Extranuclear Lipid Bodies, Elicited by CCR3-mediated Signaling Pathways, Are the Sites of Chemokine-enhanced Leukotriene C4 Production in Eosinophils and Basophils*

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Eosinophils and basophils, when activated, become major sources of cysteinyl leukotrienes, eicosanoid mediators pertinent to allergic inflammation. We show that the C-C chemokines, eotaxin and RANTES (regulated upon activation normal T cell expressed and secreted), activate eosinophils and basophils for enhanced leukotriene C4 (LTC4) generation by distinct signaling and compartmentalization mechanisms involving the induced formation of new cytoplasmic lipid body organelles. Chemokine-induced lipid body formation and enhanced LTC4 release were both mediated by CCR3 receptor G protein-linked downstream signaling involving activation of phosphoinositide 3-kinase, extracellular signal-regulated kinases 1 and 2, and p38 mitogen-activated protein kinases. Chemokine-elicited lipid body numbers correlated with increased calcium ionophore-stimulated LTC4 production; and as demonstrated by intracellular immunofluorescent localization of newly formed eicosanoid, lipid bodies were the predominant sites of LTC4 synthesis in both chemokine-stimulated eosinophils and chemokine-primed and ionophore-activated eosinophils. Eotaxin and RANTES initiated signaling via phosphoinositide 3-kinase and mitogen-activated protein kinases both elicit the formation of lipid body domains and promote LTC4 formation at these specific extranuclear sites.

Central to the pathogenesis of allergic diseases are both the recruitment and subsequent activation of specific leukocytes, including notably eosinophils and basophils, at sites of allergic inflammation (1–3). Eosinophils and basophils are major potential sources of cysteinyl leukotrienes (LTs)1 (LTC4 and its extracellular derivatives, LTD4 and LTE4), products of the 5-lipoxygenase (5-LO) pathway of arachidonic metabolism (4). Cysteinyl LTs, as paracrine mediators, cause bronchoconstriction, mucous hypersecretion, increased microvascular permeability, bronchial hyperresponsiveness, and eosinophil infiltration (5–7) and, as autocrine mediators, prolong eosinophil survival (8). Eosinophils and basophils contain the single LTC4-synthesizing enzyme, LTC4 synthase, and eosinophils are the predominant cellular source of this enzyme among resident and recruited cells in the bronchial tissues of asthmatics (9, 10). In all cells, the highly regulated generation of LTs is dependent on activation of specific phospholipases and LT-synthesizing enzymes and involves small molecules (e.g. Ca2+) and activation-dependent redistribution of 5-LO to specific membranous compartments within cells (11). One major candidate mechanism potentially involved in regulating LT formation is the translocation of 5-LO to the nuclear envelope (10, 11), but in eosinophils this nuclear translocation has been associated with both increased and decreased LTC4 formation (10, 12).

Although mechanisms that activate specific leukocytes to generate LTs currently focus on the perinuclear envelope as the site of regulated LT formation (11), eosinophils and other leukocytes associated with inflammatory reactions in vivo characteristically contain increased numbers of extranuclear lipid-rich domains in the form of cytoplasmic lipid bodies (13). These enigmatic organelles, often overlooked if their defining lipid content is lost during cell staining, have been implicated in many cell types associated with inflammation (14, 15). Although lipid bodies lack a delimiting membrane, their lipid content overcomes a poorly understood honeycomb membranous matrix (16). Neither the genesis nor function of these organelles is well defined. Although eicosanoid-forming enzymes have been localized to lipid bodies, including 5-LO and LTC4 synthase in eosinophil lipid bodies (16, 17), to date there is no direct evidence that lipid bodies are sites of eicosanoid synthesis.

Both C-C chemokines, eotaxin and RANTES (regulated upon activation normal T cell expressed and secreted), signaling via CCR3 receptors expressed on eosinophils (18) and basophils (19), are active in recruiting these leukocytes to sites of allergic inflammation (20). Since other chemoattractants can enhance LT formation (21), we investigated the intracellular pathways by which these chemokines may activate leukocytes to enhance their regulated formation of cysteinyl LTs. Notably, chemokine engagement of CCR3 receptors initiated G protein-linked downstream signaling involving phosphoinositide 3-kinase (PI3K) and the mitogen-activated protein (MAP) kinases, extracellular signal-regulated kinases (ERK) 1/2 and p38, to induce the formation of new lipid body organelles and to enhance eosinophil LTC4 release. Moreover, these cytokines elicited and promoted LTC4 formation via PI3K and MAP kinase signaling not at the perinuclear envelope but rather specifically at these extranuclear lipid body domains.

