Supporting Information

Norris and Dennis 10.1073/pnas.1200189109

SI Materials and Methods

Animals. Male C57bl/6 mice were purchased from Charles River Laboratories. Mice exhibiting skin lesions or visible tumors were excluded from the study. All experiments were carried out according to protocols approved by the Institutional Animal Care Committee of the University of California, San Diego.

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 and resuspended in RPMI medium 1640 supplemented with 10% heat inactivated FBS and 100 units/mL penicillin/streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Cells were plated in six-well culture plates with 2.0 mL of 10% FBS media with or without 10 μM EPA supplement (1×10^6 cells per well) and allowed to adhere for 24 h. Nonadherent cells were removed by aspiration and were then stimulated with Kdo_2-Lipid A (KLA) or ATP (using the same protocol as for RAW264.7, except RPMI 1640 medium was used instead of DMEM) (*Materials and Methods*).

Fatty Acid Uptake and Purity. Fatty acids (Cayman) were analyzed with GC-MS and found to be >99% pure of other fatty acids. A 24-h time course of uptake of each deuterated fatty acid was used to determine that >99% of deuterated fatty acid disappeared from the media by 24 h using GC-MS analysis.

Extraction and Analysis of Fatty Acids from Cell Membrane Phospholipids. After 24-h supplementation, cell media were aspirated, cells were washed twice with ice-cold PBS (VWR), scraped into 1 mL ice cold PBS, centrifuged, and supernatant aspirated before storage at −80 °C. Phospholipids were extracted using a modified Bligh and Dyer procedure as previously described (1). Cell pellets were extracted with 800 μL of 0.1 N HCl:MeOH (1:1) and 400 μL $CHCl_3$. The samples were vortexed for 1 min and centrifuged (5 min, 18,000 × *g*). The lower phase was then isolated and evaporated. Then, 250 μL of MeOH (containing 0.005% (wt/vol) butylated hydroxytoluene) and 250 μL of 15% (wt/vol) KOH were added and vortexed for 1 min. Samples were incubated for 30 min at 37 °C for chemical saponification. The samples were brought to ≤ pH 3 by adding 1 N HCl and then fatty acid internal standards were added and fatty acids were similarly extracted and analyzed by the protocol below.

Fatty Acid Extraction from Cell Media and Saponified Phospholipids. Fatty acids were extracted from samples as described by Zarini et al. (2). A 500-μL aliquot of sample media was supplemented with 400 μL of MeOH, 100 μL deuterated fatty acid internal standards (in MeOH), and 25 μL of 1 M HCl. Samples were then supplemented with 2 mL of iso-octane, vortexed for 30 s, and centrifuged at 1,000 × *g* for 5 min. The upper (iso-octane) layer was removed and placed into a 75 × 15 mm silica tube. The iso-octane extraction was repeated twice and stored at −20 °C. Before analysis by GC-MS, samples were evaporated using a Speed-Vac and derivatized using 25 μL of pentafluorobenzene (PFB 1% vol/vol in acetonitrile) and 25 μL of diisopropylethylamine (1% vol/vol in ACN) (Sigma). Samples were allowed to sit at 23 °C for 30 min, evaporated by Speed-Vac, and reconstituted in 50 μL iso-octane for analysis, with 5 μL injected on column.

GC-MS of Fatty Acids. Fatty acids were analyzed by GC-MS as described by Zarini et al. (2), whose work was expanded to cover additional fatty acids by Quehenberger et al. (3). Fatty acids were separated using a gas chromatograph (Agilent 6890N; Hewlett Packard) containing a 15 m (15 m × 0.25 mm inner diameter × 0.10-mm film thickness) Zebron dimethylpolysiloxane capillary column and analyzed by MS. The injector temperature was maintained at 250 °C and run in pulsed splitless mode, and the sample transfer line was maintained at 280 °C. A constant flow of helium carrier gas was set at 0.9 mL/min. Fatty acids eluted with a temperature gradient starting at 150 °C, increasing 10 °C/min until 270 °C, 40 °C/min until 310 °C and held for 1 min. Fatty acids were analyzed using a single quadrupole mass spectrometer (Agilent 5975; Hewlett Packard) via selected ion monitoring in negative ion chemical ionization mode. Methane was used as the reagent gas. The source was maintained at 280 °C and 200 eV, and the quadrupole was maintained at 150 °C. Fatty acids were identified in samples by matching their selected ion monitoring signal and GC retention time with those of a pure standard.

