# Supporting Information

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### SI Materials and Methods

**Cell Culture and Stimulation.** RAW264.7 cells (RAW) were cultured in DMEM (HyClone) containing 10% (vol/vol) FBS (HyClone) and 100 units/mL penicillin/streptomycin (Life Technologies) at 37 °C in a humidified 5% $CO_2$ atmosphere. Cells were plated in six-well culture plates with 2.0 mL of medium containing 10% FBS ($5 \times 10^5$ cells per well) and allowed to adhere for 24 h. Medium was aspirated and cells were washed twice with fresh serum-free medium, and 1.0 mL of serum-free medium was added to each well and incubated for 30 min. General toll-like receptor 4 (TLR4) priming protocol: 800 μL of medium containing $Kdo_2$ lipid A (KLA; Avanti Polar Lipids; 225 ng/mL; 2.25×) was added to the existing 1.0 mL (bringing well volume to 1.8 mL with 100 ng/mL KLA); an additional 200 μL of medium containing ATP (20 mM; 10×; Sigma) was then added for purinergic stimulation (bringing total well volume to 2.0 mL with 2 mM ATP); reactions were quenched by collecting the medium and adding EtOH [10% (vol/vol) total concentration, and internal standards were also added as described below for eicosanoid extraction]. Stimulation of TLR4 with KLA was 100 ng/mL, and purinergic receptor stimulation with ATP was 2 mM unless otherwise indicated in figure caption. Aspirin (ASA) and celecoxib were purchased from Sigma, pyrrophenone from Cayman Chemicals, and varespladib (LY315920) from Selleck Chemicals. Stock concentrations of KLA and ATP were 10 ng/μL and 200 mM, respectively, in PBS; celecoxib, pyrrophenone, and LY315920 stocks were 10 mM in DMSO; 15(R)-HETE and ASA stocks were 100 ng/μL and 40 mg/mL, respectively, in ethanol (aliquots of stocks in ethanol were dried under a stream of argon and resuspended in medium to working concentrations).

**Resident Peritoneal Macrophage Harvesting and Preparation.** Mice were killed via $CO_2$ asphyxiation. Peritoneal exudate cells were obtained by peritoneal lavage with cold $Ca^{2+}$- and $Mg^{2+}$-free PBS. Cells were centrifuged at $400 \times g$ for 5 min and resuspended in ice-cold RBC lysis buffer (Affymetrix eBioscience) for 10 min; then they were centrifuged and resuspended in RPMI medium 1640 supplemented with 10% heat FBS and 100 units/mL penicillin/streptomycin. Cells were plated and allowed to adhere at 37 °C in a humidified 5% $CO_2$ atmosphere for 16 h; nonadherent cells were then aspirated and remaining cells were washed with serum-free RPMI medium 1640. Cells were incubated from this point on in serum-free RPMI medium 1640, but were otherwise treated the same during stimulation as RAW cells described above.

**Bone-Marrow–Derived Macrophage Preparation and Cytosolic Phospholipase $A_2$ Deletion.** Bone-marrow–derived macrophages (BMDMs) were generated from femurs and tibiae (as previously described in ref. 1) of group IVA cytosolic phospholipase $A_2$ (GIVA cPLA$_2$) ($^{-/-}$) and ($^{+/+}$) mice (generated as previously described in ref. 2) from a C57BL/6 and Sv/129 mixed background. Mice were 8-wk-old male littermates. BMDM cells were stimulated in serum-free RPMI medium 1640, but otherwise conditions identical to RAW cell experiments were used.

**Eicosanoid Extraction from Cell Medium.** After stimulation endpoints, the entire 2.0 mL of medium was removed, and each sample was supplemented with 50 μL of internal standards (200 pg/μL, EtOH; Cayman). Eicosanoids were purified using solid-phase extraction and were prepared for analysis as previously described (1).

**Extraction and Analysis of Eicosanoids from Cell Membrane Phospholipids.** Cells were washed two times with ice-cold PBS and were scraped into 1 mL ice-cold PBS before centrifugation; supernatant was aspirated before storage at −80 °C. Phospholipids were extracted and then saponified as previously described (3). Eicosanoid internal standards were added and eicosanoids were similarly extracted and analyzed by the protocol above.

