Supplemental Material

METHODS

Measurements of endothelial monolayer permeability. To measure agonist-induced changes in transendothelial electrical resistance (TER) in endothelial cell (EC) cultures, the electrical cell substrate impedance-sensing (ECIS) system available from Applied BioPhysics (Troy, NY). Cells were cultured on small gold electrodes (10^{-2} mm^2), and culture media were used as electrolyte. The total electrical resistance was measured dynamically across the monolayer and was determined by the combined resistance between the basal surface of the cell and the electrode, reflective of focal adhesion and the resistance between cells. As cells adhere and spread out on the microelectrode, the transmonolayer electrical resistance increased (maximal at confluence), whereas cell retraction, rounding, or loss of adhesion was reflected by a decrease in TER. The small gold electrodes and the larger counter electrodes (100 mm^2) were connected to a phase sensitive lock-in amplifier (5301A; EG&G Instruments, Princeton, NJ) with a built in differential preamplifier (5316A; EG&G Instruments). A 1-V, 4,000-Hz alternating current signal was supplied through a 1-MΩ resistor to approximate a constant-current source. Voltage and phase data were stored and processed with PC that controlled the output of the amplifier and relay switches to different electrodes. Experiments were conducted only on wells that achieved >1,000 Ω (10 microelectrodes/well) of steady-state resistance. Resistance was expressed by the in-phase voltage (proportional to the resistance), which was normalized to the initial voltage and expressed as a fraction of the normalized resistance value.

Endothelial permeability to macromolecules was monitored by express permeability testing assay (XPerT) available from Millipore (Vascular Permeability Imaging Assay, cat. #17-10398). This assay is based on high affinity binding of cell impermeable avidin-conjugated FITC-labeled tracer to the biotinylated extracellular matrix proteins immobilized on the surface covered with EC monolayers. In permeability visualization experiments, 15 min after EC stimulation with HGF, FITC-avidin solution was added directly to the culture medium for 3 min before termination of the experiment. Unbound FITC-avidin was washed out with PBS (pH 7.4, 37°C), cells were fixed with 3.7% formaldehyde in PBS (10 min, room temperature) and visualization of FITC-avidin on the bottoms of coverslips was performed using Nikon imaging system Eclipse TE 300 (Nikon, Tokyo, Japan) equipped with a digital camera (DKC 5000, Sony, Tokyo, Japan); 10× objective lenses were used. Images were processed with Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA). For the permeability assay in 96-well plates, cells were seeded on biotinylated gelatin-coated plates ($3x10^4$ cells/well) and grown for 48-72 h prior to testing. FITC-avidin solution was added directly to the culture medium at the final concentration 25 μg/ml for 3 min before termination of the experiment unless otherwise specified. Unbound FITC-avidin was washed out with 200 μl PBS, pH 7.4, 37°C (two cycles, 10 sec each). Finally, 100 μl PBS was added to each well, and the fluorescence of matrix-bound FITC-avidin was measured on Victor X5 Multilabel Plate Reader (Perkin Elmer, Waltham, MA) using an excitation wavelength of 485 nm and emission wavelength of 535 nm.

Immunofluorescence staining. EC were fixed in 3.7% formaldehyde solution in PBS for 10 min at 4°C, washed 3 times with PBS, permeabilized with 0.1% Triton X-100 in PBS for for 30 min at room temperature and blocked with 2% BSA in PBS for 30 min. Incubation with VE-cadherin antibody was performed in blocking solution (2% BSA in PBS) for 1 hr at room temperature followed by staining with Alexa 488-conjugated secondary antibodies. Actin filaments were stained with Texas Red-conjugated phalloidin diluted in the blocking solution. After immunostaining, the slides were analyzed using a Nikon video imaging system consisting of a inverted microscope Nikon Eclipse TE300 connected to SPOT RT monochrome digital camera and image processor (Diagnostic Instruments, Sterling Heights, MI). The images were acquired using SPOT 3.5 acquisition software (Diagnostic Instruments) and processed with Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

