

Endogenous secreted phospholipase A₂ group X regulates cysteinyl leukotrienes synthesis by human eosinophils

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Background: Phospholipase A₂s mediate the rate-limiting step in the formation of eicosanoids such as cysteinyl leukotrienes (CysLTs). Group IVA cytosolic PLA₂α (cPLA₂α) is thought to be the dominant PLA₂ in eosinophils; however, eosinophils also have secreted PLA₂ (sPLA₂) activity that has not been fully defined.

Objectives: To examine the expression of sPLA₂ group X (sPLA₂-X) in eosinophils, the participation of sPLA₂-X in the formation of CysLTs, and the mechanism by which sPLA₂-X initiates the synthesis of CysLTs in eosinophils.

Methods: Peripheral blood eosinophils were obtained from volunteers with asthma and/or allergy. A rabbit polyclonal anti-sPLA₂-X antibody identified sPLA₂-X by Western blot. We used confocal microscopy to colocalize the sPLA₂-X to intracellular structures. An inhibitor of sPLA₂-X (ROC-0929) that does not inhibit other mammalian sPLA₂s, as well as inhibitors of the mitogen-activated kinase cascade (MAPK) and cPLA₂α, was used to examine the mechanism of *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-mediated formation of CysLT.

Results: Eosinophils express the mammalian sPLA₂-X gene (*PLA2G10*). The sPLA₂-X protein is located in the endoplasmic reticulum, golgi, and granules of eosinophils and moves to the granules and lipid bodies during fMLP-mediated activation. Selective sPLA₂-X inhibition attenuated the fMLP-mediated release of arachidonic acid and CysLT formation by eosinophils. Inhibitors of p38, extracellular-signal-regulated kinases 1/2 (p44/42 MAPK), c-Jun N-terminal kinase, and cPLA₂α also attenuated the fMLP-mediated formation of CysLT. The sPLA₂-X inhibitor reduced the phosphorylation of p38 and extracellular-signal-regulated kinases 1/2 (p44/42 MAPK) as

well as cPLA₂α during cellular activation, indicating that sPLA₂-X is involved in activating the MAPK cascade leading to the formation of CysLT via cPLA₂α. We further demonstrate that sPLA₂-X is activated before secretion from the cell during activation. Short-term priming with IL-13 and TNF/IL-1β increased the expression of *PLA2G10* by eosinophils.

Conclusions: These results demonstrate that sPLA₂-X plays a significant role in the formation of CysLTs by human eosinophils. The predominant role of the enzyme is the regulation of MAPK activation that leads to the phosphorylation of cPLA₂α. The sPLA₂-X protein is regulated by proteolytic cleavage, suggesting that an inflammatory environment may promote the formation of CysLTs through this mechanism. These results have important implications for the treatment of eosinophilic disorders such as asthma. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Eosinophil, leukotriene, phospholipase A₂, mitogen-activated kinase, asthma, allergy

Cysteinyl leukotrienes (CysLTs, C₄, D₄, and E₄) are generated in increased quantities in the airways of patients with asthma.¹⁻³ Eosinophils accumulate in the airways of many patients with asthma and serve as a key source of CysLTs.⁴ The rate-limiting step in the formation of CysLT is phospholipase A₂ (PLA₂)-mediated release of arachidonate from the *sn*-2 position of membrane phospholipids. It has been assumed that group IVA cytosolic PLA₂α (ie, cPLA₂α) serves as the key regulator of endogenous CysLT synthesis in myeloid cells such as eosinophils^{5,6}; however, 10 mammalian-secreted PLA₂s (sPLA₂s) have been identified, and in transfected cells, several sPLA₂s have been found to coordinate eicosanoid synthesis with cPLA₂α.⁷⁻⁹ Among the sPLA₂s, groups V and X (ie, sPLA₂-V and sPLA₂-X) have unique functional capacities to cleave phospholipids and initiate cellular eicosanoid synthesis.^{10,11} We have demonstrated that sPLA₂-X is elevated in the airways of patients with asthma,^{12,13} and serves to activate CysLT synthesis in eosinophils when the recombinant enzyme is added exogenously to eosinophils.¹⁴

Eosinophils also have endogenous sPLA₂ activity that contributes to surfactant dysfunction through the cleavage of surfactant phospholipids.^{15,16} The gene expression of sPLA₂ group IIA (sPLA₂-IIA, *PLA2G2A*) and sPLA₂-X (*PLA2G10*) was previously identified in human eosinophils.¹⁶ The sPLA₂-IIA protein was identified in eosinophils,¹⁷ but it does not contribute to the formation of leukotrienes in these cells.^{18,19} The other high-activity sPLA₂, sPLA₂-V, is not present in eosinophils.²⁰ In the present study, we investigated the contribution of the sPLA₂-X enzyme to sPLA₂ activity in human primary eosinophils and the role of this enzyme as a key control point in the formation of CysLT. The results support an important role

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Abbreviations used

5-LO: 5-Lipoxygenase
 cPLA₂α: Cytosolic phospholipase A₂α
 CysLT: Cysteinyl leukotriene
 DMSO: Dimethyl sulfoxide
 ER: Endoplasmic reticulum
 ERK1/2: Extracellular-signal-regulated kinases 1/2 (p44/42 MAPK)
 HL-60-C15: HL-60 clone-15
 JNK: c-Jun N-terminal kinase
 MAPK: Mitogen-activated protein kinase
 PLA₂: Phospholipase A₂
 PLA2G10: Mammalian sPLA₂-X gene
 pla2g10: Murine sPLA₂-X gene
 sPLA₂: Secreted phospholipase A₂
 sPLA₂-IIA: sPLA₂ group IIA
 sPLA₂-V: sPLA₂ group V
 sPLA₂-X: sPLA₂ group X
 TSLP: Thymic stromal lymphopoietin

of sPLA₂-X in endogenous CysLT synthesis in eosinophils, and that sPLA₂-X is involved in the activation of the mitogen-activated protein kinase (MAPK) cascade leading to the activation of cPLA₂α.

METHODS**Materials**

Ficoll-Paque PLUS (d 1.077) was purchased from GE Healthcare (Piscataway, NJ). Antibodies for immunomagnetic selection were purchased from Miltenyi Biotec (Auburn, Calif). [³H]Arachidonate was purchased from American Radiolabeled Chemicals (St Louis, Mo). *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) was purchased from Sigma-Aldrich (St Louis, Mo). Antibodies against phospho-cPLA₂α [Ser505], cPLA₂α, phospho-p44/42 MAPK (Erk 1/2) [Thr202/Tyr204], p44/42 MAPK (Erk 1/2), phospho-p38 MAPK [Thr180/Tyr182], p38 MAPK, phospho-stress-activated protein kinase/c-Jun N-terminal kinase (JNK) [Thr183/Tyr185], and stress-activated protein kinase/JNK were purchased from Cell Signaling Technologies (Beverly, Mass). Inhibitors of MEK 1/2 (U0126), p38 (SB203580), JNK (SP600125), Furin I (344930, D-RVKK-CMK), and Furin II (344931, 6-D-R) were purchased from EMD Biosciences (San Diego, Calif). Rabbit polyclonal anti-sPLA₂-X antibody was developed and characterized in our laboratory.²¹ Dr James J. Lee (Mayo Clinic, Scottsdale, Ariz) provided the murine monoclonal anti-EPX antibody. Murine monoclonal anti-GM130 (35/GM130) was purchased from BD Bioscience (San Jose, Calif). Murine monoclonal anti-PDI (RL90) was purchased from Abcam (Cambridge, Mass). Murine monoclonal anti-ADRP (AP125) was purchased from Fitzgerald (Acton, Mass). Rabbit anti-5-lipoxygenase (5-LO) antiserum was purchased from Cayman Chemical (Ann Arbor, Mich), and rabbit anti-cPLA₂α antiserum was purchased from Santa Cruz Biotechnology (Dallas, Texas). Cy3-conjugated goat anti-rabbit IgG was purchased from Jackson ImmunoResearch (West Grove, Pa). Alexa Fluor 647 goat anti-mouse IgG was purchased from Invitrogen (Grand Island, NY).

Isolation of human blood eosinophils and differentiation of HL15 cells

The University of Washington Institutional Review Board approved the study, and written informed consent was obtained from all participants. Peripheral blood eosinophils were obtained from volunteers with a physician diagnosis of asthma and/or allergy and 1.2×10^5 or more eosinophils/mL of peripheral blood. Granulocytes were isolated from peripheral blood by density gradient centrifugation followed by hypotonic lysis of red blood cells.

Eosinophils were removed from the granulocyte fraction by negative immunomagnetic selection. The purity of eosinophils was determined by differential counts of Romanowski-stained (Diff-Quick) cytospin preparations. Eosinophil viability was assessed by trypan blue exclusion.

HL-60 clone-15 (HL-60-C15) and AML14.3D10-CCR3 cells were purchased from ATCC (Manassas, Va). Cells were cultured in RPMI with 10% FCS. HL-60-C15 cells were treated with and without 0.5 mM sodium butyrate for 7 days.

Selective PLA₂ inhibitors

Because human eosinophils contain sPLA₂ group IIA (sPLA₂-IIA),¹⁷ we used an sPLA₂ inhibitor, known as ROC-0929, that is selective for sPLA₂-X and does not inhibit other mammalian sPLA₂s at nanomolar concentrations.²² The compound ROC-0929 is an analogue of the well-known sPLA₂ inhibitor LY315920.²² Docking studies revealed that the isobutyl group of ROC-0929 sterically excludes this compound from the active site of sPLA₂-IIA, but not the active site of sPLA₂-X, resulting in more than 80-fold difference in the inhibitory potency of ROC-0929 between the sPLA₂-X and sPLA₂-IIA enzymes.²² To test for off-target effects, we used a control compound, known as ROC-0428, that differs from ROC-0929 by 1 methyl group and is essentially devoid of sPLA₂ inhibition.²² We also used a pan-sPLA₂ inhibitor (0509A) and an inhibitor that is selective for group IIA/III enzymes (0320). Inhibitory activities of the sPLA₂ inhibitors and the control compound are presented in Table E1 in this article's Online Repository available at www.jacionline.org. Inhibition of cPLA₂α was achieved with Pyr-2 (pyrrophenone) and Wyeth-2 (giflpladib).^{23,24}

Secreted PLA₂ activity assay

³H-labeled *Escherichia coli* membranes were resuspended with reaction buffer at a final concentration of 2.0×10^6 dpm/mL.²⁵ The eosinophil lysate was treated with inhibitors or dimethyl sulfoxide (DMSO) control for 10 minutes at room temperature. The radiolabeled *E coli* membrane substrate was added to each reaction tube and incubated at 37°C for 1 hour. The reaction was stopped by the addition of 0.1 mol EDTA and the sample centrifuged at 14,000g. The supernatant was submitted to scintillation counting.