EXPERIMENTAL PROCEDURES

Eosinophil and Basophil Purification—Peripheral blood was obtained with informed consent from 12 normal donors, and eosinophils

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The abbreviations used are: LT, leukotriene; PI3K, phosphoinositide-3 kinase; 5-LO, 5-lipoxygenase; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; EIA, enzyme immunoassay; HBSS, Hank’s balanced salt solution; PAF, platelet activating factor; RANTES, regulated upon activation normal T cell expressed and secreted; IL, interleukin; EDAC, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide; mAb, monoclonal antibody.
Lipid Body Induction and Treatments—Eosinophil or basophil suspensions (10^6/ml) were incubated (37 °C) with eotaxin (1–100 ng/ml), RANTES (1–100 ng/ml), IL-8 (500 ng/ml) (R&D Systems), PAF (1 μM) (Calbiochem, La Jolla, CA), or medium alone for 1 h in HBSS−− and then cyt centrifuged (500 rpm, 5 min) onto glass slides. For inhibitor studies, cells were pre-treated for 30 min with anti-CCR3 mAb (clone 6E7; Sigma) (Alexa488-labeled rat anti-cysteinyl LT detection mAb) or with the isotype-matched rabbit IgG, goat serum, slides were incubated for 30 min with Alexa488-labeled rat anti-cysteinyl LT detection mAb (clone 6E7; Sigma) (Alexa488-labeled rat anti-cysteinyl LT detection mAb) with HBSS, the Alexa488-labeled rat anti-cysteinyl LT detection mAb (clone 6E7; Sigma) (Alexa488-labeled rat anti-cysteinyl LT detection mAb) was added (400 μl of 10 μg/ml for 1 h. Reactions were stopped by washing with 0.05% saponin, washed with HBSS, and then fixed with 2% paraformaldehyde (5 min) and stained with OsO4 as described above. To obtain a measure of polarized redistribution of lipid bodies, cells were divided approximately into two halves, one of which was pre-treated with eotaxin and the other with HBSS. Slides were viewed by both phase-contrast and fluorescence microscopy, and photography and image analysis were performed as above. Two hundred eosinophils were scored, and the percentages of those exhibiting green staining for intracellular immunoreactive LTC4 were calculated.

As a specificity control for the immunolocalization of LTC4, Alexa488-labeled rat IgG1 (Sigma) was routinely included as a non-immune isotype control for the anti-cysteinyl LT detection antibody. In addition, four other control conditions were evaluated: (i) substituting parafomaldehyde fixation with and without saponin permeabilization for the EDAC cross-linking and fixation step; (ii) using neutrophils rather than eosinophils embedded in the gel-matrix; (iii) pre-treating eosinophils in suspension for 30 min with the 5-LO activating protein inhibitor MK886 (10 μM) prior to chemokine stimulation, and (iv) pre-treating eosinophils with P33K inhibitor, wortmannin (1 μM), for 30 min prior to chemokine stimulation.

Analysis of Intracellular Distribution of Lipid Bodies in Agarose Matrix-embedded Eosinophils—To monitor changes in cell morphology, eosinophils were embedded in the agarose matrix, as described above, with or without chemokines. Following incubation of 1 h, cells were fixed with 2% parafomaldehyde (5 min) and stained with OsO4 as described above. To obtain a measure of polarized redistribution of lipid bodies, cells were divided approximately into two halves, one of which contained the nucleus. Lipid bodies were enumerated in the two halves, and those cells found to have >75% of their lipid bodies in the nuclear half of the cell were scored as exhibiting polarized lipid body distribution. In four experiments, 25–50 cells were analyzed.

Statistical Analysis—Data were expressed as mean ± S.D. Statistical comparisons were done by analysis of variance followed by Newman-Keuls Student’s test. Differences were considered significant when p < 0.05. Correlation coefficients were determined by linear regression with significance (F test) at p < 0.05.