Immunoblotting. After stimulation, cells were washed with PBS and lysed with cell lysis buffer containing 10 mM Tris (pH 7.4), 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 0.2 mM EGTA, 0.2 mM vanadate, 0.2 mM PMSF, and 0.5% phosphatase inhibitor cocktail. Total cell lysates were cleared by centrifugation and boiled with the same amount of 3 x SDS sample buffer for 5 min. Protein extracts were separated by SDS-PAGE on 7.5% gels for detection of IkBα, ICAM1, VCAM1, and β-tubulin; and 15% gels for detection of MLC. The separated proteins were transferred to nitrocellulose membranes by electrotransfer (30 V for 18 h, or 90 V for 2 h). The blots were subsequently blocked with 5% non-fat dry milk in PBST at room temperature for 1 h, and then incubated at 4°C overnight with primary specific antibodies of interest. After washing three times for 10 min with PBST, the membrane was incubated with HRP-linked IgG secondary antibodies at room temperature for 1 h, followed by washing three times for 10 min with PBST. Immunoreactive proteins were detected using an enhanced chemiluminescent detection system according to the manufacturer's protocol (Amersham, Little Chalfont, UK).

siRNA transfection of EC cultures. Pulmonary EC were treated with gene-specific siRNA duplexes. Pre-designed human Stealth™ Select siRNA sets of standard purity were ordered from Invitrogen (Carlsbad, CA) in ready-to-use, desalted, deprotected, annealed double-strand form. Non-specific, non-targeting siRNA duplex #1 (Dharmacon Research, Lafayette, CO) was used as a control treatment. HPAEC were grown to 70% confluence, and siRNA transfection (final concentration 50 nM) was performed using siPORTamine^{TM} transfection reagent (Ambion, TX) according to the manufacturer's protocol. Cells were serum-starved for 1 hour followed by incubation with 1.5 μM of target siRNA or control non-specific RNA for 6 hours in serum-free media. The serum-containing media was then added (10% serum final concentration). After 72 hrs of transfection, cells were used for experiments or harvested for RT-PCR and western blot verification of specific protein depletion. The initial testing of non-transfected and siRNA-transfected EC monolayers did not reveal adverse effects of non-specific or FPR-specific siRNA on cell viability or significant differences in basal TER levels.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Analysis of FPR1, FPR2/ALX, FPR3, 15-LO, and ICAM1 mRNA expression by human pulmonary EC was performed by quantitative real-time RT-PCR. Total RNA was isolated from ECs using TRIzol reagent, per manufacturer's instructions (Life Technology, Rockville, MD). RNA samples were quantified by measuring optical density at 260 nm using the NanoDrop ND 1,000 Spectrophotometer (Thermo Scientific, Wilmington, DE). cDNA was synthesized from 1 mg/ml of total RNA, per the manufacturer's instructions, using the Invitrogen SuperScript III First- Strand Synthesis SuperMix for qRT-PCR kit (cat. no. 11752-050). The following primers were used for RT-PCR reactions: FPR1 sense: 5′-TTG CAG TGG ATG TGA CAA GTG C-3′, antisense: 5′-ATA AGC AGG AAA TGC CTG TGG C-3′, FPR2/ALX sense: 5′-AGA GGA TAA ATG TGG CCG TTG C-3′, antisense: 5′-AAG CCT TGC TTG TGG ATC TTG G-3′, FPR3 sense: 5′- ATG GCC AAG GTC TTT CTG ATC C-3′, antisense: 5′- AAT TTT GGC AGC GAT GAT CCC-3′, 15-LO sense: 5'-GGC CGG CCA GCT TAT ACA C-3', antisense 5'-TAG ACA CTT GAG CTC GGG CA-3'; ICAM-1 sense: 5'-GGC CGG CCA GCT TAT ACA C-3', antisense 5'-TAG ACA CTT GAG CTC GGG CA-3'. RT-PCR was performed using the Bio-Rad Thermal Cycler system (Bio-Rad, Hercules, CA) and AmpliTaq Gold(R) DNA Polymerase (cat. no. 4,311,806; Applied Biophysics) using the following PCR conditions: 10 minutes at 93^{o}C, followed by 40 cycles of 30 seconds at 95^{o}C, 45 seconds at 60^{o}C, 50 seconds at 72^{o}C, and 10 minutes at 72^{o}C. PCR products were separated by electrophoresis in a 1.5% agarose gel, and then stained with ethidium bromide.