**Cell Quantitation.** Eicosanoid and fatty acid levels were normalized to cell number. After extracellular media were removed, cells were scraped into 500 μL of PBS and live cells were then counted using Trypan blue exclusion with a hemocytometer. Cell numbers were consistent between different sample groups.

**Reverse-Phase Liquid Chromatography Tandem Mass Spectrometry.** Eicosanoid analysis was performed by LC-MS/MS as previously described (4) using a tandem quadrupole mass spectrometer (ABI 4000 QTrap; Applied Biosystems) via multiple-reaction monitoring (MRM) in negative-ion mode. Eicosanoids were identified in samples by matching their MRM signal and LC retention time with those of a pure standard.

**Chiral Chromatography Tandem Mass Spectrometry.** Liquid normal-phase chiral chromatography was carried out using an adaptation of a method previously described (5). Separation was carried out on a 4.6 × 250-mm Chiral Technologies derivatized amylose column (Chiralpak AD-H) held at 35 °C and at a flow rate of 0.3 mL/min. Buffer A was hexane/anhydrous ethanol/water/formic acid: 96/4/0.08/0.02, vol/vol; buffer B was 100% anhydrous ethanol. Gradient elution was achieved using 100/0, A/B at 0 min to 2 min and linearly ramped to 90/10, A/B by 13 min; linearly ramped to 85/15, A/B by 15 min and held until 25 min; then linearly ramped to 50/50, A/B by 26 min and held until 41 min; then linearly ramped back to 100/0, A/B by 42 min and held there until 57 min to achieve column reequilibration. The chiral chromatography effluent was coupled to the same mass spectrometer as described above for reversed-phase LC/MS, except the ion source was operated in atmospheric pressure chemical ionization (APCI) mode using previously described settings (5).

**RNA Isolation and Quantitative RT-PCR.** Total RNA was isolated from macrophage cells using the RNeasy mini kit (Qiagen) and DNase 1 (Invitrogen). First-strand cDNA was synthesized using SuperScript III and random hexamers (Invitrogen). Samples were run in 20-μL reactions using an ABI 7300 (Applied Biosystems). Samples were incubated at 95 °C for 15 min, followed by 40 cycles at 95 °C for 10 s, 56 °C for 20 s, and 72 °C for 30 s. SYBR Green oligonucleotides were used for detection and quantification of a given gene, expressed as relative mRNA level compared with a standard housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase) using the ΔCT method, as described by the manufacturer (Invitrogen). RT-PCR primers for 5-lipoxygenase (5-LOX) (alox5; forward: ACTACATCTACCTCAGCCTCATT; reverse: GGTGACATCGTAGGAGTCCAC) and 5-LOX–activating protein (FLAP) (alox5ap; forward: AGCATGAAAGCAAGGCGCATA; reverse: GTACGCATCTACGCAGTTCTG) were purchased from PrimerBank. Further details have been previously described (1).

1. Norris PC, Reichart D, Dumlao DS, Glass CK, Dennis EA (2011) Specificity of eicosanoid production depends on the TLR-4-stimulated macrophage phenotype. *J Leukoc Biol* 90(3):563–574.
2. Bonventre JV, et al. (1997) Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A2. *Nature* 390(6660):622–625.
3. Norris PC, Dennis EA (2012) Omega-3 fatty acids cause dramatic changes in TLR4 and purinergic eicosanoid signaling. *Proc Natl Acad Sci USA* 109(22):8517–8522.
4. Dumlao DS, Buczynski MW, Norris PC, Harkewicz R, Dennis EA (2011) High-throughput lipidomic analysis of fatty acid derived eicosanoids and N-acylethanolamines. *Biochim Biophys Acta* 1811(11):724–736.
5. Harkewicz R, Fahy E, Andreyev A, Dennis EA (2007) Arachidonate-derived dihomoprostaglandin production observed in endotoxin-stimulated macrophage-like cells. *J Biol Chem* 282(5):2899–2910.

