Supplementary information for

Synergy between 15-lipoxygenase and secreted phospholipase A_2 promotes inflammation by formation of TLR4 agonists from extracellular vesicles

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Supplementary Information Material and Methods

Reagents. LPS (from Salmonella abortus equi HL83) was received from K. Brandenburg (Forschungszentrum Borstel, Germany). Lipid IVa was obtained from Peptide Institute Inc. Paclitaxel, Ca-ionophore A23187, N-acetyl cysteine, porcine group IB secreted phospholipase A_2 (sPLA$_2$-IB), L-a-lysoPC, sphingomyelin, L-a-PC, L-α-lysoPE and L-α-PE were from Sigma. L-α-lysoPS, L-α-PS, L-a-PI, 2-arachidonoyl-lysoPI (20:4 lysoPI), 1,2-diarachidonyl-*sn*-glycero-3-phosphoethanolamine (AAPE) were from Avanti Polar Lipids. 15-lipoxygenase, Inhibitor I and 15S-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15(S)-HpETE) were purchased from Cayman. Varespladib (LY315920) was from Selleck Chemicals. The following specific antibodies were used: anti15-LO (1:1000), anti-annexin V (1:10000), anti-calnexin (1:500) from Abcam, anti-Tsg101 (1:300), anti-CD81 (1:200) from Santa Cruz Biotechnology and secondary goat anti-mouse (1:3000) or anti-rabbit (1:4000) IgG antibodies conjugated with horseradish peroxidase (HRP) from Jackson Immuno Research and Abcam. TLR4/MD2 recombinant protein was from R&D system/Biomedis MB. Magnetic beads were from GE Healthcare Life Sciences.

Dynamic light scattering (DLS) measurements. The composition and size distribution of EVs was measured on a ZetasizerNano (Malvern, UK) at 20 ^{o}C using an angle of 173^{o} and 633-nm laser.

CRISPR/Cas9. The target sites for genome editing of human *ALOX15* gene using the CRISPR/Cas9 system was determined with the CRISPR Design Tool from MIT (1). sgRNA with target sequence CCGCATCCGCGTGTCCACTG was cloned into the BbsI site of a pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid (Addgene plasmid 42230) using annealed forward primer CACCGCCGCATCCGCGTGTCCACTG and reverse primer AAACCAGTGGACACGCGGATGCGGG. The px330 plasmid (500 ng) and eGFP expressing plasmid (100 ng) were transfected into HEK293 cells (2 x 10^5 cells/well). GFP-positive single cells were selected at 48 h post-transfection and were serial diluted in order to obtain cells, derived from a single cell. 2-3 weeks later the genomic DNA was isolated according to the manufacturer's protocol for the DNeasy Blood and Tissue Kit (Qiagen). The genomic region around DSB was PCR-amplified and then agarose gel purified. 200 ng of isolated genomic DNA (20 µl of purified PCR product in 1x NEB 2. Buffer (NEB)) were then denatured and reannealed to form heteroduplexes. After the reannealing process, products were treated with 1 µl of T7 Endonuclease I (NEB) for 20 min at 37° C and analyzed on 10–15 % native PAGE

gel. Gels were run in 1x TBE buffer at a constant 120 V for 80–90 min (Bio-Rad). Gels were stained with SYSYBR Gold DNA stain (Life Technologies) for 30 min under gentle agitation and imaged with a DNA bioimaging system (Bio-Rad). The phenotype of positive colonies were confirmed by WB and expanded for further experiments.

Nanospray Mass Spectrometry. For lipid extraction, dry samples were resuspended in water followed by addition of 10 volumes of choloroform/methanol (2:1). The samples were kept on ice for 6 min with periodical vortexing. The phases were separated by addition of 2.5 volumes of 0.7 M formic acid. The lower organic phase was collected and dried under argon flow. For MS analysis, extracted and dried lipid was dissolved in methanol/water (85:15) to a final concentration of 40 pmol/µl (calculated regarding to the amount of starting lipid applied for extraction). Offline nanospray MS analysis in negative ion mode was performed with metal-coated borosilicate emitters connected to a LTQ XL mass spectrometer with the Nanospray Flex Ion Source (Thermo Scientific). A spray voltage of 1.5 kV was applied to the emitter and data analysis was performed in the profile mode using a heated capillary temperature of 200°C. Data analysis was performed with the Xcalibur software (Thermo Scientific).