RESULTS

Eotaxin and RANTES Increase and Redistribute Lipid Bodies—Resting eosinophils exhibited a spherical shape and a normal content of osmiophilic lipid bodies (9.2 ± 1.0 lipid bodies/cell, mean ± S.D., n = 12) distributed generally throughout the cytoplasm (Fig. 1A). As fully expected from prior fluid phase assays (18), eotaxin (Fig. 1B) and RANTES (Fig. 1C), but not the C-X-C chemokine, IL-8 (Fig. 1D), elicited dramatic changes in eosinophils, including cytoplasmic veiling, increased polarization of their overall shape and nuclear location, and prominent cytoplasmic projections (lamellipodia and uropodia). Not anticipated by prior results, eotaxin and RANTES also elicited changes in intracellular lipid bodies within eosinophils. Both chemokines dose-dependently induced new lipid body formation (Fig. 2A). The magnitude of lipid body
induction with each C-C chemokine (at 100 ng/ml) was comparable to that with 1 µM PAF, a recognized stimulus for leukocyte lipid body formation (16, 23, 26). In contrast, IL-8 failed to elicit lipid body formation. In eotaxin- and RANTES-stimulated eosinophils, lipid bodies were located in cytoplasmic projections and especially in the perinuclear area (Fig. 1, B and D). In C-C chemokine polarized cells, many lipid bodies became aligned in groups proximate to the nuclear membrane and in the margin of the trailing uropod. Eosinophils in which >75% of lipid bodies were in the perinuclear half of the cell were 56 ± 9% with eotaxin (100 ng/ml) (p < 0.05 versus unstimulated, n = 4) and 59 ± 5% with RANTES (100 ng/ml) (p < 0.05 versus unstimulated, n = 3) in contrast to only 16 ± 7% with unstimulated eosinophils (mean ± S.D., n = 4) and 19 ± 6% with IL-8 (500 ng/ml) (n = 3). Thus, eotaxin and RANTES induced both the formation of new lipid bodies and their polarized redistribution within eosinophils.

The capacity of the two chemokines to elicit new formation of intracellular lipid body organelles was not restricted to eosinophils. Basophils share with eosinophils several functional features including CCR3 expression and the ability to produce LTC₄ (19). Unstimulated basophils contained 3.1 ± 1.0 lipid bodies/cell (mean ± S.D., n = 4). Both eotaxin and RANTES, but not IL-8, stimulated new lipid body formation in basophils with magnitudes comparable to that with 1 µM PAF (Fig. 3, A and B).

The induction of lipid body formation in eosinophils and basophils by eotaxin and RANTES was mediated through the G protein-linked CCR3 chemokine receptor. Pre-treatment of eosinophils or basophils with either pertussis toxin or a blocking anti-CCR3 mAb (but not an isotype control antibody) significantly inhibited lipid body formation induced by both C-C chemokines (Table I, Fig. 3B). CCR3-initiated downstream signaling pathways active in lipid body formation included mobilization of intracellular pools of Ca²⁺ and PI3K and ERK 1/2 and p38 MAP kinases. Eotaxin-induced lipid body formation occurred in a Ca²⁺-free medium and was blocked (by 82 ± 1%; n = 3, p < 0.05) by pre-treatment with the cell permeable Ca²⁺ chelator, BAPTA-AM (25 µM 77), but not by its impermeable analog BAPTA free acid (8 ± 8%). Wortmannin and LY294002, the PI3K inhibitors, PD98059 and U0126, two specific inhibitors of the ERK1/2 activating kinase (MAP ERK kinase), and SB203586, a p38 MAP kinase inhibitor, each inhibited eotaxin-stimulated lipid body formation (Fig. 4A). Thus, eotaxin stim-

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**Fig. 1. Intracellular distribution of lipid bodies in chemokine-stimulated eosinophils.** Eosinophils were incubated for 1 h with medium alone (A), eotaxin (100 ng/ml) (B), RANTES (100 ng/ml) (C), or IL-8 (500 ng/ml) (D). Lipid bodies were stained with osmium, and eosinophils were visualized by both phase-contrast (left panels) and light microscopy (right panels). Arrows indicate representative lipid bodies localized in lamellipodia, and arrowheads indicate lipid bodies closely localized around the nucleus in eotaxin- and RANTES-stimulated eosinophils. Bar, 5 µm. Images are representative of five independent experiments.
Correlation of LTC₄ production with increased lipid body formation

**Results**

**Chemokine-induced Lipid Bodies Are Sites of LTC₄ Formation**

Eosinophils were incubated with 0.1 M A23187 for 15 min for LTC₄ production. To normalize for differences in baseline LTC₄ release between different donors, results are presented as percentage of increase in the LTC₄ released by A23187-stimulated cells. Results are means ± S.D. from three to five independent assays. *, p < 0.05; **, p < 0.01, compared with negative controls.

**Fig. 2.** Lipid body formation (A) and priming for LTC₄ production (B) by C-C chemokine-stimulated human eosinophils. Eosinophils were incubated with indicated chemokines or PAF for 1 h for induction of lipid bodies. Thereafter, eosinophils were incubated with 0.1 μM A23187 for 15 min for LTC₄ production. To normalize for differences in baseline LTC₄ release between different donors, results are presented as percentage of increase in the LTC₄ released by A23187-stimulated cells. Results are means ± S.D. from three to five independent assays. *, p < 0.05; **, p < 0.01, compared with negative controls.