Assessment of arachidonate release by eosinophils

The release of arachidonate and CysLT from eosinophils was determined in 24-well plates coated with 0.01% BSA. Eosinophils (1.8×10^5 per well) were resuspended in RPMI with 0.01% BSA, and the cells were incubated for 24 hours with [³H]arachidonate (0.1 μCi/well). After the unincorporated arachidonate was washed 3 times, the cells were preincubated with inhibitors for 20 minutes at 37°C and then stimulated with fMLP for 20 minutes. Supernatants were submitted to scintillation counting after being centrifuged to remove detached cells. The remaining eosinophils were detached with 0.25% trypsin with EDTA for 30 minutes at 37°C, pelleted, and submitted to scintillation counting. Arachidonate release was expressed as a percentage of counts/min in the supernatant to the total counts/min in the cells and the supernatant.

Assessment of CysLT formation by eosinophils

Eosinophils were resuspended in HBSS with Ca²⁺ and Mg²⁺ at a concentration of 1.5×10^5 per well in a 5% CO₂ incubator at 37°C. The cells were preincubated with either inhibitor or DMSO control for 20 minutes at 37°C and then stimulated with fMLP for 20 minutes. The synthesis of eicosanoids was stopped by the addition of 4 volumes of ice-cold methanol with 0.2% formic acid. An ELISA measured LTC₄ or total CysLT levels after removal of the methanol by evaporation (Cayman Chemical).

Selective kinase inhibitors

To examine the role of the MAPKs in the synthesis of LTC₄, we used a series of inhibitors that are specific with little cross-inhibition of the other

kinases.²⁶⁻²⁸ Eosinophils (1.5×10^5 per well) resuspended in HBSS with Ca^{2+} and Mg^{2+} were preincubated with either DMSO control or inhibitors of MEK1/2 (U0126, 10 μM), p38 (SB203580, 30 μM), or JNK (SP600125, 20 μM) for 20 minutes at 37°C in a 5% CO_2 incubator and then stimulated with fMLP (100 nM). The synthesis of eicosanoids was stopped by the addition of 4 volumes of ice-cold methanol with 0.2% formic acid after 20 minutes.

Measurement of MAPK and cPLA₂ α phosphorylation

Phosphorylation of extracellular-signal-regulated kinases 1/2 (p44/42 MAPK) (ERK1/2), p38, JNK, and cPLA₂ α in eosinophils (2.0×10^6 cells) pretreated with DMSO control or ROC-0929 and then stimulated with 100 nM fMLP for 20 minutes was determined by Western blots of the phosphorylated protein relative to the total protein. The cells were pelleted and treated with lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, with protease and phosphatase inhibitors) on ice for 30 minutes. Equal amounts of cell lysate were heated for 10 minutes in LDS loading buffer with dithiothreitol and β -mercaptoethanol, and separated on 4% to 12% Bis-Tris gel under reducing conditions. The resolved proteins were transferred onto a polyvinylidene fluoride membrane using a semidry apparatus, blocked with 5% nonfat milk, and incubated with the primary antibody overnight at 4°C. ECL visualized the proteins of interest.

Propeptide convertase inhibitors

Eosinophils (1.5×10^5 per well) resuspended in HBSS with Ca^{2+} and Mg^{2+} were preincubated with either DMSO control or Furin I (D-RVKR-CMK, 10 μM) and Furin II (344931, 6-D-R, 10 μM) for 20 minutes at 37°C in a 5% CO_2 incubator and then stimulated with fMLP (100 nM). The synthesis of eicosanoids was stopped by the addition of 4 volumes of iced methanol with 0.2% formic acid after 20 minutes.

Confocal microscopy

Eosinophils (1.0×10^6 per well) were allowed to adhere to cover glasses coated with 0.01% BSA and treated with DMSO vehicle control or ROC-0929 for 20 minutes, and then treated with either vehicle control or fMLP (100 nM) for 20 minutes at 37°C in a 5% CO_2 incubator. The cells were fixed in 3.7% formaldehyde in PBS at room temperature for 30 minutes. Eosinophils were permeabilized with 0.1% Triton X-100 and blocked with 10% goat serum. Immunostained cells were visualized with a Zeiss LSM510 Confocal Microscope.

Quantitative PCR

Real-time PCR analysis was conducted using TaqMan primer-probe sets with quantification relative to an endogenous control gene using the delta Ct method. Primer-probe sets were obtained from Applied Biosystems (Foster City, Calif) using a FAM probe for *PLA2G10* (Hs00358567_m1) and a primer-limited VIC probe for *HPRT1* (4326321E) as an endogenous control (Applied Biosystems). Real-time PCR was performed using a Mastercycler ep realplex system (Eppendorf, Hauppauge, NY). In some experiments, eosinophils were placed in RPMI at 37°C in a 5% CO_2 incubator for 4 hours during treatment with buffer control, or TNF α , IL- β , IL-4, IL-5, IL-13, GM-CSF, IL-33, and/or thymic stromal lymphopoietin (TSLP) (Peprotech, Rock Hill, NJ). Following cytokine treatment, Trizol-isolated RNA was used to assay *PLA2G10* gene expression.

Statistical analysis

A 1-way ANOVA assessed differences in eicosanoid formation with fMLP stimulation in the presence of specific inhibitors. *Post hoc* comparisons were made between fMLP-treated cells and other conditions with Dunnett's multiple comparison test. A paired *t* test assessed differences between *PLA2G10* gene expression when 2 conditions were present.

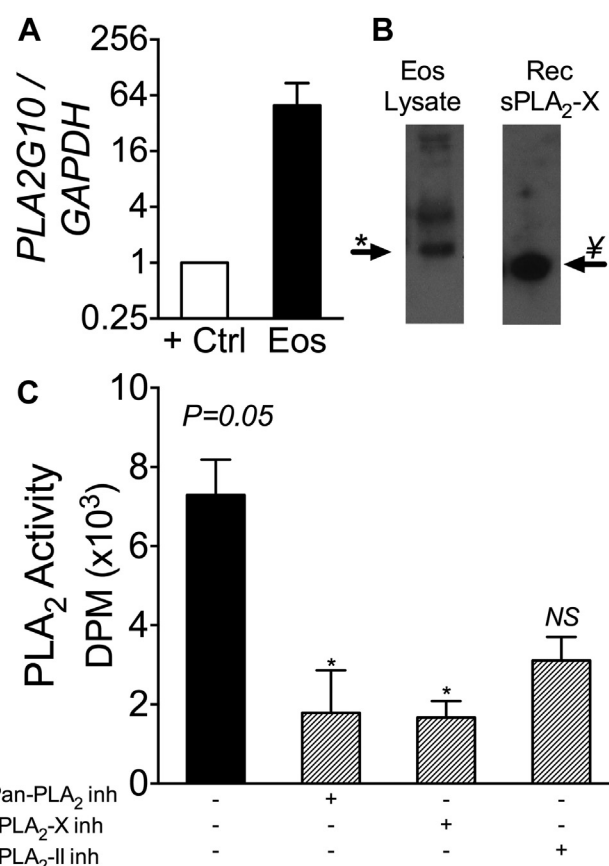


FIG 1. Identification of sPLA₂-X in human eosinophils (Eos). **A**, Human eosinophils strongly express the sPLA₂-X gene *PLA2G10* relative to the positive control (Ctrl) consisting of RNA isolated from an epithelial cell line. **B**, Western blot of eosinophil lysate demonstrates the sPLA₂-X protein running as a single band at 13.5 kDa just above the mature recombinant protein. **C**, Total sPLA₂ activity in eosinophils lysates was inhibited by an sPLA₂-X-specific inhibitor (ROC-0929) or a pan-sPLA₂ inhibitor (0509A), but the inhibition by an sPLA₂ group IIA/IIIE inhibitor did not reach statistical significance ($P = .05$ overall ANOVA; $*P < .05$ by *post hoc* tests). DPM, Disintegrations per minute; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NS, not significant; Rec, recombinant.