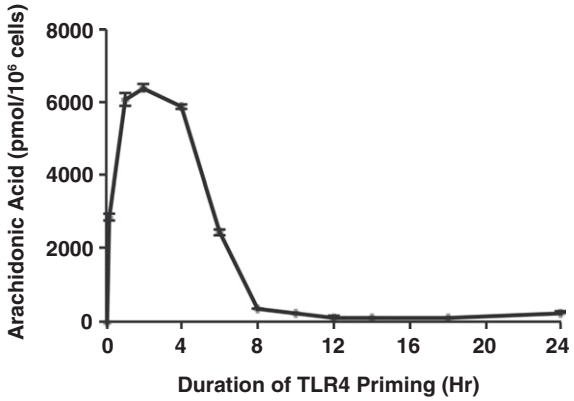


**Fig. S1.** Purinergic-stimulated arachidonic acid release is maximal with short-term TLR4 priming, and is drastically reduced with long-term priming. Extracellular arachidonic acid (AA) levels in medium after priming RAW cells with KLA for the indicated durations and final 10-min stimulation with ATP. Data are mean values of three separate experiments ± SEM (and from the same experiments as in Fig. 1*A*).

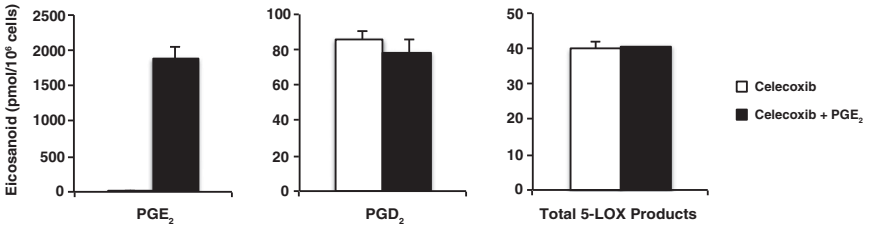


**Fig. S2.** The metabolism of AA by 5-LOX after long-term TLR4 priming is not diminished in the presence of exogenous prostaglandin $E_2$ ($PGE_2$). Levels in medium of the indicated eicosanoid species from RAW cells after incubation with KLA in the presence of only 50 nM celecoxib (white bars) or presence of 50 nM celecoxib and 1 μM $PGE_2$ (black bars) for 7.5 h before stimulation with ATP for the final 30 min; levels of $PGD_2$ and total 5-LOX products (5-HETE, $LTC_4$, 11-trans $LTC_4$, $LTB_4$, 6-trans, 12-epi $LTB_4$, 6-trans $LTB_4$, and 12-epi $LTB_4$) were not significantly different between conditions. Data are mean values of three separate experiments ± SEM.

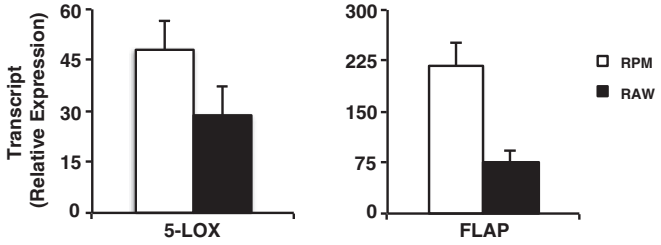


**Fig. S3.** Comparison of constitutive 5-LOX and FLAP mRNA levels in resident peritoneal macrophages (RPM) vs. RAW macrophages. Levels of 5-LOX and FLAP mRNA measured with qPCR from (white bars) RPM and (black bars) RAW264.7 cells (RAW). Data are mean values of three separate experiments ± SEM.