Both exogenous PAF (16, 23) and PAF formed endogenously in eosinophils in response to IL-5 or Fcγ receptor engagement (26) also signal via pertussis toxin-inhibitable G protein-linked PAF receptors to elicit lipid body formation. For PAF-induced lipid body formation, post-PAF receptor downstream signaling is obligately dependent on 5-LO activation to form 5(S)-hydroxyeicosatetraenoic acid (23). We, therefore, evaluated whether CCR3 downstream signaling to form lipid bodies was mediated either by autocrine PAF formation or dependent on endogenous 5-LO activation. Concentrations of the PAF receptor antagonist CV6209, the 5-LO inhibitor AA861, and the 5-LO activating protein inhibitor MK886 that blocked PAF-induced lipid body formation in eosinophils did not inhibit lipid body formation induced by the C-C chemokines (Table I). Other downstream signaling for lipid body formation also differed for PAF- and CCR3-mediated responses. Although PAF-elicited lipid body formation is inhibitable by the protein kinase C inhibitors, chelerythrine and calphostin C (27, 28), these inhibitors did not block CCR3-mediated lipid body formation in eosinophils (Fig. 4A). The tyrosine kinase inhibitors, herbimycin and genistein, inhibited neither CCR3-elicited (Fig. 4A) nor PAF-elicited (27) lipid body formation. Thus, the intracellular signaling pathways leading to lipid body induction differ between the PAF and CCR3 chemokine receptors, although both are G protein-linked, seven-transmembrane-spanning receptors that are expressed on eosinophils and basophils and can initiate lipid body formation.

**Eotaxin and RANTES Enhance Eosinophil and Basophil LTC₄ Production**—Both eotaxin and RANTES very effectively primed eosinophils for increased LTC₄ release in response to a submaximal 0.1 μM concentration of calcium ionophore A23187 (Fig. 2B). Pre-stimulation of eosinophils for 1 h with eotaxin or RANTES, but not with IL-8, dose-dependently evoked increases in A23187-induced LTC₄ production. At 100 ng/ml, eotaxin- and RANTES-pre-stimulated eosinophils released about 2.2- and 3.2-fold as much LTC₄ as did eosinophils challenged with A23187 alone. The increased quantities of LTC₄ generated by eosinophils primed with increasing concentrations of the C-C chemokines correlated highly with the increased numbers of elicited lipid bodies (r = 0.91 (p < 0.05) and r = 0.93 (p < 0.05), for eotaxin and RANTES, respectively) (Fig. 2, A and B). Moreover, inhibition of PI3K with wortmannin and LY294002, ERK1/2 with PD98059 or U0126, and p38 with SB203580, at concentrations that inhibited eotaxin-induced lipid body formation (Fig. 4A), also inhibited LTC₄ production by eosinophils (Fig. 4B). Again, inhibitors of protein kinase C or tyrosine kinase, which did not inhibit eotaxin-induced lipid body formation (Fig. 4A), also failed to affect the consequent LTC₄ production (Fig. 4B).

Although eosinophils stimulated for 1 h with 100 ng/ml eotaxin or RANTES formed more lipid bodies, quantities of LTC₄ released extracellularly in supernatants from eosinophils not activated by the calcium ionophore were not sufficient to be detectable by enzyme immunoassays (EIA) (data not shown). Supernatants of eotaxin- or RANTES-stimulated basophils also did not contain sufficient levels of LTC₄ to be detectable by EIA, but both C-C chemokines effectively primed basophils for increased LTC₄ release in response to A23187. At 100 ng/ml, eotaxin- and RANTES-pre-stimulated basophils released 8.1- and 9.3-fold, respectively, as much LTC₄ as did basophils challenged with A23187 alone. Moreover, pertussis toxin and anti-CCR3 neutralizing antibody blocked both lipid body induction (Fig. 3B) and enhanced LTC₄ production by basophils stimulated with eotaxin and RANTES (data not shown).