RESULTS

Subject characteristics

We isolated peripheral blood eosinophils 54 times from a total of 45 distinct donors for these studies. The mean viability was $99.5\% \pm 1.58\%$, and purity was $99.4\% \pm 0.64\%$ eosinophils. The mean age of the donors was 30.2 years, and 55.6% were women. In the study population, 8.9% were Hispanic, 62.2% were white, 8.9% were black, and 6.7% were Asian. Both asthma and allergic diseases were present in 53.3% of the population, whereas 11.1% had asthma alone and 35.6% had allergic diseases. The mean concentration of eosinophils in peripheral blood was 3.0×10^5 eosinophils/mL, and the mean percentage of eosinophils was 4.5%.

sPLA₂-X is expressed by human eosinophils and localizes to structures involved in leukotriene formation

We assessed the expression of the sPLA₂-X gene *PLA2G10* in human eosinophils from 3 donors and found that human

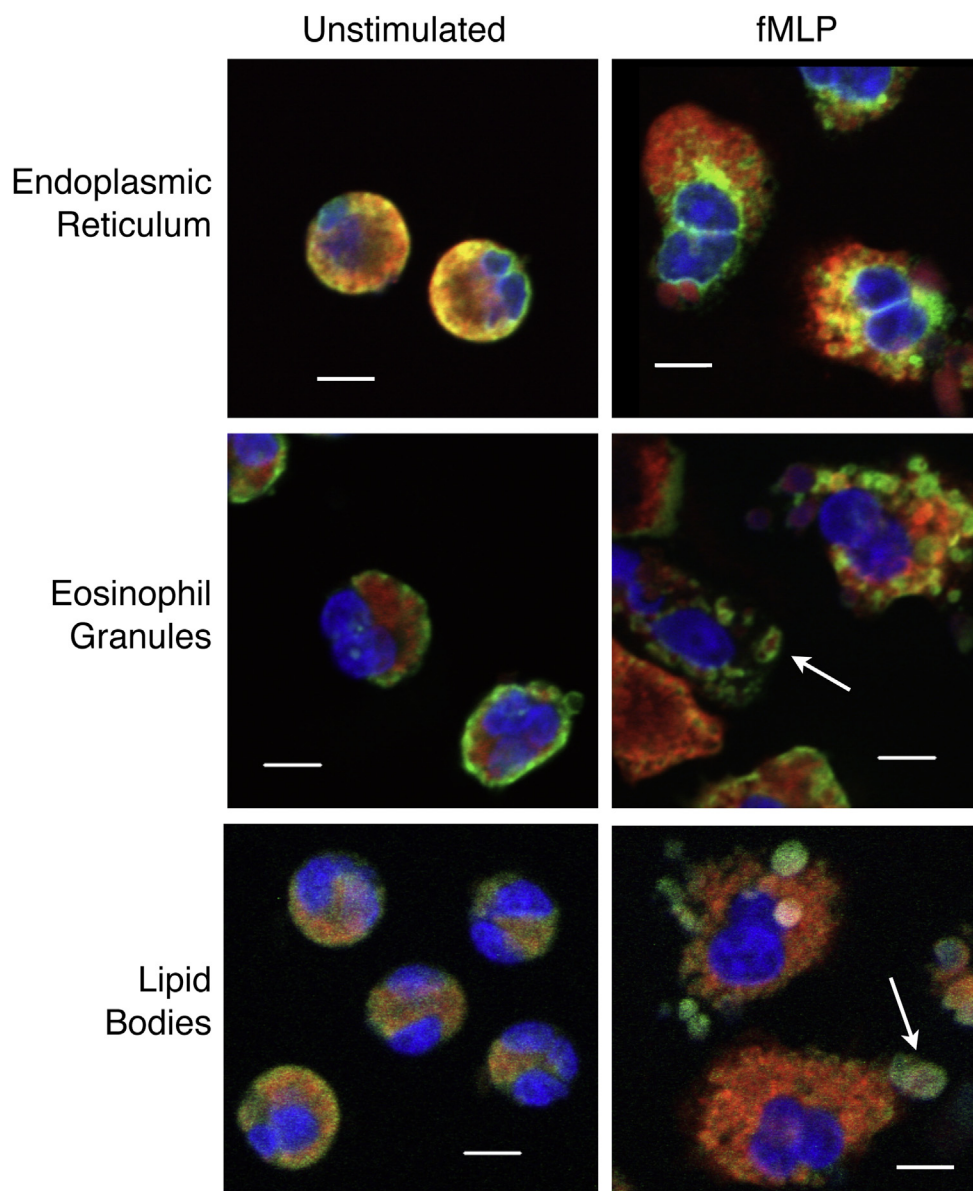


FIG 2. Localization of sPLA₂-X in human eosinophils by confocal microscopy. Immunostaining demonstrates colocalization in *yellow* using antibodies directed against sPLA₂-X (*red*) and endoplasmic reticulum (ER, anti-PDI), granules (anti-EPX), and lipid bodies (anti-ADRP), all in *green*. sPLA₂-X colocalized predominantly to the ER in unstimulated eosinophils. Following fMLP stimulation, there is persistent colocalization to the ER and some colocalization to granules (*arrow*, the *middle panel*), and lipid bodies (*arrow*, the *lower panel*) that form after stimulation with fMLP. Some of the lipid bodies containing sPLA₂-X appear extracellular. Further details of the immunostaining for sPLA₂-X and eosinophil structures are presented in [Figs E1 and E2](#). Scale bar is 5 μ m.

eosinophils strongly express *PLA2G10* ([Fig 1, A](#)). The positive control for *PLA2G10* expression was RNA obtained from a human epithelial cell line because epithelial cells strongly express *PLA2G10*.¹³ In cell lysates of eosinophils, the sPLA₂-X protein can be identified on Western blot using a rabbit polyclonal antibody that is specific for sPLA₂-X ([Fig 1, B](#)).^{21,29} The native sPLA₂-X protein runs on the gel at about 13.5 kDa, slightly larger than the recombinant mature sPLA₂-X protein, indicating that some posttranslational modification of the protein has occurred. Treatment with N-Glycosidase A to cleave N-glycans did not significantly change the gel shift of the protein (data not shown).

A second larger band identified in the blot appears to be a dimer of the sPLA₂-X enzyme that is also seen with the recombinant protein when run at a higher concentration (see [Fig E3, E](#), in this article's Online Repository at www.jacionline.org). Either an sPLA₂-X-specific inhibitor (ROC-0929) or a pan-sPLA₂ inhibitor (0509A) significantly decreased the total sPLA₂ activity in lysates from human eosinophils; however, the inhibitory activity of the sPLA₂-IIA/III inhibitor (0320) did not reach statistical significance ([Fig 1, C](#); [Table E1](#)). It should be noted that the high-sensitivity PLA₂ assay used in the present study has a specific activity for sPLA₂-X that is relatively low due to

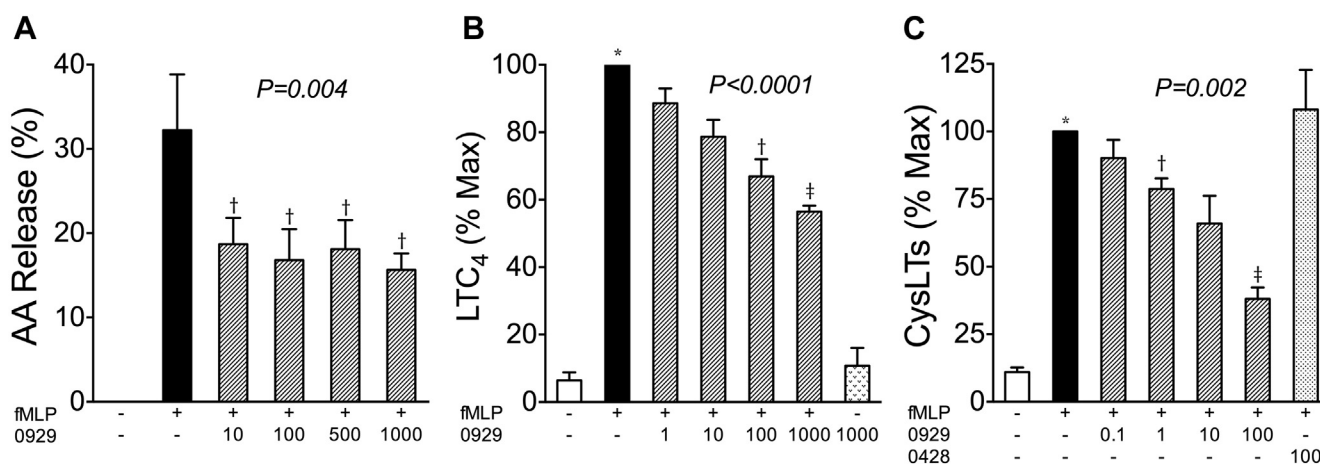


FIG 3. Eicosanoid formation mediated by endogenous sPLA₂-X. **A**, An active-site-directed sPLA₂-X inhibitor (0929) reduced the amount of arachidonic acid (AA) released in response to fMLP across all concentrations of the inhibitor ranging from 10 to 1000 nM. **B**, The fMLP-mediated formation of LTC₄ was reduced in a dose-related manner by the sPLA₂-X inhibitor, but had no effect on basal LTC₄ synthesis. **C**, The formation of total CysLTs was inhibited by the sPLA₂-X inhibitor, but a structural analogue that is not active as an inhibitor (0428) did not affect the formation of CysLT. **P* < .05, †*P* < .01, and ‡*P* < .001.

the anionic phospholipid composition in the *E coli* radiometric assay.³⁰ In contrast, the release of free arachidonate from mammalian HEK293 cells is approximately 1000 times higher for sPLA₂-X over the sPLA₂-IIA enzyme.¹¹ These results indicate that sPLA₂-X is expressed in human eosinophils and comprises most of the sPLA₂ activity in these cells.

We used confocal microscopy to characterize the location of sPLA₂-X in resting and fMLP-stimulated eosinophils. In resting cells, the sPLA₂-X protein colocalized predominantly to the endoplasmic reticulum and golgi (Fig 2; see Fig E1 in this article's Online Repository at www.jacionline.org). There may also be some colocalization to eosinophil granules identified by immunostaining for EPX. The antibody used to identify lipid bodies (ie, anti-ADRP) demonstrated only background immunostaining on the plasma membrane in resting cells.

Following stimulation of the cells with fMLP, there was persistent colocalization to the endoplasmic reticulum and less colocalization to the golgi (Fig 2; see Fig E2 in this article's Online Repository at www.jacionline.org). In stimulated cells, the granule staining by anti-EPX became more distinct and there was some colocalization with sPLA₂-X to the granules (arrow in the middle panel of Fig 2). The immunostaining for sPLA₂-X in granules is further delineated in Fig E2, demonstrating red immunostaining for sPLA₂-X in structures labeled with the anti-EPX antibody for granules in green (arrows, the third panel). With the anti-ADRP immunostaining of lipid bodies, fMLP stimulation caused the formation of lipid bodies that could be observed both within the cytoplasm of eosinophils and some lipid bodies that appear extracellular. Although there was limited colocalization of sPLA₂-X to lipid bodies (arrow in the lower panel of Fig 2), it was apparent that the sPLA₂-X protein resides in these structures, but in a slightly different location from the target for the anti-ADRP (arrows in the lower panel of Fig E2). These results indicate that sPLA₂-X is located predominantly in the endoplasmic reticulum of resting eosinophils, but enters the secretory system and lipid bodies on stimulation and may be externalized during activation either through release or eosinophil necrosis.

