

1 **Supplement**

2
3 Endogenous secreted phospholipase A₂ group X regulates cysteinyl leukotrienes synthesis by
4 human eosinophils

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14 **Supplemental Results**

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

16 We differentiated HL-60-C15 cells with the HDAC inhibitor sodium butyrate (SB, 0.5 mM)
17 for 7 days and found that the morphology of the cells attained characteristics of mature
18 eosinophils, including an increase in the ability to synthesize CysLTs in response to fMLP (**Fig**
19 **E3A**, $P=0.003$). In such differentiated HL-60-C15 cells the effect of the sPLA₂-X inhibitor was
20 modest as opposed to the effects identified in primary human eosinophils (**Fig E3B**, $P=0.04$).
21 Further investigation of the effects of the HDAC inhibitor in these cells revealed that the HDAC
22 inhibitor markedly suppressed the gene expression of *PLA2G10* (**Fig E3C**, $P=0.001$).

23 Because HL-60-C15 cells require the HDAC inhibitor to attain a mature morphology, we
24 also evaluated the potential use of the AML14.3D10-CCR3 eosinophil-like cell line. We found
25 that the gene expression of *PLA2G10* was much lower in either undifferentiated HL-60-C15 cells
26 or AML14.3D10-CCR3 relative to primary human epithelial cells (**Fig E3D**). Kwatia et al further
27 corroborate these findings of differences in the level of expression in primary eosinophils and
28 the AML14.3D10 cell line (6). We further validated these findings with a western blot of cell
29 lysates from primary human eosinophils, undifferentiated HL-60-C15 cells and AML14.3D10-
30 CCR3 cells in addition to recombinant version of the sPLA₂-X pro-enzyme and the mature sPLA₂-
31 X enzyme (**Fig E3D**). The results show that there is significantly more sPLA₂-X protein in primary
32 human eosinophils, than in either of the two cell lines tested. Further, there is a gel shift in the
33 protein suggesting that there are post-translational modifications of the protein structure, as
34 we have seen in prior studies (2,7). In our experience, the *PLA2G10* expression in these cells is
35 not sufficient to serve as an adequate target for shRNA studies. These results indicate that

36 eosinophil-like cell lines do not serve as an adequate model for further examining the
37 mechanism of sPLA₂-X mediated regulation of leukotriene synthesis that occurs in primary cells.

38 We also evaluated the ability to maintain primary human eosinophils in culture in the
39 laboratory over a prolonged period of time to conduct siRNA studies. We found that after 24
40 hours in cell culture, primary eosinophils lose the capacity for strong fMLP-mediated eicosanoid
41 generation either with or without the addition of fetal calf serum (FCS) to the medium (**Fig E4**).
42 *sPLA₂-X may be secreted during eosinophil activation.*

43 As we found that sPLA₂-X resides at least in part in the secretory apparatus of
44 eosinophils, and it is known that the ROC-0929 inhibitor is not readily cell permeable (1), we
45 examined whether sPLA₂-X is secreted during activation. We found that the sPLA₂-X protein
46 could be identified in the supernatant of eosinophils, but the level is low based on western blot
47 and there was no apparent difference in the amount of sPLA₂-X in the eosinophil supernatant
48 with activation (**Fig E6A**). One checkpoint in the activation of sPLA₂-X is the cleavage of a pro-
49 peptide that occurs at a furin consensus sequence (2,3). We found that a competitive inhibitor
50 of furin-like propeptide convertases that acts intracellularly or extracellularly as a substrate
51 mimic (6-D-R) (4) inhibited fMLP-mediated LTC₄ formation in the same range of magnitude as
52 the cell impermeant ROC-0929 sPLA₂-X inhibitor (**Fig E6B**). In contrast an irreversible pro-
53 peptide convertase inactivator (D-RVKR-CMK) (5) did not alter LTC₄ formation. Although these
54 results are not definitive, they suggest that the sPLA₂-X enzyme is secreted during activation,
55 and that it may be activated by proteolytic cleavage prior to secretion.

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57

58 **Supplemental Figure Legends:**

59 **Figure E1.** Localization of sPLA₂-X in unstimulated human eosinophils by confocal microscopy.