**Lipid Bodies Are Sites for Eotaxin- and RANTES-enhanced LTC₄ Synthesis in Eosinophils**—Since eotaxin- and RANTES-initiated signaling led to correlative quantitative increases in both lipid body formation and enhanced LTC₄ formation, we employed a new strategy for direct *in situ* immunolocalization of intracellular LTC₄ to ascertain the intracellular compartmentalization of cysteinyl LT synthesis. Unstimulated eosino-
strain the C-C chemokines induce formation of lipid bodies containing 5-LO in human basophils. Basophils were incubated for 1 h with medium alone, eotaxin (100 ng/ml), RANTES (100 ng/ml), or IL-8 (500 ng/ml). In A, images of representative basophils stained with osmium and visualized by both phase-contrast (left panels) and light microscopy (right panels) are shown. In B, lipid bodies were enumerated in 50 consecutive cells, and each value represents the mean ± S.D. of lipid bodies from one experiment representative of three experiments. C shows immunolocalization of 5-LO at lipid bodies in eotaxin-stimulated basophils. Focal, punctate staining of lipid bodies is present with 5-LO localization (Fig. 3B, arrowheads), and some focal staining at extranuclear sites, the pattern of LTC4 localization was different in eotaxin- and RANTES-primed eosinophils. In these C-C chemokine-primed and A23187-stimulated eosinophils, only occasional LTC4 immunofluorescence was detected with a linear perinuclear rim pattern (Fig. 6C, arrowhead). More extensive anti-LTC4 staining exhibited a punctate pattern, with very distinct focal staining proximate to, but separate from, the nucleus (Fig. 6B, asterisk) and within the uropod and lamellipodia (Fig. 6, B and C, arrows) of polarized eosinophils, fully consistent in size, form, number, and distribution with cytoplasmic lipid bodies (Fig. 1, B and C). Again, pre-treatment of eosinophils with the 5-LO pathway inhibitor, MK886, completely abolished all LTC4 immunostaining (Fig. 6, B and C). The PI3K inhibitor, wortmannin, which inhibited chemokine enhanced release of LTC4 from eosinophils (Fig. 4), inhibited chemokine-elicted LTC4 production only at all (both preformed and chemokine-elicted) lipid body sites, with no inhibitory effect on the immunoreactive LTC4 generated at the perinuclear membrane of eosinophils (Fig. 6). Notably, in A23187-activated eosinophils, wortmannin inhibited lipid body LTC4 formation, but inhibited neither LTC4 production at the perinuclear membrane (Fig. 6) nor overall LTC4 generation, as assessed by immunofluorescence microscopy (Table II) and ELISA assays of released LTC4 (data not shown).

In basophils, LTC4 localization at lipid bodies was not possible since these cells were destroyed during EDAC cross-linking and fixation, but basophil lipid bodies were sites of 5-LO localization (Fig. 3C).

DISCUSSION

Our results elucidate mechanisms whereby chemoattractant chemokines may activate a major functional response in eosinophils and basophils, specifically their capacity to generate cysteinyl LTs, and also provide novel findings pertinent to the regulated intracellular compartmentalization of eicosanoid
formation by multiple cell types. The C-C chemokines, eotaxin and RANTES, acting via CCR3 receptors on eosinophils and basophils, may not only recruit these leukocytes to sites of allergic inflammation but also activate distinct intracellular signaling and compartmentalization mechanisms within these recruited cells to enhance their capacity to form cysteinyl LTs. The means whereby these chemokines enhance eosinophil and basophil LT formation were suggested by the finding that these two C-C chemokines, but not IL-8, stimulated the formation of new cytoplasmic lipid bodies, organelles previously implicated in eicosanoid synthesis (17). This induction of lipid body formation was mediated via G protein-linked CCR3 chemokine receptors and was dependent on downstream activation of PI3K and the ERK1/2 and p38 MAP kinases. Activation of ERK and p38 MAP kinases in eosinophils in response to chemoattractants (21) and of PI3K in human basophils in response to IgE-mediated stimulation (29) has been shown to participate in the regulated activation of LTC4 formation, but the means for such regulation has been undefined. Inhibitors of PI3K, ERK1/2, and p38 MAP kinases inhibited both CCR3-mediated lipid body formation and CCR3-mediated priming for enhanced eosinophil LTC4 release. CCR3-mediated downstream signaling, in contrast to IL-5 and Fcy receptor-mediated stimulation of eosinophil lipid body formation (26), did not require endogenous PAF formation and, distinct from PAF, which signals through its own heterotrimeric G-protein-linked, seven-transmembrane-spanning receptor, was not dependent on intermediate 5-LO activity or PKC activation (23).