An active-site-directed inhibitor of sPLA₂-X inhibits fMLP-mediated eicosanoid synthesis

We activated human eosinophils with fMLP based in part on the observation that well-established allergens, house dust mite proteins, activate eosinophils via the formyl peptide receptors.³¹ After eosinophils were incubated with radiolabeled arachidonate, the percentage of [³H]arachidonate released in response to fMLP was attenuated by pretreatment of the cells with the sPLA₂-X-specific inhibitor ROC-0929 (Fig 3, A; *P* = .004). In this assay, there was no significant difference between the effects of the inhibitor at concentrations ranging from 10 to 1000 nM. The release of the CysLT LTC₄ from fMLP-activated eosinophils was reduced in a dose-dependent manner by increasing concentrations of ROC-0929 ranging from 1 to 1000 nM, although only the 100 and 1000 nM concentrations of the inhibitor caused a statistically significant reduction in the formation of LTC₄ based on *post hoc* tests (Fig 3, B; *P* < .0001). The ROC-0929 inhibitor did not have any effect on the basal release of LTC₄ from human eosinophils. We further quantified the total CysLT release from eosinophils following fMLP stimulation and found that there was dose-dependent inhibition of the formation of CysLT starting at a concentration of just 1 nM (Fig 3, C). As a further control for this experiment, we found that there was no alteration in CysLT formation mediated by the control inhibitor (ROC-0428) that is structurally similar to ROC-0929, but is unable to bind to the active site of sPLA₂-X because of the addition of a methyl group.^{14,22}

We assessed the potential use of HL-60-C15 and AML14.3D10-CCR3 cells as potential cell lines that could be used to further understand the biology of sPLA₂-X in eosinophils but found that *PLA2G10* expression in these cells was much lower than in primary human eosinophils (see this article's Results section in the Online Repository at www.jacionline.org; Fig E3, D). However, the HL-60-C15 cell line provides a further control for our results in primary human eosinophil (Fig 3) studies because this cell line has minimal expression of *PLA2G10* and the ROC-0929 inhibitor had a negligible impact on the formation of CysLT in these cells (Fig E3, B). We also assessed the

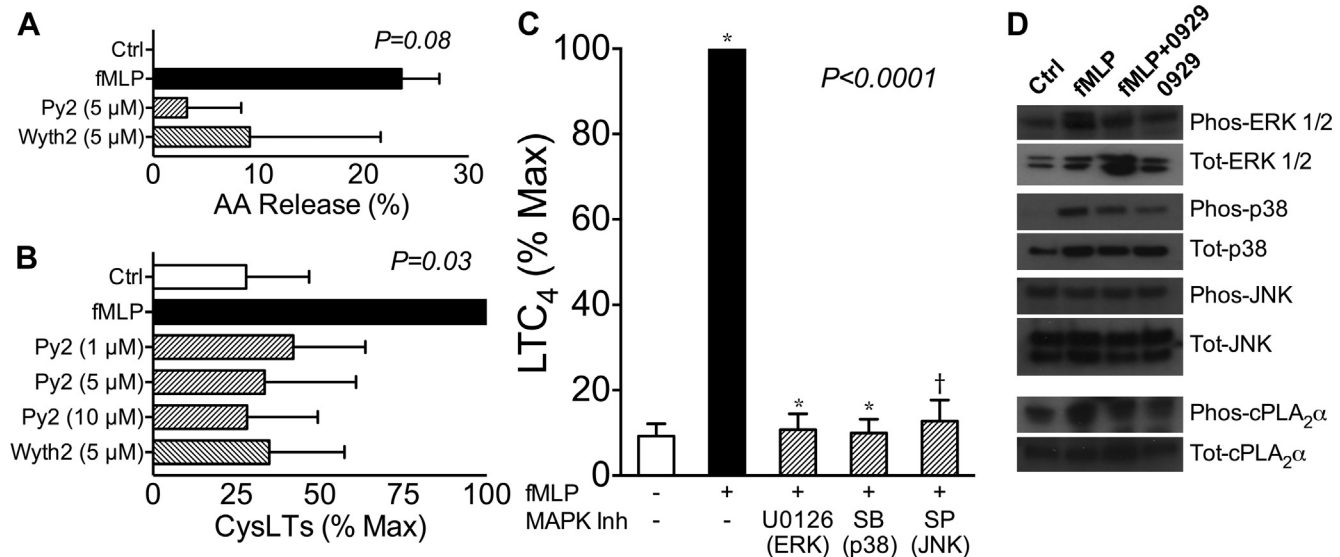


FIG 4. Effects of sPLA₂-X mediated through the MAPK cascade. **A**, The fMLP-mediated release of [³H]arachidonate was attenuated by the cPLA₂α inhibitors Pyr-2 (5 μM) and Wyeth-2 (5 μM). **B**, The formation of CysLTs in response to fMLP was also decreased by Pyr-2 (1-10 μM) and Wyeth-2 (5 μM). **C**, The fMLP-mediated formation of LTC₄ was inhibited by a MEK 1/2 inhibitor (U0126 10 μM), a p38 inhibitor (SB203580 30 μM), and a JNK inhibitor (SP600125 20 μM). **D**, Western blots of cell lysates from eosinophils treated with buffer control (Ctrl), fMLP, or fMLP plus the sPLA₂-X inhibitor (0929) demonstrate that the 0929 inhibitor attenuated the phosphorylation of ERK1/2 and p38 in response to fMLP. There were no changes in the phosphorylation of JNK, whereas the phosphorylation of cPLA₂α at Ser⁵⁰⁵ relative to total cPLA₂α was modestly reduced.

prolonged maintenance of primary eosinophils in culture for potential RNA interference studies and found that although the cells appeared viable, they failed to strongly activate in response to fMLP after 24 hours in culture, either with or without the addition of FCS to the medium (see Fig E4 in this article's Online Repository at www.jacionline.org). We further found that treatment of eosinophils with IL-5 and GM-CSF to prolong survival would not serve as an adequate model because of the impact on *PLA2G10* expression (see below and Fig 5).

sPLA₂-X is involved in the activation of the MAPK cascade and cPLA₂α

Because we have demonstrated that exogenously added sPLA₂-X acts in part via the activation of the MAPK cascade leading to the phosphorylation at Ser⁵⁰⁵ of cPLA₂α,¹⁴ we investigated the possibility that endogenous sPLA₂-X serves as an initiator of the MAPK cascade and cPLA₂α phosphorylation. The role of cPLA₂α was examined using 2 cPLA₂α inhibitors Pyr-2 (pyrrophenone) and Wyeth-2 (girfiladib) during fMLP-mediated activation of eosinophils. Treatment of eosinophils with either cPLA₂α inhibitor at a concentration of 5 μM decreased the release of radiolabeled arachidonate from eosinophils (Fig 4, A; $P = .08$). The generation of CysLTs in response to fMLP was also significantly inhibited in a dose-dependent manner by Pyr-2 in concentrations ranging from 1 to 10 μM, and by 5 μM Wyeth-2 (Fig 4, B; $P = .03$). Selective inhibitors of MEK 1/2 (U0126 10 μM), p38 (SB203580 30 μM), and JNK (SP600125 20 μM) each suppressed the formation of LTC₄ initiated by fMLP-treated eosinophils (Fig 4, C; $P < .0001$).

To connect the activation of CysLT formation by cPLA₂α and the MAPK pathway to the endogenous role of sPLA₂-X in this process, we examined Western blots of eosinophil lysates following stimulation with fMLP, with or without the selective sPLA₂-X inhibitor ROC-0929. We found that fMLP strongly initiated ERK1/2 and p-38 phosphorylation, and further that pretreatment of the cells with ROC-0929 inhibited the phosphorylation of ERK1/2 and p-38 (Fig 4, D). We were unable to identify any effects of fMLP or the ROC-0929 inhibitor on JNK phosphorylation, but we did find that JNK was phosphorylated in these cells before fMLP treatment. We also found that the sPLA₂-X inhibitor decreased the phosphorylation of cPLA₂α at Ser⁵⁰⁵ by approximately half as compared with fMLP-stimulated cells without this inhibitor (Fig 4, D). These results indicate that sPLA₂-X initiates the phosphorylation of ERK1/2 and p-38, which is known to initiate the phosphorylation of cPLA₂α, leading to eicosanoid synthesis.³²

To further examine the sPLA₂-X-mediated activation of CysLT synthesis, we examined the effects of sPLA₂-X inhibition on the localization of 5-LO and cPLA₂α, which form a complex in proximity to nuclear membranes and lipid bodies during eosinophil leukotriene formation.³³⁻³⁵ Following fMLP-mediated activation, there was an increase in the signal for 5-LO and cPLA₂α in the cytoplasmic space surrounding the nucleus and lipid bodies (see Fig E5 in this article's Online Repository at www.jacionline.org). Pretreatment of the cells with the sPLA₂-X inhibitor ROC-0929 resulted in partial attenuation of the changes in 5-LO and cPLA₂α immunostaining in these cells. These results further indicate that sPLA₂-X is involved in the cascade of events leading to the formation of leukotriene. Because we found that sPLA₂-X resides in the secretory apparatus, and the ROC-0929 inhibitor is not readily