60 Immunostaining was used to co-localize the sPLA₂-X (red) immunostaining to the endoplasmic
61 reticulum (ER, anti-PDI), golgi (anti-GM130), granules (anti-EPX) and lipid bodies (anti-ADRP), all
62 in green. In unstimulated eosinophils, sPLA₂-X co-localized predominantly to the endoplasmic
63 reticulum (ER) and golgi (first and second panels).

64

65 **Figure E2.** Localization of sPLA₂-X in fMLP stimulated human eosinophils by confocal

66 microscopy. Immunostaining was used to co-localize sPLA₂-X immunostaining to other

67 structures as outlined in Figure E1. Following fMLP stimulation, there was an increase in

68 colocalization to the ER and granules, and formation of lipid bodies that have immunostaining

69 for sPLA₂-X. Arrows in the figure delineate sPLA₂-X immunostaining (red) within structures that

70 clearly immunostain with markers for granules (anti-EPX in green, third panel down) and lipid

71 bodies (anti-ADRP in green, lower panel). Some of the lipid bodies containing sPLA₂-X appear

72 extracellular.

73

74 **Figure E3.** Characterization of *PLA2G10* in primary eosinophils and eosinophil-like cell lines. A,

75 Differentiation of HL-60-C15 cells with the HDAC inhibitor sodium butyrate increased the ability

76 of these cells to synthesize CysLTs in response to fMLP. B, The effect of the sPLA₂-X inhibitor

77 was modest in these cells in contrast to the effects of the cPLA₂α inhibitor Pyr-2. C, The HDAC

78 inhibitor markedly suppressed the gene expression of *PLA2G10* in HL-60-C15 cells. D,

79 Comparison of the expression of *PLA2G10* in primary eosinophils demonstrates that while

80 primary eosinophils strongly express *PLA2G10*, undifferentiated HL-60-C15 cells and
81 AML14.3D10-CCR3 cells have extremely low expression of *PLA2G10*. E, A western blot with
82 recombinant human sPLA₂-X zymogen and mature enzyme demonstrates that there is more
83 sPLA₂-X protein in primary human eosinophils relative to undifferentiated HL-60-C15 cells and
84 AML14.3D10-CCR3 cells. In this blot, the endogenous enzyme has a gel shift from the
85 recombinant protein, and there is also a band that may represent a dimer of both the
86 recombinant protein and the native protein.

87

88 **Figure E4.** Effects of prolonged cell culture on the ability to activate primary eosinophils. In
89 freshly isolated eosinophils, there is a marked increase in LTC₄ production in response to fMLP
90 (100 nM) 20 minutes after treatment with fMLP. In contrast, following 24 hours of cell culture
91 in RPMI with or without FCS, eosinophils failed to generate LTC₄ following the same fMLP
92 stimulus.

93

94 **Figure E5.** Effect of the sPLA₂-X inhibitor on the translocation of cPLA₂ and 5-LO in response to
95 fMLP. Eosinophils were allowed to adhere to BSA coated cover slips and treated with vehicle
96 alone (unstimulated), fMLP or fMLP with the sPLA₂-X inhibitor 0929. A, Immunostaining for 5-
97 LO (upper panel) and cPLA₂α (lower panel) changes from faint and diffuse to more focal staining
98 in peri-nuclear spaces and focal intracytoplasmic locations. The changes in 5-LO and cPLA₂α
99 were attenuated by pre-treatment of the eosinophils with the sPLA₂-X inhibitor 0929.