**Table I**

| Conditions | Lipid bodies/cell | Inhibition |
|------------|------------------|------------|
| Eotaxin (100 ng/ml) | 19.3 ± 1.2 | 100% |
| + Control IgG2a | 8.2 ± 1.5* | 0% |
| + Pertussis toxin | 19.7 ± 2.0 | 97% |
| + CV6209 | 9.1 ± 2.3* | 13% |
| + AA861 | 17.9 ± 1.4 | 0% |
| + MK886 | 20.1 ± 1.5 | 0% |
| RANTES (100 ng/ml) | 16.1 ± 1.0 | 11% |
| + α-CCR3 mAb | 21.6 ± 3.0 | 11% |
| + Control IgG2a | 9.7 ± 2.1* | 93% |
| + Pertussis toxin | 19.5 ± 1.3 | 6% |
| + CV6209 | 20.0 ± 1.2 | 12% |
| + AA861 | 19.7 ± 2.0 | 16% |
| + MK886 | 22.4 ± 2.3 | 91% |
| PAF (1 μM) | 18.0 ± 3.0 | 100% |
| + CV6209 | 7.5 ± 1.1* | 0% |
| + AA861 | 10.1 ± 2.0* | 86% |
| + MK886 | 8.2 ± 1.7* | 100% |

**Figure 4.** Signaling via activation of PI3K and ERK1/2 and p38 MAP kinases mediates lipid body formation (A) and priming for LTC4 production (B) by C-C chemokine-stimulated human eosinophils. Eosinophils were pretreated for 30 min with kinase inhibitors (as indicated) and then incubated with eotaxin for 1 h for induction of lipid bodies. Thereafter, eosinophils were incubated with 0.1 μM A23187 for 15 min for LTC4 production. To normalize for differences in base-line LTC4 release between different donors, results are presented as percentage of increase in the LTC4 released by A23187-stimulated cells. Results are means ± S.D. from four independent assays. **, p < 0.01 compared with eotaxin.
That the formation of new lipid body organelles elicited by CCR3-mediated signaling provided a distinct intracellular compartment for the regulated generation of LTC₄ was indicated by several findings. The numbers of eosinophil- and RANTES-elicited lipid bodies correlated with the magnitudes of the priming responses for increased extracellular release of LTC₄ elicited by prior exposure of eosinophils to eosinax and RANTES. Inhibitors of PI3K and ERK1/2 and p38 MAP kinases inhibited both CCR3-mediated lipid body formation and enhanced LTC₄ release by eosinophils. More directly, newly formed LTC₄ was localized almost exclusively to lipid bodies in eosinophil- and RANTES-stimulated eosinophils, in contrast to perinuclear localization of LTC₄ in eosinophils activated solely with calcium ionophore A23187. In ionophore-activated eosinophils, the PI3K inhibitor, wortmannin, failed to inhibit the overall eosinophil LTC₄ generation that was shown to occur at perinuclear membranes. In contrast, wortmannin, which inhibited the chemokine-enhanced extracellular release of LTC₄, blocked chemokine-stimulated LTC₄ formation at both preformed and chemokine-elicited lipid bodies, but not at the perinuclear membrane. These findings indicated that chemokine-elicited activation of PI3K was involved in both the induction of new lipid body formation and the regulated activation of LTC₄ formation at all lipid body domains, providing additional evidence that chemokine-elicited lipid bodies were the principal sites of enhanced LTC₄ synthesis.

Heretofore, the intracellular sites of eicosanoid formation in any cell have not been directly demonstrated, but rather have been inferred based on the immunolocalization of specific eicosanoid-forming enzymes. Hence, the translocation of 5-LO from the cytosol to the nucleus in eosinophils in response to calcium ionophore has suggested that enhanced LTC₄ is formed at perinuclear sites (12) (consistent with our findings in eosinophils solely activated by ionophore). Indeed, in many cells, major sites of synthesis of both 5-LO- and cyclooxygenase pathway-derived eicosanoids are believed to be the perinuclear membranes (11, 30, 31). Based on calcium ionophore-elicited translocation of 5-LO from either the nucleus or the cytosol, redistribution of 5-LO to the nuclear envelope has been found in neutrophils, eosinophils, alveolar macrophages, blood monocytes, mast cells, and the rat basophilic leukemia mast cell-like cell line (11). A role for extranuclear sites for 5-LO catalyzed LT formation, however, would be compatible with the earlier finding that enucleate neutrophil cytoplasts generate LTB₄ in response to A23187 (32). More recently, we established that enucleate eosinophil cytoplasts formed lipid bodies in response to PAF stimulation and that the numbers of lipid bodies in cytoplasts correlated with levels of primed LTC₄ and PGE₂ released by cytoplasts following submaximal A23187 challenge (16). Moreover, lipid bodies in enucleate cytoplasts were sites of immunolocalized 5-LO, cyclooxygenase, and LTC₄ synthase proteins (16). Our findings extend knowledge of the regulated intracellular compartmentalization of eicosanoid formation. Although perinuclear membranes may be sites of eicosanoid formation in cells singularly activated with calcium ionophore A23187 (as we confirmed), in leukocytes first stimulated with specific chemokines or other leukocyte agonists, e.g. PAF, lipid bodies (as present in vivo in inflammation-associated leukocytes) are the predominant sites of enhanced eicosanoid formation.