Human PMNs isolation. Venous blood from normal human subjects was collected based on a protocol approved by the University of Chicago Institutional Review Board and in compliance with the University of Chicago and U.S. governmental guidelines for studies in which donors are not participating subjects. Informed consent was obtained from all volunteers in this study before participation. PMNs were isolated

by Ficoll-Paque sedimentation. Cells were resuspended in HBSS buffer + Ca^{++}/0.2% BSA prior to counting. Purity of PMN on H and E-stained cytoslides was ~90-95%. Cells were kept on ice until use.

Mass spectrometry analysis of LXA4. Lipids were extracted from cell culture conditioned media using a modified Bligh and Dyer approach [33] with 0.1N HCl to induce phase separation and improve lipoxin A4 recovery in chloroform layer. Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) of lipoxin A4 was performed using Sciex 6500 QTRAP triple quadrupole ion trap hybrid mass spectrometer interfaced either with Agilent 1290 UHPLC system or Shimadzu Nexera-X2 UHPLC system. Lipoxin A4 was separated from other components either on Ascentis Express C18 column (2.1 x 50 mm, 2.7 μm particle size) or on Ascentis Express RP-Amide column (2.1 x 50 mm, 2.7 μm particle size). Chromatography was performed using gradient elution from solvent A (methanol:water:formic acid 40:60:0.5, with 5 mM ammonium formate) to solvent B (methanol:chloroform:water:formic acid, 90:10:0.5:0.5, with 5 mM ammonium formate) at 0.3 ml/min flow rate. Deuterated lipoxin A4 (lipoxin A4-D5, Cayman Chemicals, Ann Arbor, MI) was added during initial steps of extraction to ensure lipoxin A4 quantitation through the isotope dilution approach. Lipoxin A4 and Lipoxin A4-D5 were detected using ESI in negative ions as a transition from m/z 351.3 to m/z 115.0 (lipoxin A4) and from m/z 356.3 to m/z 115.0 (lipoxin A4-D5) with declastering potential (DP) of -65V and collision energy (CE) of -19V.