Eicosanoid Cell Media Sample Preparation. Media samples were analyzed for extracellular eicosanoid release. After stimulation with KLA (4) (Avanti Polar Lipids) or ATP (Sigma), 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 Chemicals). Samples were centrifuged for 5 min at 1,000 × *g* to remove cellular debris, and were then purified. Eicosanoids were extracted using Synergy C18 Strata-X SPE columns (Phenomenex). Columns were washed with 3 mL of MeOH and then 3 mL of H_2O. After applying the sample, the columns were washed with 10% MeOH, and the eicosanoids were then eluted with 1 mL of MeOH and stored at −20 °C. The eluent was dried under vacuum and redissolved in 100 μL of LC solvent A [water/acetonitrile/acetic acid (70:30:0.02; vol/vol/vol)] for LC/MS/MS analysis.

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.

LC-MS. Eicosanoid analysis was performed by LC-MS/MS as previously described (5). Briefly, eicosanoids were separated by a 25-min reverse-phase LC gradient using Solvent A [water-acetonitrile-acetic acid (70:30:0.02; vol/vol/vol)] and solvent B [acetonitrile-isopropyl alcohol (50:50; vol/vol)]. Eicosanoids were subsequently analyzed using a tandem quadrupole mass spectrometer (ABI 4000 Q-Trap; Applied Biosystems) via multiple-reaction monitoring in negative-ion mode. Eicosanoids were identified in samples by matching their multiple-reaction monitoring signal and LC retention time with those of a pure standard.

Western Blotting. Cells were washed twice with cold PBS and scraped into 250 μL of ice cold PBS containing Complete Mini protease inhibitor mixture (Roche) at pH 7.4. Protein concentrations were determined and normalized using the BCA Protein Assay Kit (Fisher Scientific). Ten micrograms of total protein was loaded onto a 4–12% Bis-Tris SDS polyacrylamide gel (Invitrogen), electrophoresed, and transferred onto a PVDF membrane (BioRad). The membrane was blocked with 100% methanol for 5 min, dried for 10 min, and then incubated with the appropriate antibody (Cell Signaling) overnight in 5% (wt/vol) milk protein in ultrapure water containing 0.1% Tween 20. Membranes were then washed three times in TBS-Tween buffer

and incubated with the appropriate secondary antibody (Cell Signaling Technology) for 1 h. All membranes were washed three times before development using the ECL Plus Western Blotting Detection System (Amersham Biosciences).

1. Milne S, Ivanova P, Forrester J, Alex Brown H (2006) Lipidomics: An analysis of cellular lipids by ESI-MS. *Methods* 39:92–103.
2. Zarini S, Gijón MA, Folco G, Murphy RC (2006) Effect of arachidonic acid reacylation on leukotriene biosynthesis in human neutrophils stimulated with granulocyte-macrophage colony-stimulating factor and formyl-methionyl-leucyl-phenylalanine. *J Biol Chem* 281:10134–10142.
3. Quehenberger O, Armando A, Dumlao D, Stephens DL, Dennis EA (2008) Lipidomics analysis of essential fatty acids in macrophages. *Prostaglandins Leukot Essent Fatty Acids* 79:123–129.
4. Raetz CR, et al. (2006) Kdo2-Lipid A of *Escherichia coli*, a defined endotoxin that activates macrophages via TLR-4. *J Lipid Res* 47:1097–1111.
5. 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* 1811:724–736.