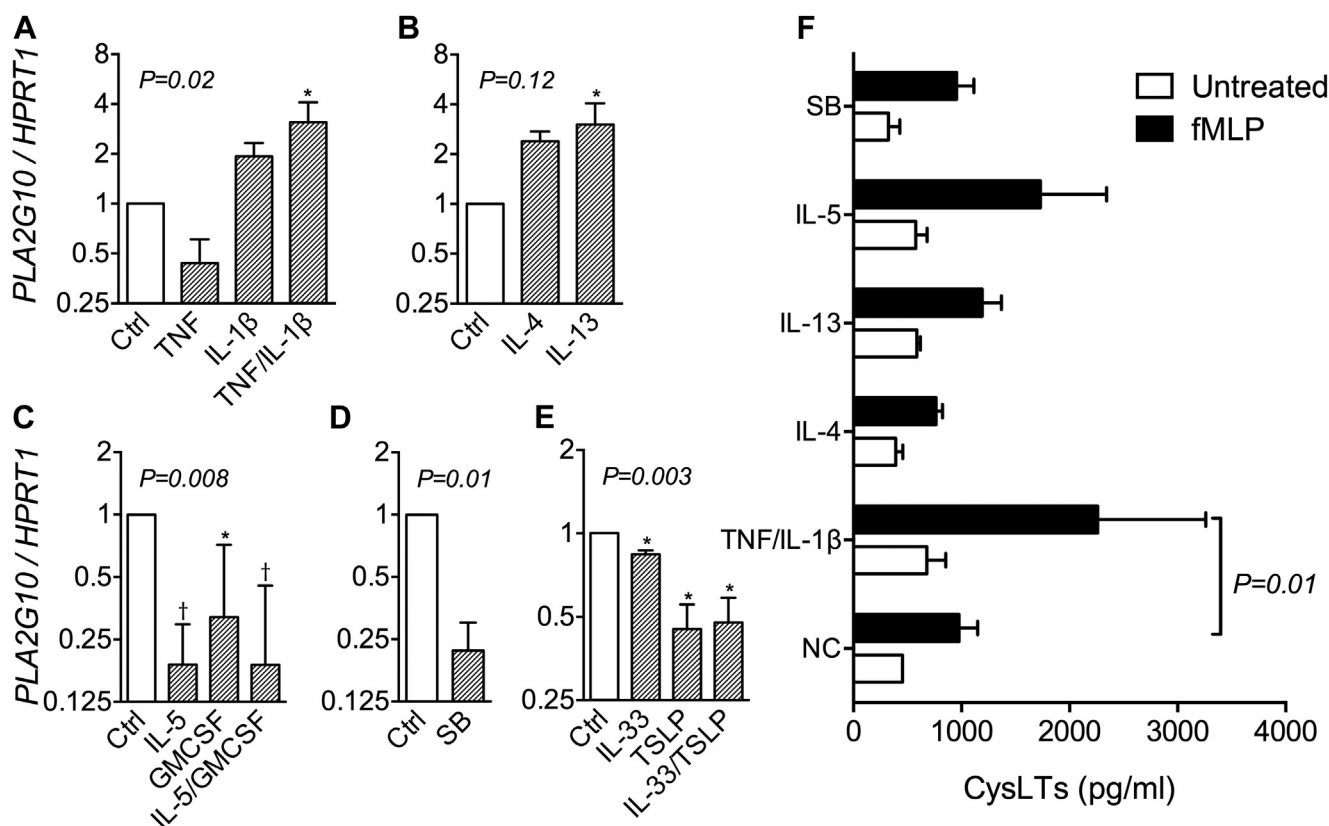


FIG 5. Consequences of short-term cytokine treatment on *PLA2G10* expression in eosinophils. **A-E**, Expression of *PLA2G10* by eosinophils was increased by TNF and IL-1 β and by IL-13 alone, whereas the expression was decreased by treatment with IL-5 with or without GM-CSF, sodium butyrate, and TSLP, IL-33, or a combination of IL-33 and TSLP. **F**, The formation of CysLTs by fMLP-stimulated eosinophils was increased after priming with TNF/IL-1 β ($P = .01$) but did not reach statistical significance for other selected cytokines. Ctrl, Control; HPRT1, Hypoxanthine phosphoribosyltransferase 1. * $P < .05$ and † $P < .01$.

cell permeable,⁸ we assessed Western blots of the sPLA₂-X protein in eosinophil supernatant and found that although the protein could be detected in extracellular fluid, there was no apparent difference in the amount of sPLA₂-X in the eosinophil supernatant with activation (see Fig E6, A, in this article's Online Repository at www.jacionline.org). One checkpoint in the activation of sPLA₂-X is the cleavage of a propeptide that occurs at a furin consensus sequence.^{36,37} We found that a competitive inhibitor of furin-like propeptide convertases (6-D-R)³⁸ inhibited fMLP-mediated formation of LTC₄ whereas an irreversible propeptide convertase inactivator (D-RVKR-CMK)³⁹ did not alter the formation of LTC₄ (Fig E6, B).

PLA2G10 expression is increased by inflammatory signals and reduced by a histone deacetylase inhibitor

Because sPLA₂-X is upregulated by inflammatory stimuli such as TNF⁴⁰ and it is well known that eosinophils are "primed" in the airways under the influence of IL-5 and GM-CSF,⁴¹ we tested the effects of cytokines implicated in asthma on the expression of *PLA2G10* in primary eosinophils. We found that the expression of *PLA2G10* was increased by treatment of the cells with TNF and IL-1 β together, but not by either cytokine alone (Fig 5, A). Although the expression of *PLA2G10* tended to be increased by both IL-4 and IL-13, the increase reached statistical significance

only for IL-13 (Fig 5, B). In contrast to our expectations, the expression of *PLA2G10* was decreased by treatment with IL-5 with or without GM-CSF (Fig 5, C), and was also decreased by treatment with TSLP and IL-33 (Fig 5, D). In accord with our expectation, treatment with the HDAC inhibitor sodium butyrate decreased the expression of *PLA2G10* in primary eosinophils (Fig 5, E). We also examined the effects of selected cytokines on the fMLP-mediated formation of CysLTs and found that priming the eosinophils with TNF/IL-1 β was the only factor that led to a statistically significant increase in CysLTs ($P = .01$; Fig 5, F), while IL-5 tended to increase the formation of CysLT but this difference was not statistically significant ($P = .17$). These data indicate that TNF/IL-1 β as well as IL-13 increases the gene expression of *PLA2G10* in eosinophils and that TNF/IL-1 β treatment increased fMLP-mediated formation of CysLTs.

DISCUSSION

Eosinophils are known to have endogenous sPLA₂ activity,^{15,16} but the only previously characterized sPLA₂ enzyme in eosinophils is sPLA₂-IIA,¹⁷ an enzyme that does not contribute to the formation of leukotrienes in these cells.^{18,19} A previous study found that the sPLA₂-X gene *PLA2G10* is expressed in eosinophils.¹⁶ In this study, we confirmed that *PLA2G10* is expressed in human eosinophils, and demonstrate for the first time that sPLA₂-X significantly contributes to sPLA₂ activity

and is involved in the endogenous release of arachidonic acid and the formation of CysLTs. On the basis of confocal microscopy, we found that sPLA₂-X resides within the secretory compartment and appears to be externalized during activation. Because it is known that fMLP-mediated formation of CysLTs is mediated by cPLA₂α, we found that sPLA₂-X was involved in the activation of the MAPK cascade, specifically ERK1/2 and p-38, which activate cPLA₂α by phosphorylation at a Ser⁵⁰⁵.^{32,42,43} These results show that sPLA₂-X serves as a key regulator of CysLT formation by eosinophils, in part through the regulation of the MAPK cascade and cPLA₂α. We further demonstrate that the gene expression of *PLA2G10* is increased in response to TNFα/IL-β, and IL-13, and that TNFα/IL-β increased the formation of CysLT by eosinophils. These findings have important implications for asthma and allergic disease through the identification of a novel PLA₂ enzyme that we show for the first time to regulate leukotriene formation by eosinophils.

Human eosinophils undergo piecemeal degranulation in response to fMLP stimulation,⁴⁴ suggesting that the sPLA₂-X that we identified in the secretory compartment should be externalized during activation. Externalization may be very important because sPLA₂-X has very high activity for phosphatidylcholine that is enriched on the extracellular-facing plasma membrane¹¹ and a large portion of eicosanoid synthesis in transfected HEK293 cells occurred after secretion on the extracellular face of the cell.⁴⁵ We did not observe immunostaining on the outer cell membrane by confocal studies probably because sPLA₂-X does not bind to the plasma membrane or accumulate on the cell surface.⁸ The relatively large volume of cell culture medium that was used *in vitro* for the experiment also reduces cell surface binding. Because the ROC-0929 sPLA₂-X inhibitor is cell impermeant and blocked the formation of CysLT, these studies support the finding that endogenous sPLA₂-X is externalized where it can act on the outer cell membrane to generate free fatty acids including arachidonic acid, generate lysophospholipids, and contribute to surfactant dysfunction. These results indicate that the endogenous sPLA₂-X acts in a manner that we have identified previously for exogenous sPLA₂-X, causing the formation of CysLT via the MAPK cascade and cPLA₂α, and also further amplifying the formation of CysLT by providing an additional source of arachidonic acid.^{14,46} The release of lysophospholipids is critical in the initiation of the MAPK cascade as we have previously demonstrated in eosinophils,¹⁴ and lysophospholipids were recently implicated as a major regulator of innate lymphoid cell responses in the periphery.⁴⁷