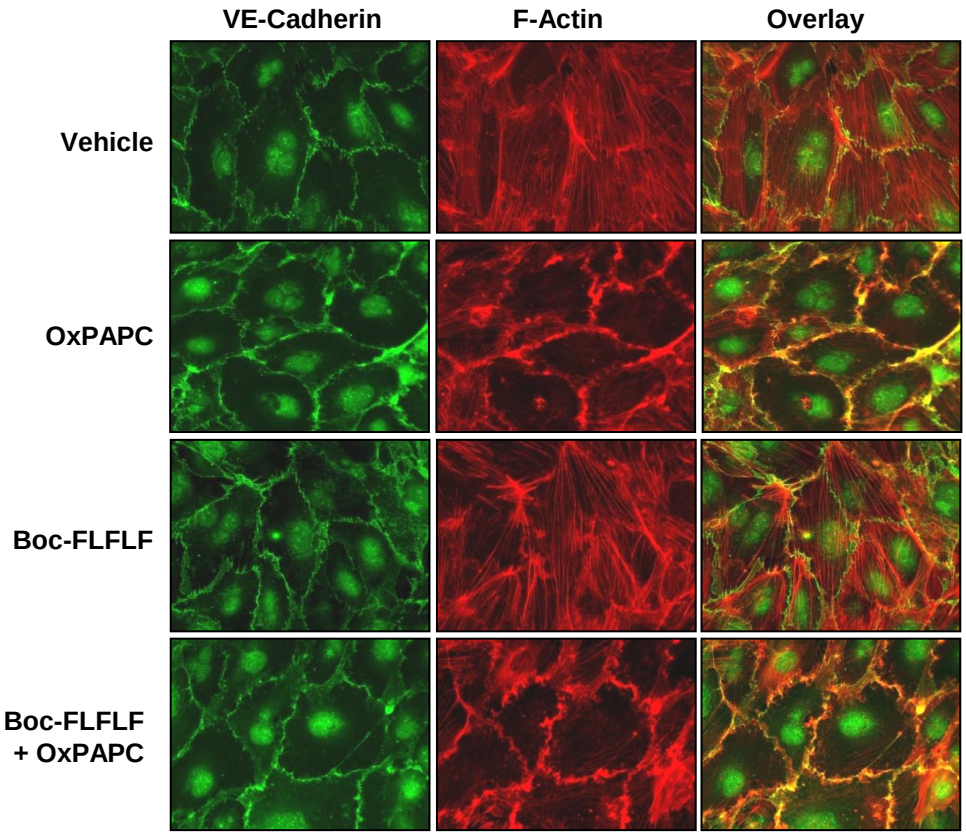
Animal studies. All animal care and treatment procedures were approved by the University of Chicago Institutional Animal Care and Use Committee. Animals were handled according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg) and acepromazine (1.5 mg/kg). Bacterial lipopolysaccharide (LPS, 0.7 mg/kg body weight; *Escherichia coli* O55:B5) was injected intratracheally in a small volume (20–30 μl). OxPAPC (1.5 mg/kg) or sterile saline solutions were administrated 5 hrs after LPS instillation by intravenous injection in the external jugular vein. Animals were sacrificed by exsanguination under anesthesia 24 hrs after LPS challenge and used for evaluation of lung injury parameters. *Bronchoalveolar lavage (BAL) analysis.* After the experiment, animals were sacrificed by exsanguination under anesthesia. Tracheotomy was performed, and the trachea was cannulated with a 20 gauge intravenous catheter, which was tied into place. BAL was performed using 1 ml of sterile Hanks Balanced Salt Buffer. The collected lavage fluid was centrifuged at 2500 rpm for 20 min at 4°C, the supernatant was removed and frozen at –80°C for subsequent protein study. The cell pellet was then resuspended in 1 ml of red blood cell lysis buffer (ACK Lysing Buffer, BioSource International) for 5 min and then re-pelleted by centrifugation at 2500 rpm for 20 min at 4°C. The cell pellet was again resuspended in 200 μl of PBS, and 20 μl of cell suspension were used for cell counting by a standard hemocytometer technique. The BAL protein concentration was determined by a modified Lowry colorimetric assay using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). The absorbance was measured at 750 nm, and protein concentration was determined using standard curves. *Assessment of pulmonary vascular leakage by Evans blue.* Evans blue was injected intravenously at dose of 30 mg/kg into the external jugular vein 2 hrs before termination of the experiment to assess vascular leak. At the end of the experiment, thoracotomy was performed, and the lungs were perfused in-situ via the left atrium with PBS containing 5 mM EDTA to flush the blood off the lungs. Left lung and right lungs were excised and imaged by a Kodak digital camera.

FIGURE LEGENDS

Online Figure I. ALX/FPR2 peptide inhibitor Boc-FLFLF does not affect enhancement of peripheral actin cytoskeleton and adherens junctions in OxPAPC- stimulated EC. Pulmonary EC pretreated with vehicle or Boc-FLFLF (20 μM, 1 hr) were stimulated with OxPAPC (15 μg/ml, 30 min). Immunofluorescence staining of actin cytoskeleton using Texas Red-conjugated phalloidin (red) and adherens junctions (VE-cadherin, green) was performed as described in Methods. Merged images are shown in right panels. Bar = 10 μm.