100

101 **Figure E6.** Effects of furin-like propeptide convertase inhibitors on LTC₄ synthesis. A, The sPLA₂-
102 X protein appears predominantly as a single band that runs in the location of the mature
103 protein after cleavage of the propeptide in both the supernatant (Sup) and cell lysates (Lys) of
104 eosinophils. The concentration in the supernatant was low, and changes with fMLP stimulation
105 were not apparent. B, Pre-treatment with a competitive inhibitor of furin-like propeptide
106 convertase (6-D-R) inhibited the formation of LTC₄ to a similar amount as the sPLA₂-X inhibitor.
107 In contrast, the irreversible furin-like propeptide convertase inactivator (6-RVKR-CMK) did not
108 attenuate LTC₄ formation.
109
110

Supplemental Table I: Selectivity of sPLA₂-X inhibitor (ROC-0929) and structurally similar control inhibitor (ROC-0428), a non-selective 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
hGIIE	>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

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

**IC₅₀ value obtained using radiolabeled *E. coli* membrane assay.

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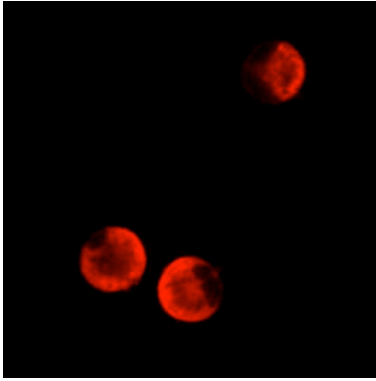
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113 **Supplemental References:**