A key control point in sPLA₂-X-mediated activation of eicosanoid synthesis is the proteolytic cleavage of a propeptide that must be removed to generate the active enzyme.^{48,49} Transgenic expression of full-length *Pla2g10* in a mouse created no alteration in phenotype in the absence of an inflammatory environment.³⁷ In contrast, transgenic expression of *Pla2g10* without the propeptide (ie, active sPLA₂-X) in macrophages causes unrestrained inflammation.⁵⁰ The propeptide contains a highly conserved RXn(K/R)R furin-recognition motif at its C-terminus.³⁶ We found that a competitive furin propeptide convertase inhibitor (ie, 6-D-R) that can act either within or following secretion from cells³⁸ inhibits the formation of LTC₄ in these cells. In the cell lysate or the extracellular fluid, only a single band for the mature protein is seen (Figs 1, B, and E4, E). These results suggest that sPLA₂-X is activated within the cell before

secretion. Intracellular inhibition of sPLA₂-X could have even greater effects on eicosanoid formation in these cells; however, this hypothesis could not be further tested because a cell permeate sPLA₂-X inhibitor is not available. As seen in Fig E3, the eosinophil-like cell lines HL-60-C15 and AML14.3D10-CCR3 have much lower expression of *PLA2G10* than do primary human eosinophils; thus, shRNA gene silencing studies were not attempted. We also found that the reduced activation of eosinophils after 24 hours in cell culture limited our ability to perform short-term siRNA studies (Fig E4). In a previous study, an irreversible furin propeptide convertase inhibitor (ie, D-RVKR-CMK) prevented the secretion of the protein in HEK293 cells transfected with full-length human *PLA2G10* or murine *Pla2g10*, suggesting that proteolytic conversion is regulating sPLA₂-X release.³⁶ This same irreversible furin propeptide convertase inhibitor did not alter the formation of LTC₄ in eosinophils, suggesting that different propeptide convertases regulate leukotriene formation in HEK293 cells and primary human eosinophils.^{38,51-53} Because furins serve as checkpoints of inflammation, these results suggest that proteolytic control of sPLA₂-X may be important in eosinophil-mediated inflammation.⁵⁴

It is well established that airway eosinophils relative to circulating eosinophils have increased *ex vivo* survival and markers of activation.^{41,55-57} Although IL-5 and GM-CSF have the most established effects on the production, differentiation, and survival of eosinophils,⁵⁸⁻⁶⁰ eosinophil migration is also regulated by IL-4 and IL-13.^{61,62} We found that IL-13 and IL-1β in combination with TNF were the major cytokines that increased *PLA2G10* expression. We also found that TNF/IL-1β priming of eosinophils increased the formation of CysLT, suggesting that such an increase in *PLA2G10* may be involved in increasing the formation of CysLT by eosinophils. A number of cytokines including IL-5 and GM-CSF actually decreased *PLA2G10* expression. Furthermore, the expression of *PLA2G10* in eosinophils was decreased by an HDAC inhibitor and by IL-33 and TSLP. These findings were unexpected because IL-33 activates eosinophils via the transmembrane ST2 receptor,⁶³⁻⁶⁵ and TSLP activates both CD34⁺ progenitor cells and mature eosinophils.^{66,67}

A major question raised by these results is whether the increased levels of sPLA₂-X identified in asthma^{12,13} could be the result of secretion of this enzyme from eosinophils as suggested by the present study. Because we have shown that the epithelium¹³ and airway macrophages²⁹ immunostain in human airway samples for sPLA₂-X, and that primary epithelial cells in organotypic culture secrete the mature sPLA₂-X protein,¹³ the precise cellular source of the enzyme in humans is unclear. The deletion of the murine sPLA₂-X gene (*pla2g10*)⁶⁸ or an active-site-directed inhibitor of human sPLA₂-X in a transgenic mouse model⁶⁹ inhibits the development of airway inflammation, hyperresponsiveness, and structural remodeling. Thus, it will be important to identify the relative importance of the epithelium, macrophages, and eosinophils in future murine studies examining the *in vivo* effects of sPLA₂-X on allergic inflammation.

These studies have important potential therapeutic implications for asthma because it is clear that lipid mediators including CysLTs play an important role in asthma,^{70,71} but therapies targeting CysLTs have been only modestly effective by virtue of blocking only 1 receptor (ie, CysLT₁ receptor that predominantly binds to LTD₄), while these lipid mediators bind

to multiple receptors (ie, CysLT₂ that preferentially binds LTC₄, and the LTE₄ receptors, CysLT_E, P2Y₁₂ and GPR99).^{72,73} In this article, we showed for the first time in a primary human cell that an inhibitor of sPLA₂-X can effectively block the formation of CysLTs. Furthermore, the products of sPLA₂s including lysophospholipids may also serve as important regulators of T_H2-mediated inflammation.⁴⁷ These findings further support sPLA₂-X as a potential therapeutic target in asthma.

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RESULTS

Eosinophil-like cell lines do not serve as an adequate model for sPLA₂-X function

We differentiated HL-60-C15 cells with the HDAC inhibitor sodium butyrate (0.5 mM) for 7 days and found that the morphology of the cells attained characteristics of mature eosinophils, including an increase in the ability to synthesize CysLTs in response to fMLP (Fig E3, A; $P = .003$). In such differentiated HL-60-C15 cells, the effect of the sPLA₂-X inhibitor was modest as opposed to the effects identified in primary human eosinophils (Fig E3, B; $P = .04$). Further investigation of the effects of the HDAC inhibitor on these cells revealed that the HDAC inhibitor markedly suppressed the gene expression of *PLA2G10* (Fig E3, C; $P = .001$).

Because HL-60-C15 cells require the HDAC inhibitor to attain a mature morphology, we also evaluated the potential use of the AML14.3D10-CCR3 eosinophil-like cell line. We found that the gene expression of *PLA2G10* was much lower in either undifferentiated HL-60-C15 cells or AML14.3D10-CCR3 relative to primary human epithelial cells (Fig E3, D). Kwatia et al^{E1} further corroborate these findings of differences in the level of expression in primary eosinophils and the AML14.3D10 cell line. We further validated these findings with a Western blot of cell lysates from primary human eosinophils, undifferentiated HL-60-C15 cells, and AML14.3D10-CCR3 cells in addition to recombinant version of the sPLA₂-X proenzyme and the mature sPLA₂-X enzyme (Fig E3, D). The results show that there is significantly more sPLA₂-X protein in primary human eosinophils than in either of the 2 cell lines tested. Furthermore, there is a gel shift in the protein, suggesting that there are post-translational modifications of the protein structure, as we have seen in previous studies.^{E2,E3} In our experience, the *PLA2G10* expression in these cells is not sufficient to serve as an adequate target for shRNA studies. These results indicate that eosinophil-like cell lines do not serve as an adequate model for further examining the mechanism of sPLA₂-X-mediated regulation of leukotriene synthesis that occurs in primary cells.

We also evaluated the ability to maintain primary human eosinophils in culture in the laboratory over a prolonged period of time to conduct siRNA studies. We found that after 24 hours in cell culture, primary eosinophils lose the capacity for strong fMLP-mediated eicosanoid generation either with or without the addition of FCS to the medium (Fig E4).

sPLA₂-X may be secreted during eosinophil activation

Because we found that sPLA₂-X resides at least in part in the secretory apparatus of eosinophils, and it is known that the ROC-0929 inhibitor is not readily cell permeable,^{E4} we examined whether sPLA₂-X is secreted during activation. We found that the sPLA₂-X protein could be identified in the supernatant of eosinophils, but the level is low based on Western blot and there was no apparent difference in the amount of sPLA₂-X in the eosinophil supernatant with activation (Fig E6, A). One checkpoint in the activation of sPLA₂-X is the cleavage of a propeptide that occurs at a furin consensus sequence.^{E2,E5} We found that a competitive inhibitor of furin-like propeptide convertases that acts intracellularly or extracellularly as a substrate mimic (6-D-R)^{E6} inhibited fMLP-mediated formation of LTC₄ in the same range of magnitude as the cell-impermeant ROC-0929 sPLA₂-X inhibitor (Fig E6, B). In contrast, an irreversible propeptide convertase inactivator (D-RVKR-CMK)^{E7} did not alter the formation of LTC₄. Although these results are not definitive, they suggest that the sPLA₂-X enzyme is secreted during activation and that it may be activated by proteolytic cleavage before secretion.

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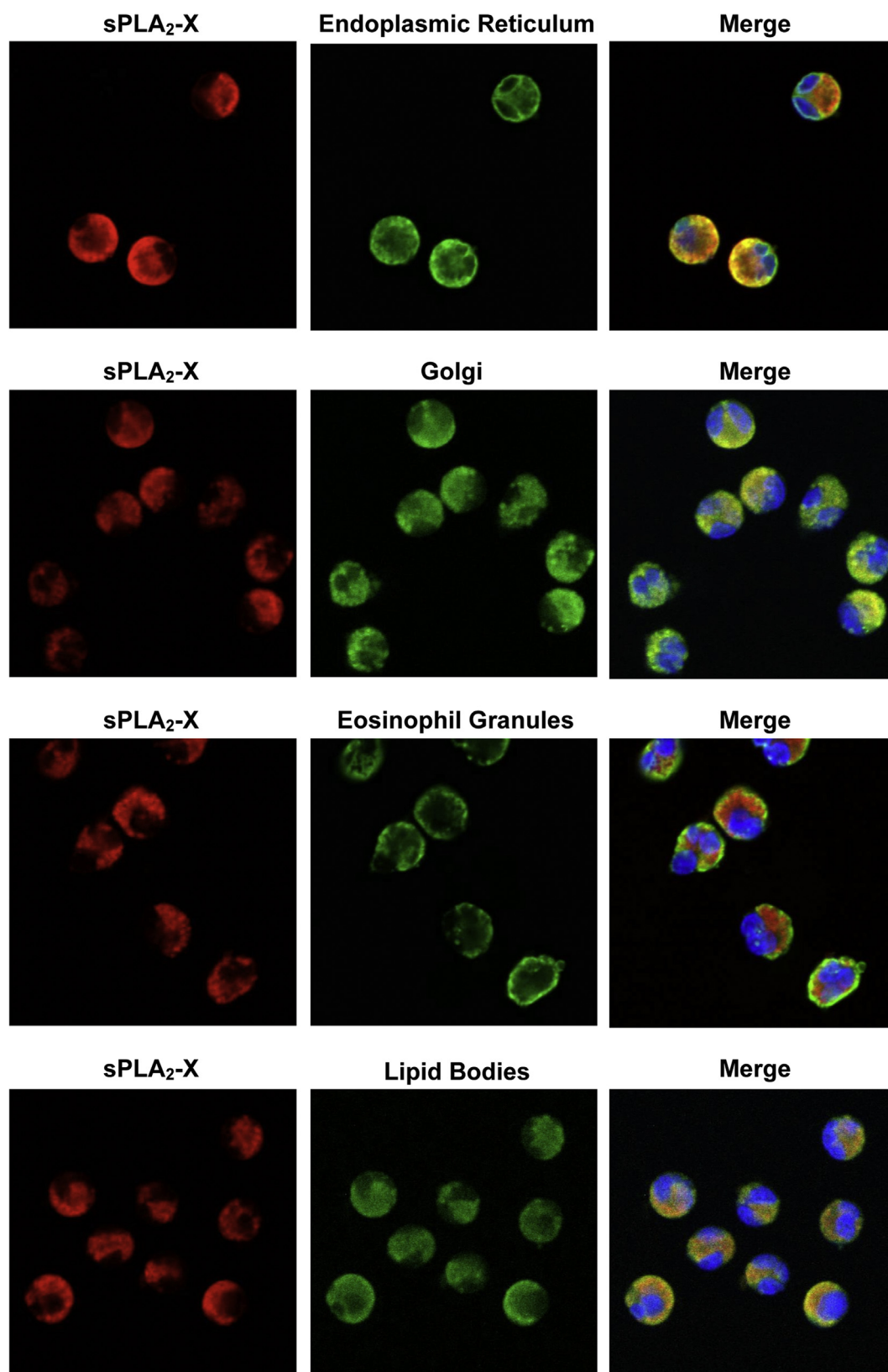