Online Figure II. Dose-dependent effects of LXA4 on TNFa-induced EC inflammatory activation. Cells were treated with TNFα (20 ng/ml, 6 hrs) alone or in the presence of indicated concentrations of LXA4. **A** - NFκB phosphorylation and VCAM1 expression was analyzed by Western blotting. Probing for β-tubulin was used as a normalization control. Numerical data depict results of quantitative densitometry; n=4; $p<0.05$ vs TNFα alone. **B** - ELISA assay of IL-8 production; n=4, $*P < 0.05$ vs TNFα alone.

Online Figure III. LC-MS/MS detection of LXA4-type molecule in the supernatant of HPAECs treated with OxPAPC but not with DMPC. A - LXA4 standard was subjected to LC separation and MS/MS detection as described in Experimental section. LXA4 molecule was identified and quantified in negative ions as a transition from m/z 351.3 to m/z 115.0. **B -** Blank medium was extracted and processed for LXA4 detection by the LC-MS/MS in a same way as the medium from treated and non-treated cells. Note very low background noise and the absence of the signal corresponding to the LXA4 m/z 155.3>115.0 transition. **C -** Medium from DMPC-treated (15 mg/ml, 6 hours) HPAECs was extracted and processed for the LC-MS/MS detection of LXA4 as a m/z 315.3>115.0 transition. Note that the medium from DMPC-treated cells does not have detectable levels of LXA4. **D -** Medium (1 ml) from OxPAPC-treated (15 mg/ml, 6 hours) HPAECs was extracted and processed for the LC-MS/MS detection of LXA4 as a m/z 315.3>115.0 transition. Note a strong LXA4 signal that coincides by the retention time with the authentic LXA4 standard (RT=2.27 min). Provided extracted ion chromatograms are direct screenshots from the Analyst 1.6.2 view of selected samples.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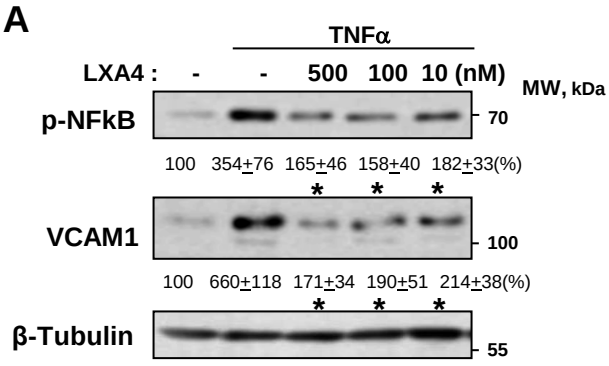
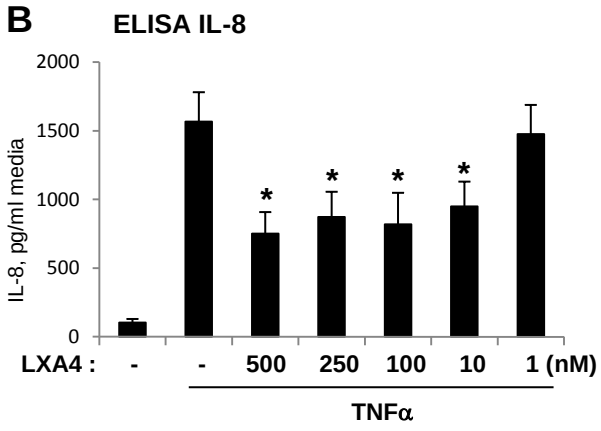
F-Actin
Overlay
Vehicle
OxPAPC
Boc-FLFLF
Boc-FLFLF
+ OxPAPC