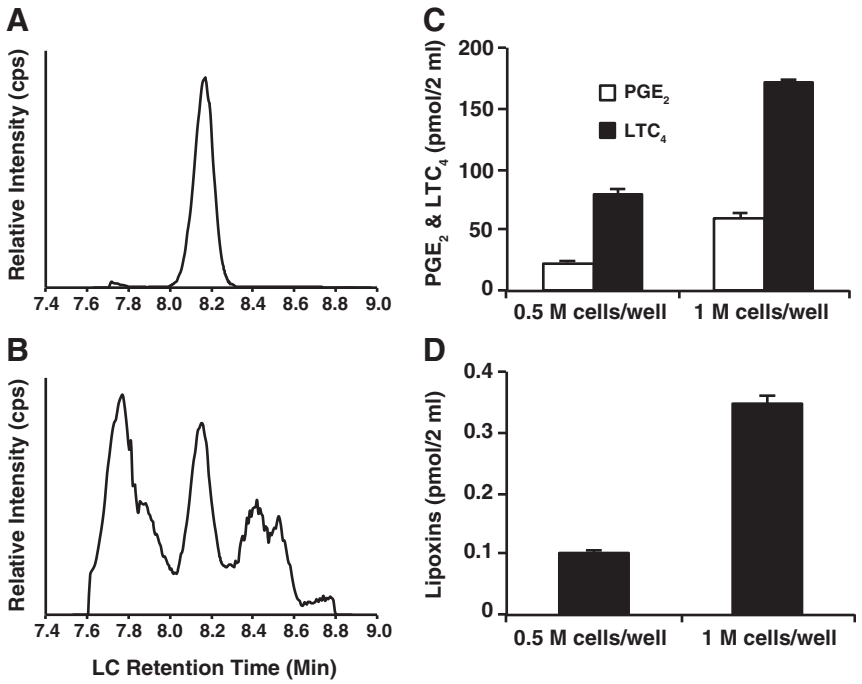


**Fig. S4.** TLR4 primed, $P2X_7$-stimulated resident peritoneal macrophages (RPMs) produce lipoxins, and 5-LOX activity is not attenuated by increased $PGE_2$ resulting from increased cell density. Reversed-phase elution of (*A*) $LXA_4$: 15-epi–$LXA_4$ standards (1:1 ratio), and of (*B*) medium from RPM cells (0.5 M cells per well, in 6-well plates) primed with KLA for 7.5 h before 30-min stimulation with ATP. Levels in medium of (*C*) $PGE_2$ (white bars), $LTC_4$ (black bars); (*D*) lipoxins (15-epi–$LXA_4$ and $LXA_4$) from RPM cells plated at 0.5 M cells per well, or 1 M cells per well in 6-well plates after stimulation as in *B*. Data in *B*–*D* are mean values of three separate experiments ± SEM.

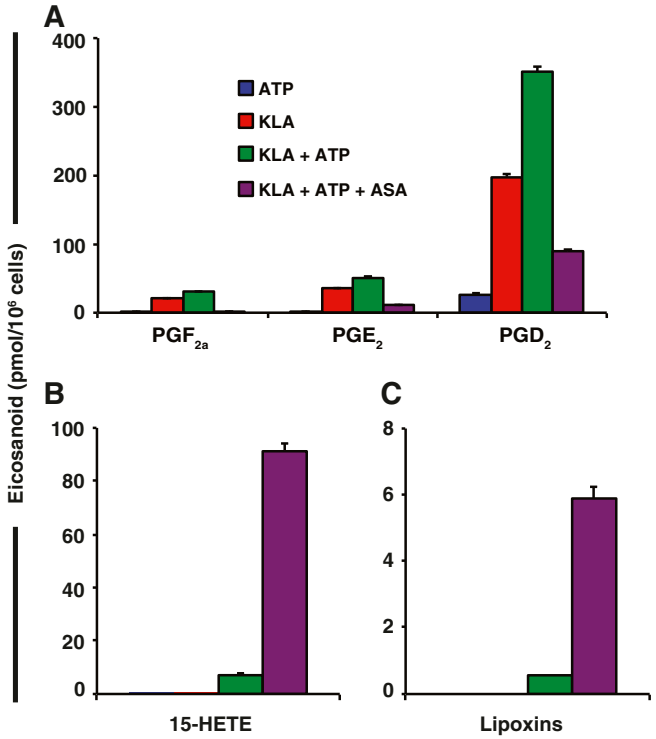


**Fig. S5.** Effects of TLR4 priming and aspirin treatment on synthesis of prostaglandins, 15-HETE, and lipoxins. Extracellular levels of (*A*) prostaglandins; (*B*) 15-HETE; and (*C*) lipoxins (15-epi–$LXA_4$ and $LXA_4$) after treatment with the indicated agonists/inhibitors are mean values of three separate experiments ± SEM (and are from the same experiment as in Fig. 2*B*).

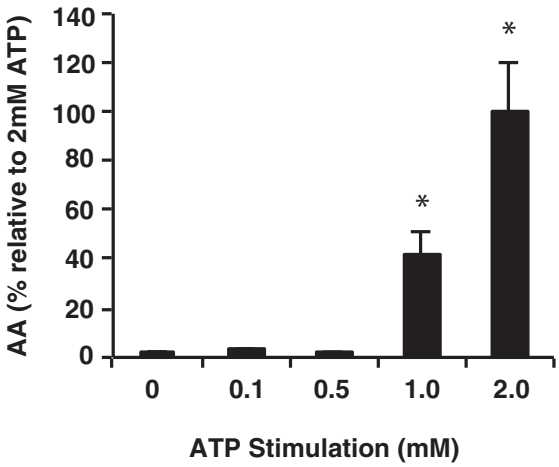


**Fig. S6.** Arachidonic acid release is significantly higher with millimolar, vs. micromolar ATP. RAW cells were incubated in the presence of 1 μM 15(R)-HETE and the indicated concentrations of ATP for 30 min. Data are mean values of three separate experiments ± SEM (from the same experiments as in Fig. 3*A*). AA release was significantly higher in 1 and 2 mM ATP-treated cells vs. 0–500 μM ATP-treated cells; $*P < 0.05$.