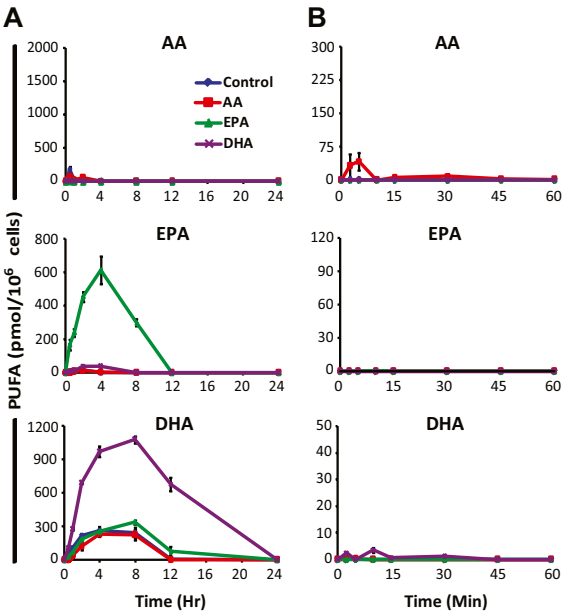

Fig. S1. Without stimulation, supplementation causes increased release of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) during long-term incubation with 10% serum and minimal fatty acid release during short-term incubation without serum. RAW264.7 cells were incubated for 24 h in non-supplemented media (blue), or 25 μM arachidonic acid (AA, red), EPA (green), or DHA (purple) before incubation (*A*) in 10% serum media for the indicated times over a 24-h period or (*B*) in serum-free media for the indicated times over a 60-min period; 0 h values were subtracted from each time point. Eicosanoid levels were determined with MS and the data are expressed as mean values ± SEM of three biological replicates.

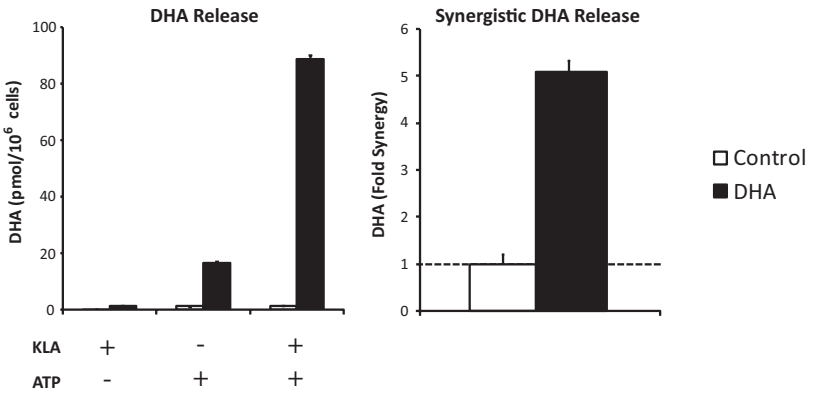

Fig. S2. DHA supplementation leads to significant release of DHA after $P2X_7$ stimulation but not Toll-like receptor 4 (TLR4) stimulation and synergistically increased DHA release with TLR4 priming and $P2X_7$ stimulation. RAW264.7 cells were incubated for 24 h in nonsupplemented media (white) or 25 μM DHA (black). Cells were then incubated in serum-free media with or without 100 ng/mL KLA for 2 h, with or without 2 mM ATP for 10 min, or with or without 100 ng/mL KLA for 1 h 50 min followed by incubation with or without 2 mM ATP for an additional 10 min before media were removed and (*Left*) DHA levels were analyzed with MS (2-h unstimulated DHA levels were subtracted from each condition). The data are expressed as mean values ± SEM of three biological replicates. (*Right*) Synergistic activation ratios were calculated with DHA values via the equation: fold synergy = (KLA + ATP)/(KLA) + (ATP). The data are expressed as mean values ± SEM of three biological replicates.