Since the immunolocalization of eicosanoid-forming proteins need not reflect the regulated activity of these enzymes, we utilized a more direct approach to detect the intracellular sites of 5-LO- and LTC₄ synthase-mediated LTC₄ formation in eosinophils. Eosinophils incubated in a gel matrix were fixed and permeabilized with a water soluble cross-linker EDAC, enabling: 1) the covalent cross-linking of eicosanoid carboxyl groups to adjacent amines in proteins at the sites of eicosanoid formation, 2) the penetration of detecting fluorochrome-conjugated anti-cysteinyl LT mAb into eosinophils, and, importantly, 3) the relative preservation of lipid body domains (which dissipate with air drying or commonly used alcohol fixation). Eosinophils were especially amenable to this strategy for localizing LTC₄, their dominant 5-LO product. In eosinophils, 5-LO and LTC₄ synthase enzymatic activities are tightly coupled, as indicated by the paucity of nonenzymatic breakdown products of intermediate LT₁₄ (33); both enzymes have been localized at eosinophil lipid bodies (16). In basophils, 5-LO was also localized at lipid bodies; but basophils, unlike eosinophils, were not durable to the LTC₄ fixation process. In contrast, in calcium ionophore-activated neutrophils, 5-LO-catalyzed formation of LTA₄ is not tightly coupled to subsequent LT₄₂ formation, which may be mediated by cytosolic leukotriene A₄ hydroxase or may occur substantially following extracellular release of LTA₄ (34). Immunofluorescent localization of newly formed LTC₄ in eosinophils activated with calcium ionophore exhibited a predominantly perinuclear staining, remarkably similar to the immunolocalization of 5-LO protein reported in ionophore-activated eosinophils (12). In contrast, eosinophils exposed to etoxaxin or RANTES prior to ionophore activation showed pre-
dominant anti-LTC₄ staining at focal lipid body structures either proximate to the nucleus or in distant lamellipod protrusions. Moreover, wortmannin inhibition of PI3K blocked both overall eotaxin-primed eosinophil LTC₄ release and lipid body (but not perinuclear) LTC₄ formation. Thus, in C-C chemokine-primed and ionophore-activated eosinophils, lipid body organelles were the predominant sites of regulated LTC₄ synthesis.

Lipid bodies are complex and as yet poorly understood organelles. They are not unique to eosinophils and basophils and are found in a diversity of cells ranging from fibroblasts to endothelial cells to leukocytes (35). Lipid body numbers characteristically increase in cells associated with various forms of inflammation (14, 15, 36). Lipid body formation is rapidly inducible by specific stimuli, including hypoxia in endothelial cells (37), cis-unsaturated fatty acids in neutrophils (38), and receptor-mediated ligands, including PAF (23) and, as shown here, specific chemokines for eosinophils and basophils. Lipid bodies contain esterified arachidonic acid, ill defined membranous structures, and in many cell types, including eosinophils, basophils, neutrophils, alveolar macrophages, mast cells, one or more enzymes involved in eicosanoid synthesis, including cyclooxygenase, 5-LO, 15-LO, and/or LTC₄ synthase (16, 39–41). Lipid bodies also contain cytosolic phospholipase A2 and several signal transducing kinases, including PI3K, ERK1, ERK2, p85, and p38 MAP kinases (42, 43). Although these findings have suggested that lipid bodies might be sites of regulated eicosanoid formation in several cell types, direct evidence for such had been lacking. Our direct demonstration that eosinophil lipid bodies are specific sites of LTC₄ synthesis has implications for many other cell types, especially those involved in diverse inflammatory responses, in which lipid bodies might likewise serve as sites of regulated eicosanoid formation.

Although eosinophils stimulated with eotaxin or RANTES did not release levels of LTC₄ adequate for detection in supernatants by EIA, the heightened sensitivity of the immunofluo-

**Fig. 5. C-C chemokines directly stimulate LTC₄ production in eosinophils.** Phase-contrast (left panels) and fluorescent (right panels) microscopy of identical fields of eosinophils incubated for 1 h, and then fixed with EDAC and stained with Alexa488-labeled anti-cysteinyl LT mAb. To facilitate intracellular localization, anti-LTC₄ immunoreactive sites (green staining) were overlaid on phase-contrast images and white lines were drawn delineating the nuclear perimeter. Unstimulated (A) and IL-8-stimulated (D) eosinophils display no fluorescent LTC₄ immunostaining. Representative eosinophils stimulated with eotaxin (100 ng/ml) (B) and RANTES (100 ng/ml) (C) exhibit perinuclear (arrowheads) and punctate cytoplasmic (arrows) immunoreactive LTC₄. Bar, 5 μm.

