophils may include both of the currently known cysLTRs, CysLT1 and CysLT2 (28, 35), were not involved. Instead, an intracellular cysLTR must be involved. A number of findings indicated that this cysLTR was distinct from either CysLT1 or CysLT2. LTC<sub>4</sub> was 10-fold more potent than LTD<sub>4</sub> in eliciting IL-4 release from permeabilized eosinophils. Because LTD<sub>4</sub> is formed extracellularly from LTC<sub>4</sub>, this is further consistent with an intracrine role preferentially for LTC<sub>4</sub>. In contrast, CysLT1 is more potently activated by LTD<sub>4</sub> than LTC<sub>4</sub>, whereas both of these cysLTs are equipotent on CysLT2 (25, 27, 28). CysLT1 can be activated by the pyriminidergic ligand UDP (29), but neither UDP nor UTP stimulated IL-4 release from permeabilized eosinophils. Moreover, the CysLT1 inhibitor LY171883 and the CysLT1 and CysLT2 dual inhibitor BAYu9773 failed to inhibit IL-4 secretion from intact eosinophils stimulated with IL-16 or eotaxin and from permeabilized eosinophils stimulated with LTC<sub>4</sub> and LTD<sub>4</sub> (Fig. 4). Thus, the intracrine activity of LTC<sub>4</sub> is likely mediated via a cysLTR distinct from either CysLT1 or CysLT2.

Known cysLTRs are seven transmembrane spanning receptors that may signal at least some responses via G proteins. LTC<sub>4</sub> elicitation of eosinophil IL-4 release was inhibited by PTX, suggesting the intracellular cysLTR functions via coupling to PTX-sensitive G<sub>α/o</sub>-like proteins. Downstream signaling via this cysLTR to elicit IL-4 secretion required activation of PI3K and genistein- and herbimycin-inhibitable tyrosine kinases, but not PKC or MAP kinases (Fig. 7). PI3K and tyrosine kinase activation were also required for eotaxin and RANTES CCR3-mediated activation of IL-4 release. Because genistein- and herbimycin-inhibitable tyrosine kinases do not block eotaxin- and

RANTES-elicited lipid body formation for enhanced LTC<sub>4</sub> formation (20), the roles of these tyrosine kinases in signal transduction leading to IL-4 release is solely downstream of LTC<sub>4</sub>-cysLTR-initiated signaling. In contrast, PI3K activation is required at two points in the chemokine-initiated signaling, PI3K inhibitors block CCR3-acting chemokines from forming new lipid bodies and generating LTC<sub>4</sub> (20), and as shown here also block the downstream signaling initiated by LTC<sub>4</sub> at its receptor (Fig. 7). The MAP kinases, ERK 1/2 and p38, act proximally in the CCR3-chemokine-initiated signaling to form LTC<sub>4</sub> (20), but do not act downstream of the cysLTR signaling for IL-4 release from eosinophils.

There is limited precedence for cysLTs eliciting cytokine secretion. With human placental cord blood-derived eosinophils, we have shown that cysLTs stimulate the vesicular transport-mediated release of IL-4 (49), but the findings with those IL-3- and IL-5-stimulated cells differed in several regards from what we demonstrate with normal blood-derived eosinophils. Cord blood-derived eosinophils responded not to LTC<sub>4</sub> but to LTD<sub>4</sub>, which acted extracellularly on plasma membrane receptors that were inhibited by two inhibitors of CysLT1 (LY171833 and MK571) and required much higher concentrations of LTD<sub>4</sub> (10<sup>-7</sup>-10<sup>-6</sup> M; reference 49). Human cord blood-derived mast cells, after exposure to IL-4, also have been shown to respond to cysLTs with the release of cytokines (30). Exogenous LTC<sub>4</sub> and LTD<sub>4</sub>, which were equipotent at 10<sup>-7</sup> M concentrations, acted on a UDP-activatable and MK571-inhibitable cysLTR. This differs from our findings with human eosinophils, in which the cysLTR was intracellular, activatable preferentially by LTC<sub>4</sub> at much lower 10<sup>-10</sup>-10<sup>-9</sup> M concentrations, and not inhibitable by CysLT1 or CysLT2 inhibitors.

In contrast to human cord blood-derived mast cells, in which cytokine release required de novo transcription and translation to generate new cytokines (30), a difference with eosinophils is that they contain preformed cytokines that can be rapidly mobilized within minutes from granule stores and released by a BFA-inhibitable, vesicular transport-mediated mechanism (Table I), which, as we have shown previously for IL-4, does not require new IL-4 transcription or protein synthesis (16). The release of granule-derived IL-4 at the surface of eosinophils was sensitively detected by EliCell assays at levels beneath the sensitivity (>15 pg/ml) of conventional ELISA assays (including with LTC<sub>4</sub>-stimulated, permeabilized eosinophils; not depicted). A role for eosinophil-derived IL-4 has been demonstrated in a murine system in which the intraperitoneal instillation of *Schistosoma mansoni* eggs leads to the release of IL-4 derived from peritoneal exudate eosinophils (50). Fundamental issues in delineating eosinophil contributions to immunologic reactions are understanding the stimuli and intracellular signaling mechanisms that may selectively release some and not other preformed cytokines and chemokines from granule stores. Eotaxin and RANTES elicit IL-4, but not IL-12, release (e.g., from eosinophils; reference 39). As demonstrated here with permeabilized eosinophils,



LTC<sub>4</sub> stimulated IL-4 but not RANTES release and likewise did not elicit IL-12 release (not depicted). Thus, some of the specificity in secreting selective cytokines and chemokines from eosinophils is based on the activation of intracellular LTC<sub>4</sub> signal-transducing pathways.

In summary, our findings support a role for a novel intracellular cysteinyl LT receptor, apparently distinct from known CysLT1 or CysLT2, that functions via coupling to PTX-sensitive G<sub>iα/o</sub>-like proteins. By means of this cys-LTR, LTC<sub>4</sub> can function as an intracrine signal transducer that activates pathways that selectively mobilize IL-4, and not RANTES, for vesicular transport-mediated extracellular release. Therefore, the generation of LTC<sub>4</sub> within eosinophils is important not only for its varied activities as an extracellular paracrine mediator of inflammation, but also for its role as an intracrine signal-transducing molecule that may regulate fundamental cellular responses, including the selective secretion of cytokines from eosinophils.

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