Flow cytometer. RAW 264.7 cells were seeded onto a 24 wells tissue culture plates then stimulated with LPS (1 µg/ml) for 4h or 6h, stressEVs (10 µg/ml) for 4h, 20:4 lysoPI, ox 20:4 lysoPI, synEVs, oxidized synEVs or oxidized and hydrolyzed sPLA$_2$-IIA synEVs (40 µg/ml) for 6h. After stimulation, cells were incubated with Mouse BD Fc Block (rat anti-mouse CD16/CD32, BD Pharmingen) for 10 min on ice, washed twice with ice-cold PBS and incubated in the dark for 20 min on ice with PE anti-mouse TLR4 (clone MTS510, BioLegend). Afterward, cells were washed with ice-cold PBS and analyzed on flow cytometer (CyFlow space, Partec). Data were analyzed with FlowJo software (Tree Star).

Preparation of BMDMs and BMDCs. Bone marrows were collected from wild-type C57BL/6, TLR4 KO, TRIF KO and MyD88 KO (all C57BL/6) mice. Bone marrow cells were plated at a density of 2×10^6 cells/ml in RPMI 1640 media containing 10% FBS, 1% penicillin-streptomycin (all Invitrogen). For differentiation in macrophages, cells were incubated with macrophage colony-stimulating factor (M-CSF) (eBioscience; 40 ng/ml). After 3 days culture media was changed for 20 ng/ml M-CSF for additional 4 days before stimulation. For differentiation to dendritic cells, cells were incubated with Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) (PreproTech; 20ng/ml). On day 3, floating cells were discarded and fresh medium containing 20 ng/ml GM-CSF was added. On day 5, half volume of fresh

media was added to the cultures. On day 7, the semi-suspended cells and loosely attached cells were collected by gently pipetting the medium against the plate. All experiments with mice were performed under protocols approved by the local ethical committee and followed guidelines for use of animal in research.

qPCR. WT, MyD88 KO macrophages, as well as TLR4 KO and TRIF KO BMDMs were seeded at 1×10^6 cells/well and left untreated or stimulated with various compounds (indicated in Figure legends) for 6 h. Total RNA was isolated using TRIZOL reagent (Roche). cDNA was prepared using high capacity cDNA reverse transcription kit (Applied Biosystems) and qPCR for mouse *Gapdh* (Fw TTCACCACCATGGAGAAGGC; Rv GGCATGGACTGTGGTCATGA), *Il6* (Fw CGGAGGCTTAATTACACATGTTC; Rv CTGGCTTTGTCTTTCTTGTTATC), *Il1b* (Fw AAGGAGAACCAAGCAACGACAAAA; Rv TGGGGAACTCTGCAGACTCAAACT), *Il23a* (Fw GACCCACAAGGACTCAAGGA; Rv CATGGGGCTATCAGGGAGTA), *Socs2* (Fw GAGGAACAGTCCCCCGAG; Rv AAGAAAGTTCCTTCTGGAGCC), *Tnfaip6* (Fw TCCTCCTTTGCTTATGCGTC; Rv CGGCGTAGGTGAGCTTGTAT), *Ccl24* (CTGTGACCATCCCCTCATCT; Rv CTTATGGCCCTTCTTGGTGA) and human *TNFA* (Fw CCTGTGAGGAGGACGAAC; Rv CGAAGTGGTGGTCTTGTTG) were performed using SYBR green I master kit (Roche) on LightCycler 480 (Roche). The data are presented as fold increase of mRNA in treated cells relative to untreated cells.

Dual luciferase test. HEK293 or HEK293T cells were growing in DMEM containing 10% heat-inactivated FBS (both Invitrogen Life Technologies) and 2 x 10^4 cells/well were transfected with expression plasmids for pFlag-CMV-hTLR4 (a gift from Carsten Kirschning, University of Duisburg-Essen,Germany) (or pUNO-hTLR4^{F440A}, pUNO-hTLR4^{F463A} (Invivogen)), pDUO-hMD-2/hCD14 (Invivogen) as well as pELAM1-luciferase (NF-κB promotor) (a gift from Carsten Kirschning) and phRL-TK *Renilla* luciferase (Promega) using PEI transfection reagent (12 µl/1 µg DNA). Instead of pELAM1-luciferase pAP-1-luciferase, pISRE-luciferase, pSRE-luciferase, pE2F-luciferase, pCRE-luciferase (all BD Clontech) or pGAS-luciferase (a gift from dr. N. Mukaida (2)) were used. Cells were stimulated with various compounds (indicated in Figure legends). Cells were lysed and analyzed for luciferase activity using dual luciferase assay on Orion luminometer (Berthold).

Native PAGE. Recombinant human TLR4/MD2 complex proteins were incubated with 20:4 lysoPI, 15-LO oxidized 20:4 lysoPI or LPS in MQ for 2.5 h at 37 °C. After incubation, samples

were subjected to 10% native PAGE and and run at 90V for 4 hours at 4°C. The dimerization of TLR4 was analyzed by Western blotting with Tetra·His Ab (1:2000) (Qiagen).