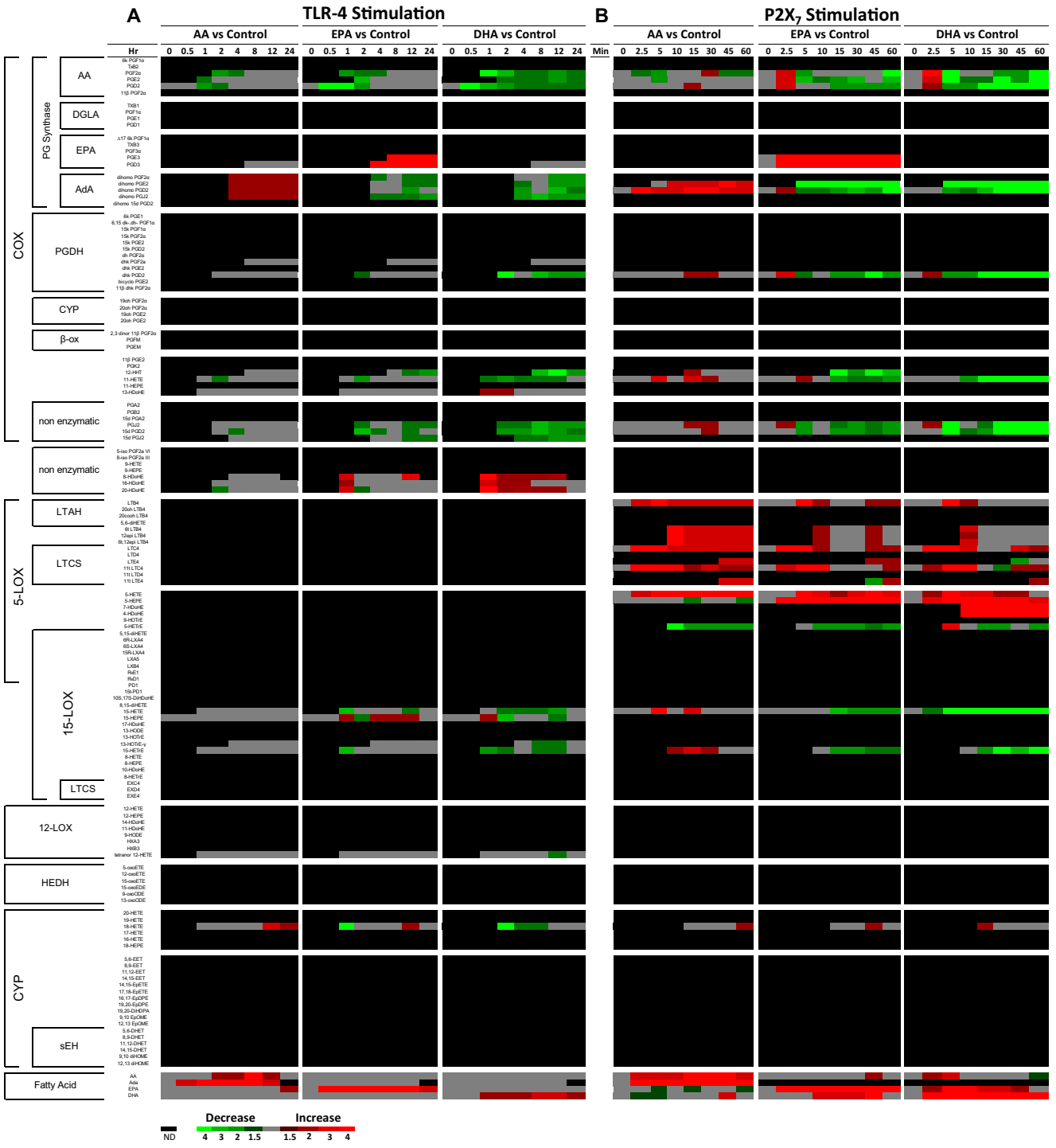

Fig. S3. Global effects of polyunsaturated fatty acids (PUFA) supplementation on TLR4-stimulated and $P2X_7$-stimulated eicosanoid production. Heat map representing fold-change of AA, EPA, and DHA supplemented vs. nonsupplemented (control) RAW264.7 cells in the extracellular medium levels of 140 eicosanoid species measured with MS after stimulation with (*A*) KLA (100 ng/mL) or (*B*) 2 mM ATP. Increases in metabolite levels are indicated by red, decreases by green, and detectable but unchanged levels by gray. Metabolites below the limit of detection are indicated by black; $n = 3$ individual biological replicates/time point/group; the data were from the same experiment as shown in Fig. 2.