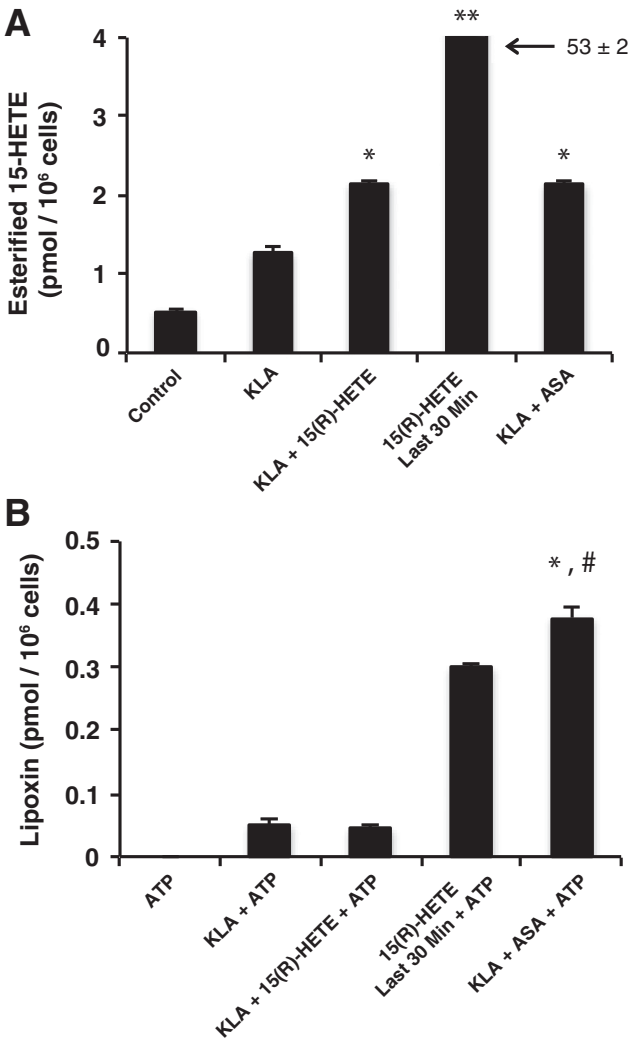


**Fig. S7.** Esterified 15-HETE generated from TLR4-mediated COX-2 activity is more efficiently converted to lipoxins than from exogenous sources. (*A*) Levels of 15-HETE in phospholipids of RAW cells after 8 h in the presence of the conditions indicated; control = untreated cells; concentrations of KLA, 15(R)-HETE, and ASA were 100 ng/mL, 1 μM, and 1 mM, respectively; 15(R)-HETE last 30 min condition = no treatment for first 7.5 h followed by incubation with 15(R)-HETE for last 30 min; esterified 15-HETE levels were increased with KLA + 15(R)-HETE and KLA + ASA vs. KLA (*$P < 0.0005$), but were not significantly different between cells treated with KLA + 15(R)-HETE vs. KLA + ASA, and were increased with cells treated with 15(R)-HETE last 30 min vs. all other conditions (**$P < 0.0001$). (*B*) Levels of lipoxins ($LXA_4$ and/or 15-epi–$LXA_4$) in medium after 8-h incubations with the indicated conditions correspond to those in *A* along with additional stimulation with 2 mM ATP for the final 30 min; lipoxin levels between cells treated with KLA + ATP vs. KLA + 15(R)-HETE + ATP were not significantly different; lipoxin levels were increased in cells treated with KLA + ASA + ATP vs. KLA + ATP and KLA + 15(R)-HETE + ATP (*$P < 0.0001$); lipoxin levels were increased with KLA + ASA vs. last 30 min 15(R)-HETE ($^{\#}P < 0.005$). Data are mean values of three separate experiments ± SEM.