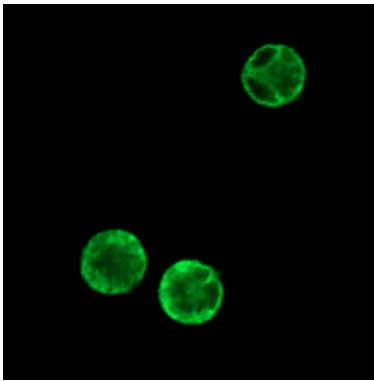
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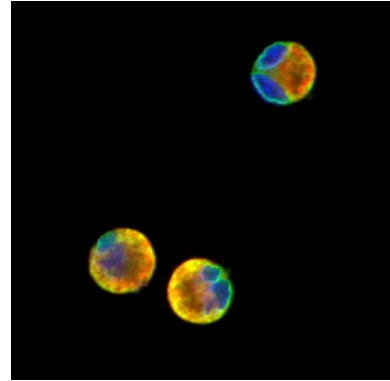
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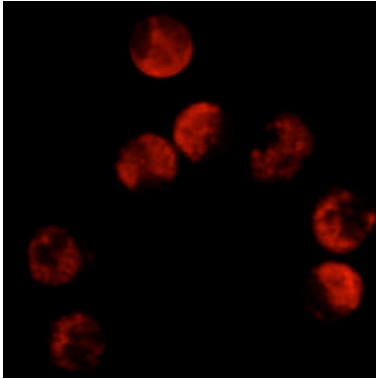
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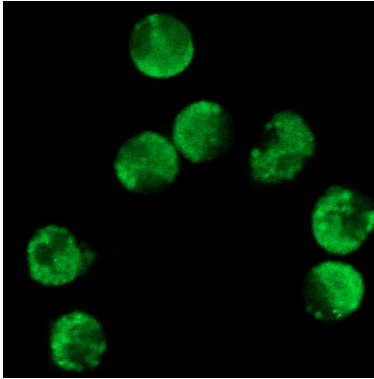
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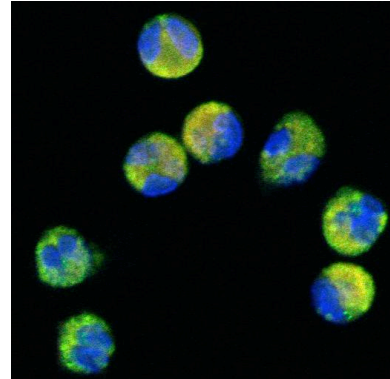
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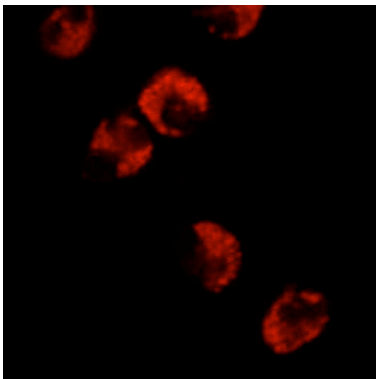
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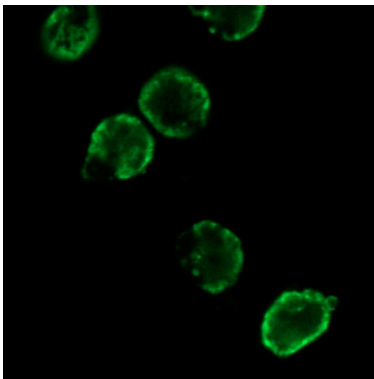
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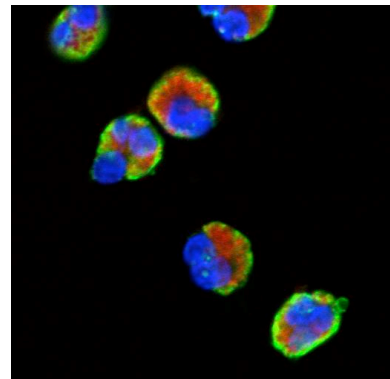
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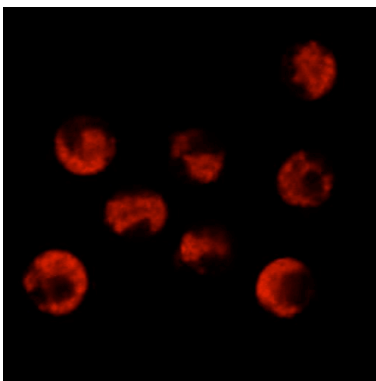
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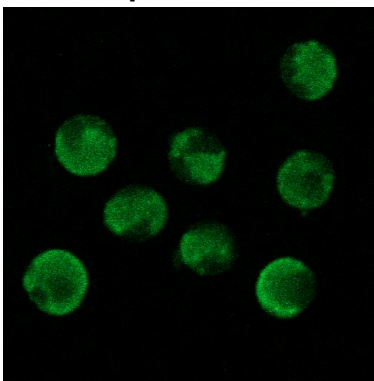
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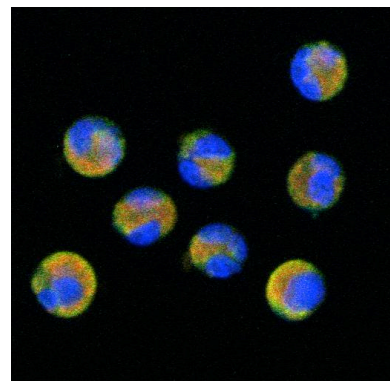
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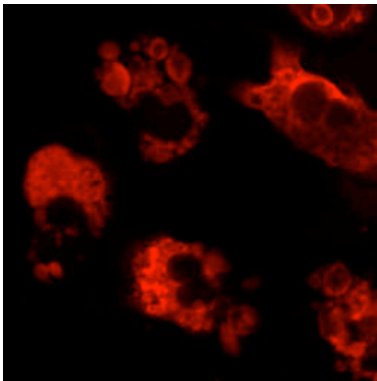
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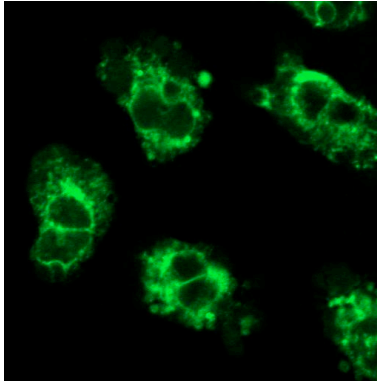
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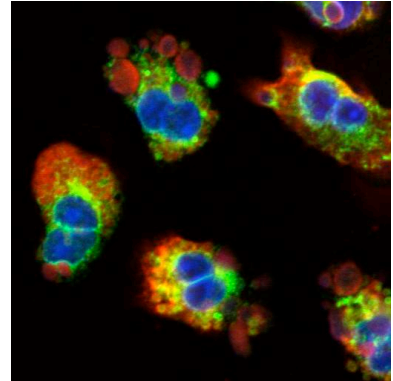
sPLA₂-X