FIG E1. Localization of sPLA₂-X in unstimulated human eosinophils by confocal microscopy. Immunostaining was used to colocalize the sPLA₂-X (*red*) immunostaining to the endoplasmic reticulum (ER, anti-PDI), golgi (anti-GM130), granules (anti-EPX), and lipid bodies (anti-ADRP), all in *green*. In unstimulated eosinophils, sPLA₂-X colocalized predominantly to the ER and golgi (*first and second panels*).

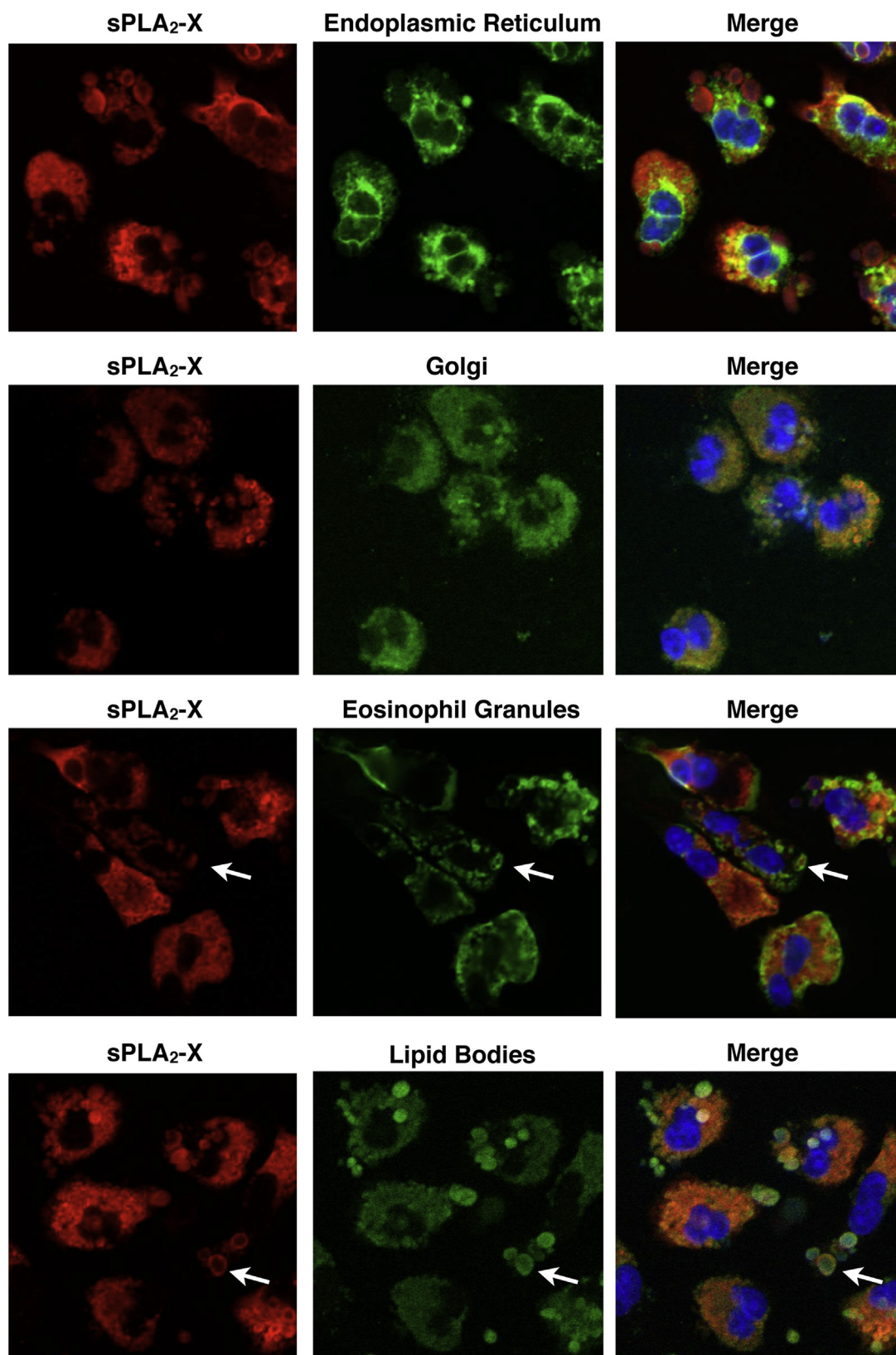


FIG E2. Localization of sPLA₂-X in fMLP-stimulated human eosinophils by confocal microscopy. Immunostaining was used to colocalize sPLA₂-X immunostaining to other structures as outlined in Fig E1. Following fMLP stimulation, there was an increase in colocalization to the endoplasmic reticulum and granules, and formation of lipid bodies that have immunostaining for sPLA₂-X. Arrows in the figure delineate sPLA₂-X immunostaining (red) within structures that clearly immunostain with markers for granules (anti-EPX in green, third panel down) and lipid bodies (anti-ADRP in green, the lower panel). Some of the lipid bodies containing sPLA₂-X appear extracellular.