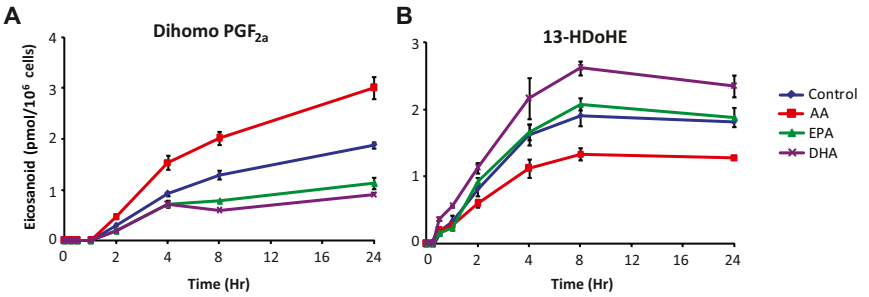

Fig. S4. Non-AA COX-2 metabolite production. RAW264.7 cells were incubated for 24 h in nonsupplemented media (blue), 25 μM AA (red), EPA (green), or DHA (purple) before stimulation with KLA (100 ng/mL) for the indicated times over a 24-h period and subsequent eicosanoid analysis. (*A*) Adrenic acid (AdA)-derived dihomo prostaglandin (PG)F_{2a} and (*B*) DHA-derived 13-hydroxy DHA (HDoHE) levels were determined with MS; 0 h values were subtracted from each time point and the data are expressed as mean values ± SEM of three biological replicates from the same experiment shown in Fig. 2.

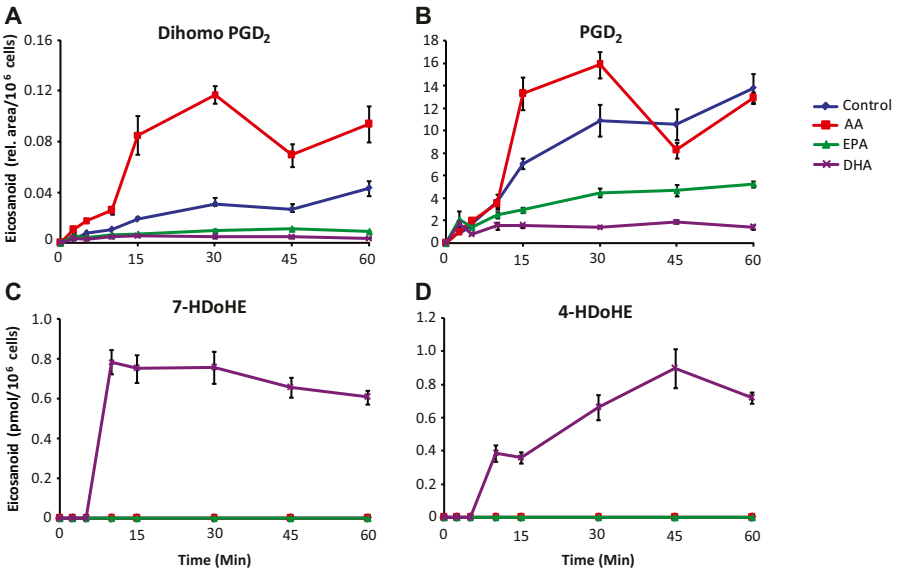

Fig. S5. Supplementation yields increased, but relatively low levels of AdA cyclooxygenase (COX)-1 prostanoids vs. AA prostanoids and substantial DHA 5-lipoxygenase (LOX) metabolite levels after $P2X_7$ stimulation. RAW264.7 cells were incubated for 24 h in nonsupplemented media (blue), or 25 μM AA (red), EPA (green), or DHA (purple) before stimulation with 2 mM ATP for the indicated times over a 60-min period. (*A*) COX-1 AdA-derived dihomo PGD_2 and (*B*) AA-derived PGD_2 are expressed as area relative to area of a deuterated PGD_2 internal standard; 5-LOX DHA-derived (*C*) 7-HDoHE and (*D*) 4-HDoHE are absolute values relative to pure standards; 0 h values were subtracted from each time point. Eicosanoid levels were determined with MS and the data are expressed as mean values ± SEM of three biological replicates from the same experiment shown in Fig. 2.