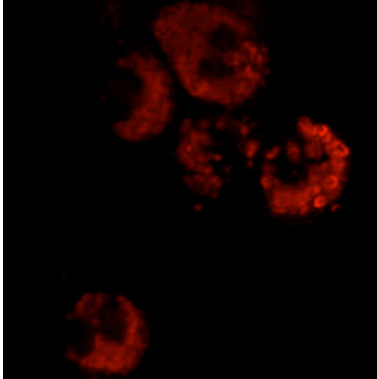
Endoplasmic Reticulum



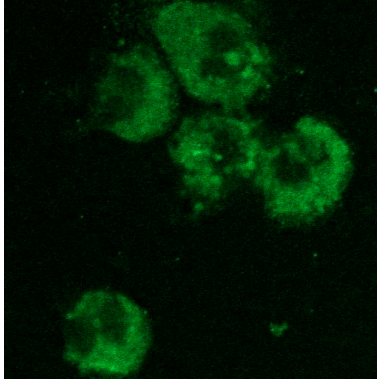
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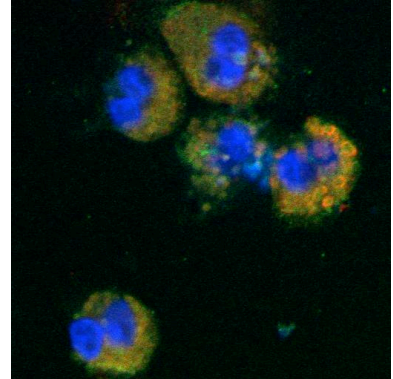
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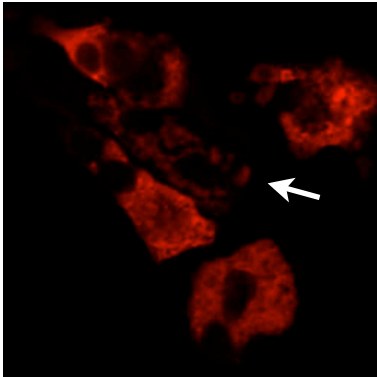
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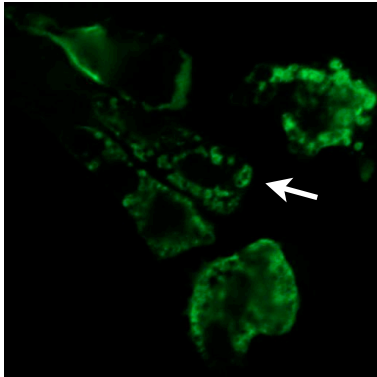
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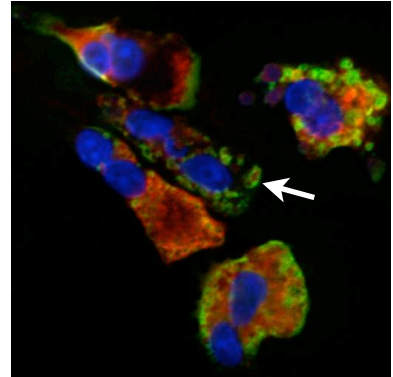
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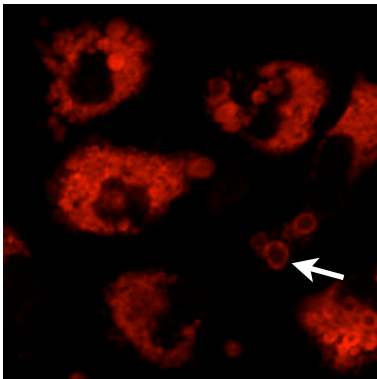
Eosinophil Granules



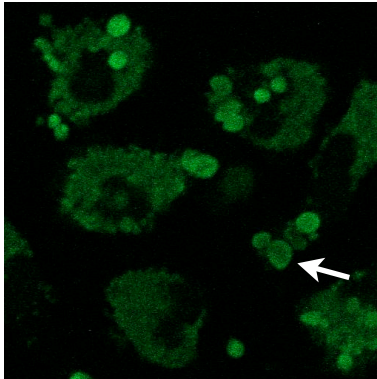
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sPLA₂-X



Lipid Bodies



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