A
TNFα
LXA4 : - - 500 100 10 (nM)
MW, kDa
p-NFkB
70
100 354±76 165±46 158±40 182±33(%)
VCAM1
100
100 660±118 171±34 190±51 214±38(%)
β-Tubulin
55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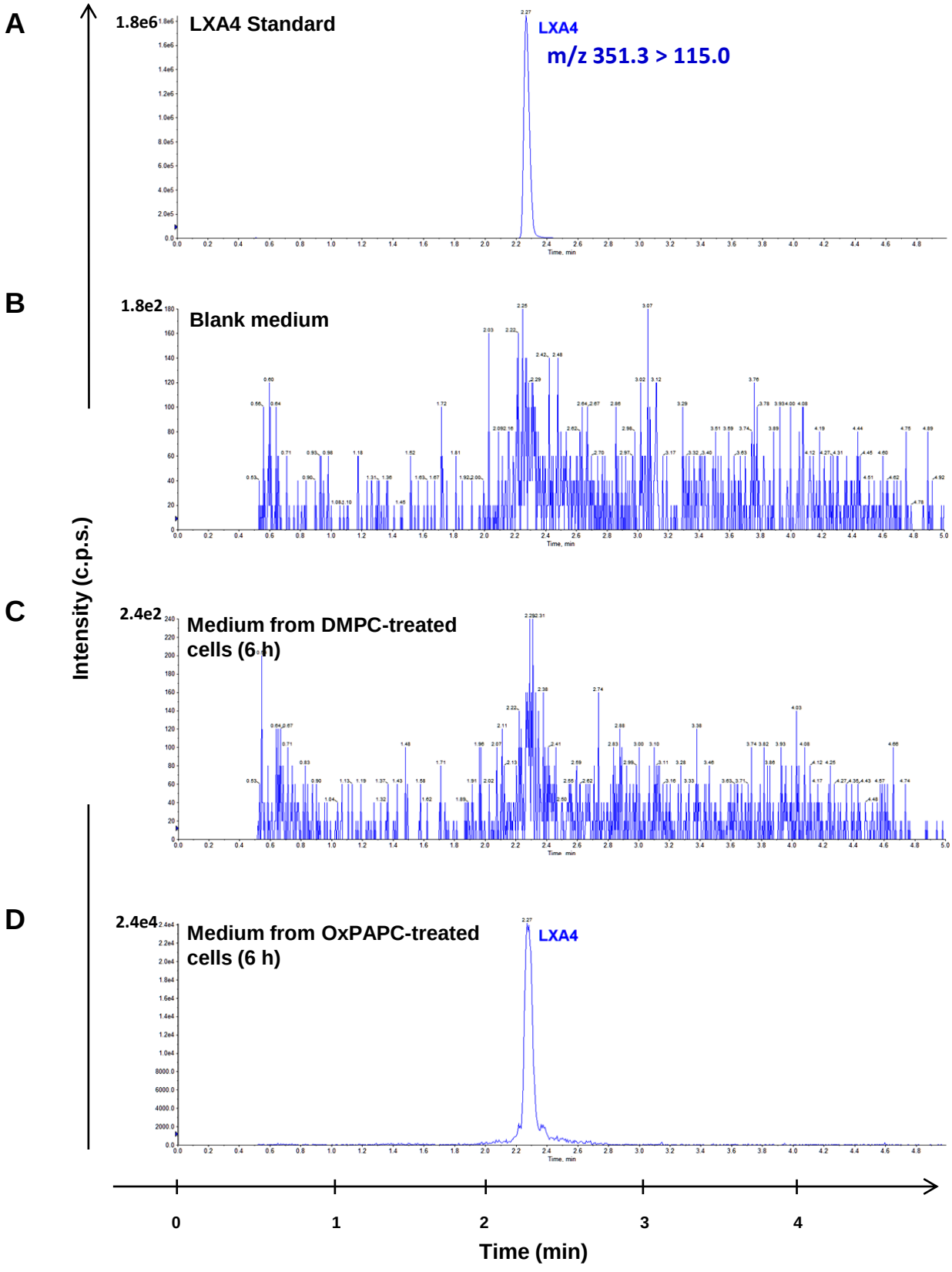
1500
1000
500
0
LXA4 : - - 500 250 100 10 1 (nM)
TNFα


A
1.8e6
LXA4 Standard
LXA4
m/z 351.3 > 115.0
B
1.8e2
Blank medium
C
2.4e2
Medium from DMPC-treated cells (6 h)
D
2.4e4
Medium from OxPAPC-treated cells (6 h)
LXA4
Intensity (c.p.s.)
Time, min
0
1
2
3
4
Time (min)