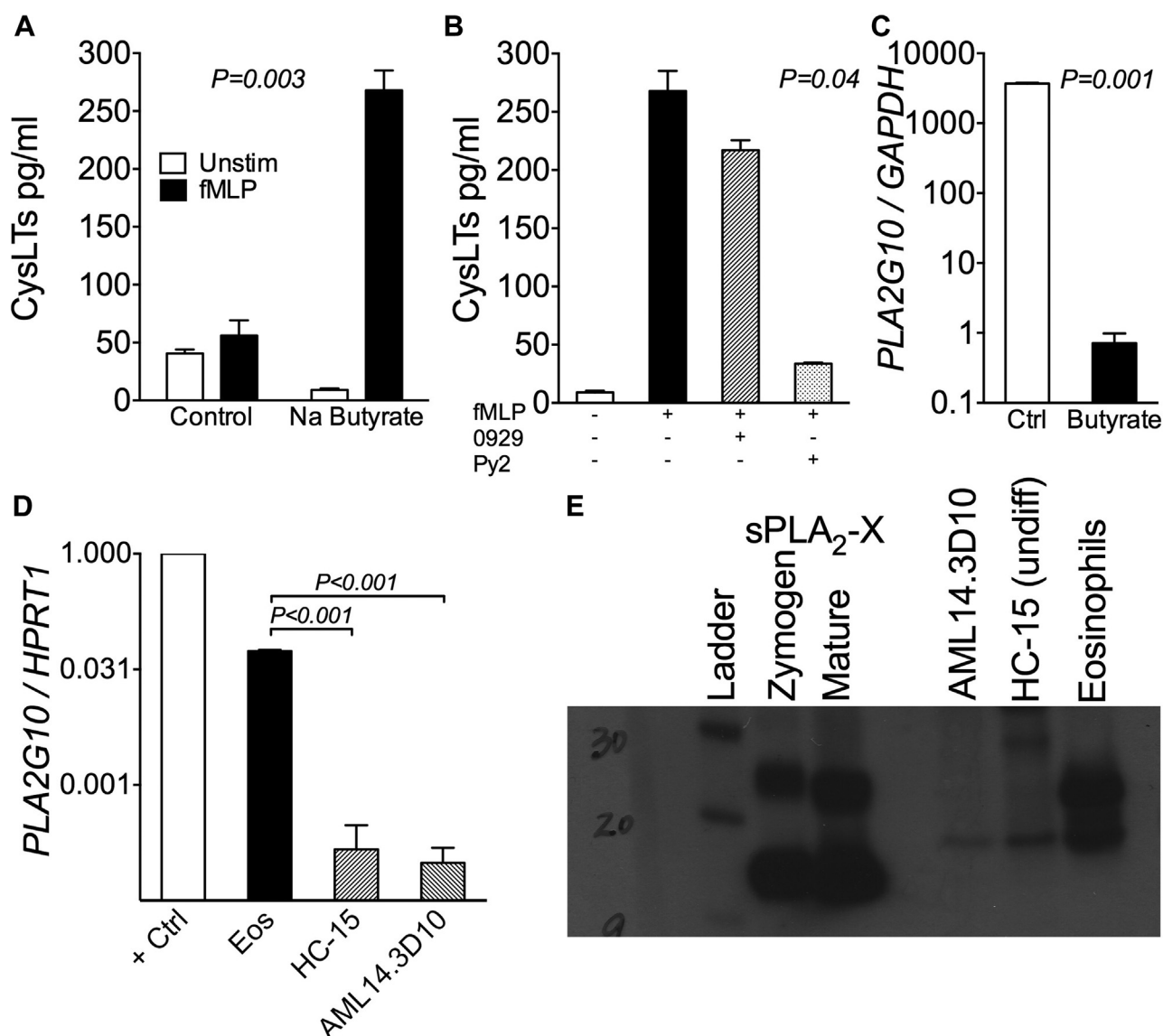


FIG E3. Characterization of *PLA2G10* in primary eosinophils and eosinophil-like cell lines. **A**, Differentiation of HL-60-C15 cells with the HDAC inhibitor sodium butyrate increased the ability of these cells to synthesize CysLTs in response to fMLP. **B**, The effect of the sPLA₂-X inhibitor was modest in these cells in contrast to the effects of the cPLA₂ inhibitor Pyr-2. **C**, The HDAC inhibitor markedly suppressed the gene expression of *PLA2G10* in HL-60-C15 cells. **D**, Comparison of the expression of *PLA2G10* in primary eosinophils demonstrates that while primary eosinophils strongly express *PLA2G10*, undifferentiated (undiff) HL-60-C15 cells and AML14.3D10-CCR3 cells have extremely low expression of *PLA2G10*. **E**, A Western blot with recombinant human sPLA₂-X zymogen and mature enzyme demonstrates that there is more sPLA₂-X protein in primary human eosinophils relative to undifferentiated HL-60-C15 cells and AML14.3D10-CCR3 cells. In this blot, the endogenous enzyme has a gel shift from the recombinant protein and there is also a band that may represent a dimer of both the recombinant protein and the native protein. Ctrl, Control; Eos, eosinophil; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPRT1, hypoxanthine phosphoribosyltransferase 1; unstim, unstimulated.

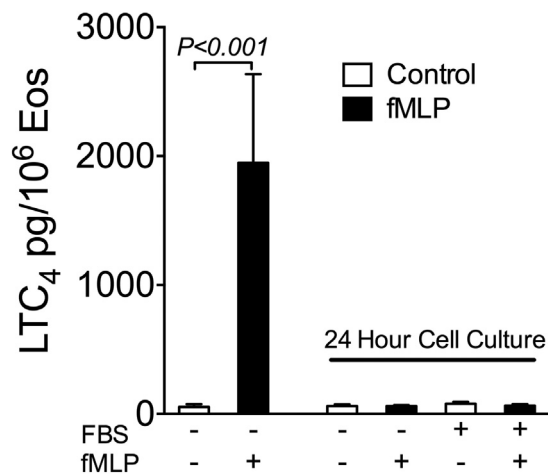


FIG E4. Effects of prolonged cell culture on the ability to activate primary eosinophils. In freshly isolated eosinophils, there is a marked increase in the production of LTC₄ in response to fMLP (100 nM) 20 minutes after treatment with fMLP. In contrast, following 24 hours of cell culture in RPMI with or without FCS, eosinophils failed to generate LTC₄ following the same fMLP stimulus. *Eos*, Eosinophil.

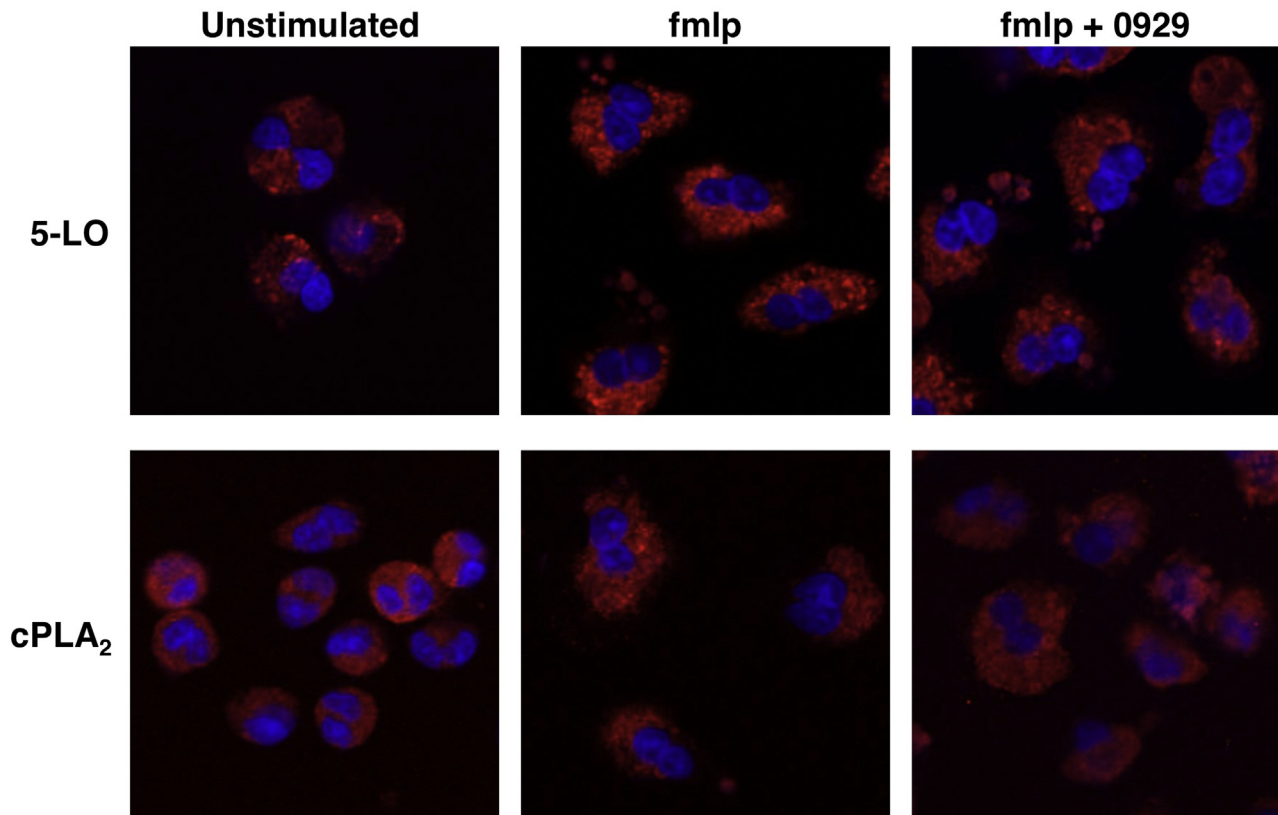


FIG E5. Effect of the sPLA₂-X inhibitor on the translocation of cPLA₂ and 5-LO in response to fMLP. Eosinophils were allowed to adhere to BSA-coated cover slips and treated with vehicle alone (unstimulated), fMLP, or fMLP with the sPLA₂-X inhibitor 0929. Immunostaining for 5-LO (*upper panel*) and cPLA₂α (*lower panel*) changes from faint and diffuse to more focal staining in perinuclear spaces and focal intracytoplasmic locations. The changes in 5-LO and cPLA₂α were attenuated by pretreatment of the eosinophils with the sPLA₂-X inhibitor 0929.

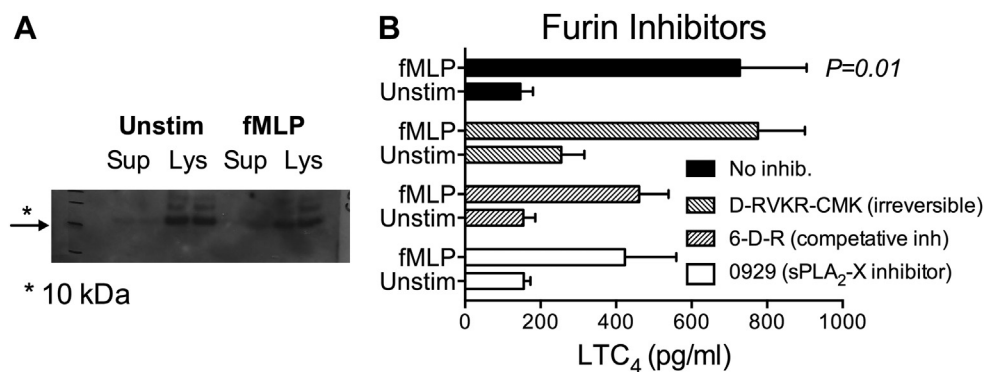


FIG E6. Effects of furin-like propeptide convertase inhibitors on the synthesis of LTC₄. **A**, The sPLA₂-X protein appears predominantly as a single band that runs in the location of the mature protein after cleavage of the propeptide in both the supernatant (Sup) and cell lysates (Lys) of eosinophils. The concentration in the supernatant was low, and changes with fMLP stimulation were not apparent. **B**, Pretreatment with a competitive inhibitor of furin-like propeptide convertase (6-D-R) inhibited the formation of LTC₄ to a similar amount as the sPLA₂-X inhibitor. In contrast, the irreversible furin-like propeptide convertase inactivator (6-RVKR-CMK) did not attenuate LTC₄ formation. *Unstim*, Unstimulated.

TABLE E1. Selectivity of the sPLA₂-X inhibitor (ROC-0929) and the structurally similar control inhibitor (ROC-0428), a nonselective sPLA₂ inhibitor (0509A), and an inhibitor predominantly active against group II sPLA₂s (0320) for the 9 mammalian sPLA₂s (IC₅₀ [nM]^{*})

sPLA ₂	Inhibitor			
	ROC-0929	ROC-0428	0509A	0320
hGIB	>1600	ND	80 ± 3	>1600
hGIIA	>1600	ND	40 ± 2	35 ± 2
hGIID [†]	700 ± 230	ND	7 ± 3	>1300
hGIIIE	>1600	ND	7 ± 2	50 ± 10
hGIIF	>1600	ND	50 ± 3	>1600
hGIII	>1600	ND	>1600	>1600
hGV	>1600	ND	35 ± 7	>1600
hGX	20 ± 10	6600 ± 900	20 ± 3	>1600
hGXIIA	>1600	ND	>1600	>1600

ND, Not determined.

^{*}IC₅₀ values obtained using fluorometric assay with pyrene-labeled phosphatidylglycerol as substrate.

[†]IC₅₀ value obtained using radiolabeled *E coli* membrane assay.