**Fig. 6. Perinuclear and extranuclear production of LTC₄ in human eosinophils.** Phase-contrast and fluorescent microscopy of identical fields of eosinophils incubated for 1 h with the C-C chemokines (as indicated) and activated for 15 min with 0.1 μM A23187. Eosinophils were fixed with EDAC and stained with Alexa488-labeled anti-cysteinyl LT mAb. To facilitate intracellular localization, anti-LTC₄ immunoreactive sites (green staining) were overlaid on phase-contrast images and white lines were drawn delineating the nuclear perimeter. Eosinophils activated with A23187 exhibit fluorescent anti-LTC₄ staining especially at the perinuclear envelope (arrowheads) as well as at some extranuclear sites (arrow). Eosinophils stimulated with eotaxin (100 ng/ml) (B) or RANTES (100 ng/ml) (C) prior to A23187 activation exhibited some perinuclear anti-LTC₄ staining (arrowheads) and abundant punctate LTC₄ immunoreactive staining both proximate to the nucleus (*) and at more distant cytoplasmic sites (arrows). Eosinophils pre-treated with the 5-LO inhibitor, MK 886 (10 μM), had no anti-LTC₄ immunofluorescent staining (right panels), whereas cells pre-treated with the PI3K inhibitor, wortmannin, showed only perinuclear staining for LTC₄. Bar, 5 μm.
recent detection of LTC₄ formation enabled the demonstration that about a quarter of eosinophils stimulated solely with these C-C chemokines, and not with IL-8, synthesized low levels of LTC₄. Since LTC₄ is being recognized to have autocrine effects on eosinophils, including prolonging their longevity (8) and regulating the vesicular transport-mediated release of preformed eosinophil granule-derived cytokines (e.g. IL-4), the capacity of the two chemokines to stimulate even low level LTC₄ synthesis intracellularly at focal extranuclear sites may augment eosinophil effector functioning. Moreover, the stimulation of eosinophils for increased LTC₄ formation by eotaxin and RANTES, based on their receptor-mediated signaling to induce and activate lipid body organelles that are sites for regulated LTC₄ synthesis, promotes cysteinyl LT generation and release by recruited eosinophils and further contributes to the pathogenesis of allergic inflammation.

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REFERENCES

1. Wardlaw, A. J. (1999) J. Allergy Clin. Immunol. 104, 917–926
2. Gleich, G. J. (2000) J. Allergy Clin. Immunol. 105, 651–663
3. Costa, J. J., Weller, P. F., and Galli, S. J. (1997) J. Am. Med. Assoc. 276, 1815–1822
4. Weller, P. F. (1993) in Immunopharmacology of Eosinophils: The Handbook of Immunopharmacology (Smith, J. H., and Cook, R. M., eds) pp. 25–42, Academic Press, London
5. Lewis, R. A., Austen, K. F., and Soberman, R. J. (1990) N. Engl. J. Med. 323, 645–655
6. Henderson, W. R., Jr. (1994) Ann. Intern. Med. 121, 684–697
7. Laitinen, L. A., Haahienda, T., Spur, B. W., Laitinen, A., Vilkkia, V., and Lee, T. H. (1993) Lancet 341, 969–970
8. Lee, E., Robertson, T., Smith, J., and Kilfeather, S. (2000) J. Exp. Med. 191, 1349–1356
9. Cowburn, A. S., Sladek, K., Soja, J., Adamek, L., Nizankowska, E., Szczeklik, A., Lam, B. K., Penrose, J. F., Austen, F. K., Holgate, S. T., and Sampson, A. P. (1998) J. Clin. Invest. 101, 834–846
10. Cowburn, A. S., Holgate, S. T., and Sampson, A. P. (1999) J. Immunol. 163, 456–465
11. Peters-Golden, M., and Brock, T. G. (2000) Am. J. Respir. Crit. Care Med. 161, S36–S40
12. Brock, T. G., Andson, J. A., Fries, F. P., Peters-Golden, M., and Sporn, P. H. (1999) J. Immunol. 162, 1689–1676
13. Weller, P. F., and Dvorak, A. M. (2000) in Asthma and Rhinitis (Busse, W. B., and Holgate, S. T., eds) pp. 351–372, Blackwell Scientific Publications, Boston

2 C. Bandeira-Melo, M. Phoofolo, and P. F. Weller, unpublished observations.
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