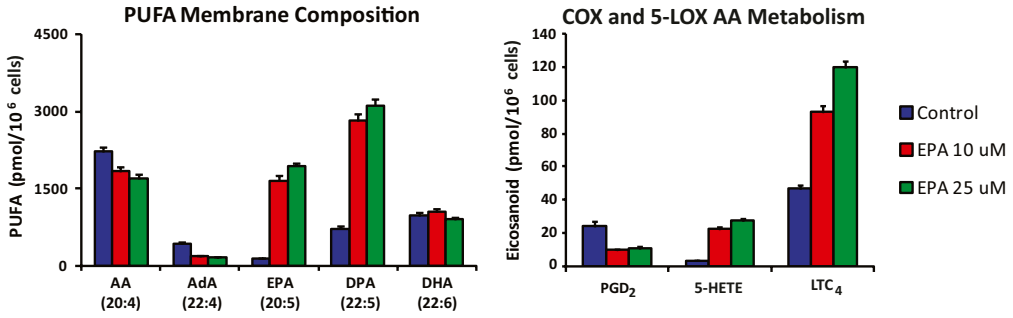

Fig. S6. Effects of 10 μM vs. 25 μM EPA supplementation on phospholipid incorporated EPA and DPA, as well as $P2X_7$-stimulated COX-1 inhibition and 5-LOX shunting are similar. RAW264.7 cells were incubated for 24 h in nonsupplemented media (blue), 10 μM EPA (red), or 25 μM EPA (green). (*Left*) Supplemented EPA along with the elongated product DPA increased in membrane phospholipids to similar extents with both concentrations relative to control as determined by GC-MS shown in Fig. 1. (*Right*) After 15-min purinergic stimulation with 2 mM ATP, similar reduced levels of PGD_2 and increased levels of 5-hydroxyeicosatetraenoic acid (HETE) and leukotriene (LT)C_4 were observed with both supplemented EPA concentrations relative to control as determined by LC-MS/MS. The data are expressed as mean values ± SEM of three biological replicates.

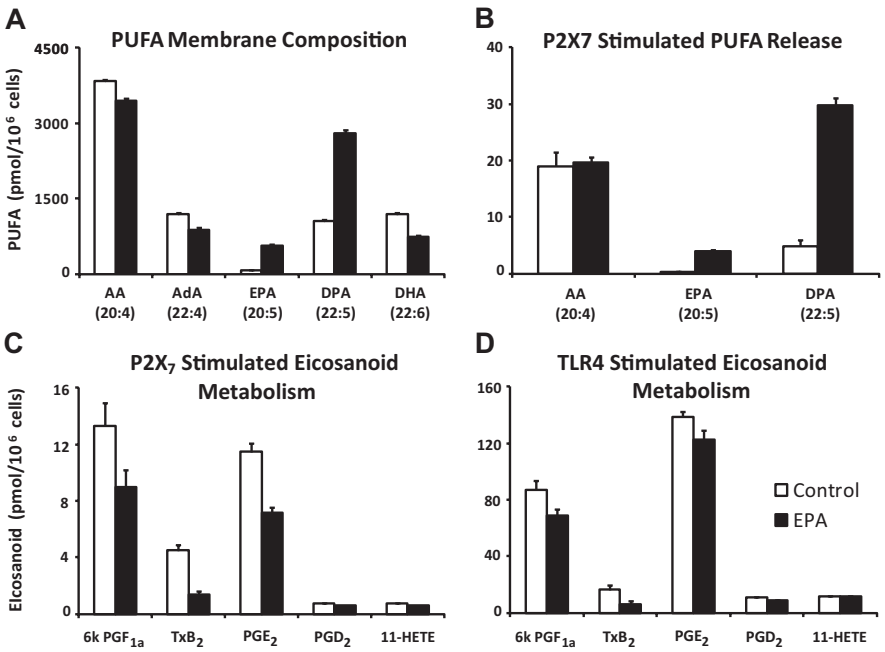

Fig. S7. EPA supplementation with resident peritoneal macrophages causes larger increased levels of DPA vs. EPA within membrane phospholipids and in media after $P2X_7$ stimulation and subsequently attenuates COX AA metabolism. Resident peritoneal macrophages were incubated for 24 h in non-supplemented media (white) or 10 μM EPA (black). (*A*) PUFA levels in membrane phospholipids were determined as in Fig. 1 using GC-MS. (*B*) Released PUFA levels and (*C*) eicosanoid levels after $P2X_7$ stimulation with 2 mM ATP and (*D*) eicosanoid levels after TLR4 stimulation with 100 ng/mL KLA were determined by LC-MS/MS (0 h levels were subtracted from each time point). The data are expressed as mean values ± SEM of three biological replicates.