ELISA. BMDMs were seeded at 1.5 x 10^5 cells per well of 96 well plate then stimulated with LPS (2 ng/ml), stressEVs (10 μg/ml), 20:4 lysoPI or ox 20:4 lysoPI (10 μg/ml). After 16h, Il-6 (Bioscience) and Ccl24 (Abcam) concentrations in supernatants or Il-23 (Raybiotech) production in cell lysate were measured by ELISA according to manufacturer's instructions.

BMDCs were seeded at 2 x 10^5 or 5 x 10^5 cells per well of 96 well plate for detecting Il-6 and Il-23 respectively, then stimulated with LPS (2 ng/ml), stressEVs (10 μg/ml), 20:4 lysoPI or ox 20:4 lysoPI (10 μg/ml). After 8 or 16h, the supernatants were collected to measure the concentration of Il-23 (Raybiotech) and Il-6 (Bioscience) ELISA according to manufacturer's instructions. Absorbance was measured on SynergyMx (BioTek).

Gradient ultracentrifugation. OptiPrep (60 w/v % iodixanol) was diluted to 40 %, 20 %, 10 % and 5 % in 0.25M sucrose in 10 mM Tris-HCl, pH 7.5) and a discontinuous gradient was formed by layering 2.5 ml of each solution in 10 ml polypropylene centrifugation tubes (Beckman Coulter). 250 μl of stressEVs was layered on the top. The samples were centrifuged at 100,000 g (rotor type 50TI, Beckman Coulter) for 16 h at 4 °C. 1 ml fractions of density gradient layers were collected from the top (fr.1-fr.9). The density of fractions was calculated based on iodixanol concentration measured by spectrophotometry at 340 nm on SynergyMx (BioTek). Western blot using anti-Tsg101, anti-CD81, anti-annexin V and anti-calnexin antobodies was performed.

LC-MS/MS analyses. The samples were protected from UV light and protected with N2 during the sample preparation. 10 μL of NaCl solution and 100 μL of ice cold acetonitrile with 100 ppm butylhydroxytoluene was added to the pellets. After vortexing, the samples were let to stand in cold for 30 min and centrifuged for 3 min at 9600 g. The supernatant was collected and analysed on Acquity UPLC (Waters Corporation, Milford, USA) using a Acquity UPLC BEH C18 (2.1 x 100mm 1.7 μm) analytical column held at 50°C. The injection volume was 10 μL. Multiple reaction monitoring was performed on a Xevo TQ-S micro tandem mass spectrometer (MS/MS) (Waters Corporation, Milford, USA) with an atmospheric electrospray interface operating in positive ion mode (*SI Appendix* Tab. S1). The source capillary was set to 3.50 kV, the source temperature was set to 150 °C, the desolvation temperature was set to 350 °C, and the desolvation gas flow was set to 650 L/Hr. The mobile phases used for the analysis were A

(water with 0.1% formic acid) and B (methanol with 0.1% formic acid). Briefly, the gradient was programmed as follows: 0–1 min, 40 % solvent B; 1–13 min, 80 % solvent B; 13-16 min, 100 % solvent B; 16-18 min, 100 % solvent B, flow rate 0.250 ml/min. Total run time was 20 minutes.

Supplementary Tab. S1. MRM settings.

Name	Precursor ion > daughter ion	Cone (V)	Collision (eV)
11/15-HETE-LPE	518.29 > 500.21	20.0	30.0
PGE2-LPE	550.27 > 532.26	20.0	30.0
11/15-HETE-LPC	560.33 > 542.32	20.0	30.0
PGE2-LPC	592.32 > 574.31	19.0	30.0
LysoPI	621.30 > 603.29	19.0	30.0
LysoHETE-PI	637.30 > 619.29	18.0	30.0

Supplementary Tab. S2: The composition of phospholipids in synEVs. Adopted from Weerheim et al (3).

Name	Solvent	%
L-a-lysophosphatidylcholine (from egg yolk), lysoPC	$CHCl_3$:MeOH; 1:1	2.05
Sphingomyelin (from brain, porcine), SM	$CHCl_3$:MeOH; 1:1	20.6
L-a-phosphatidylcholine (from egg yolk), PC	$CHCl_3$	59.2
L-α-lysophosphatidylserine (from brain, porcine), lysoPS	$CHCl_3$	1.05
L-a-phosphatidylserine (from brain, porcine), PS	$CHCl_3$	3.63
L-a-phosphatidylinositol (from soya), PI	$CHCl_3$	3.21
L-a-phosphatidylethanolamine (from egg yolk), PE	$CHCl_3$	9.4
L-α-lysophosphatidylethanolamine (from egg yolk), lysoPE	$CHCl_3$	0.83

Supplementary Fig. 1

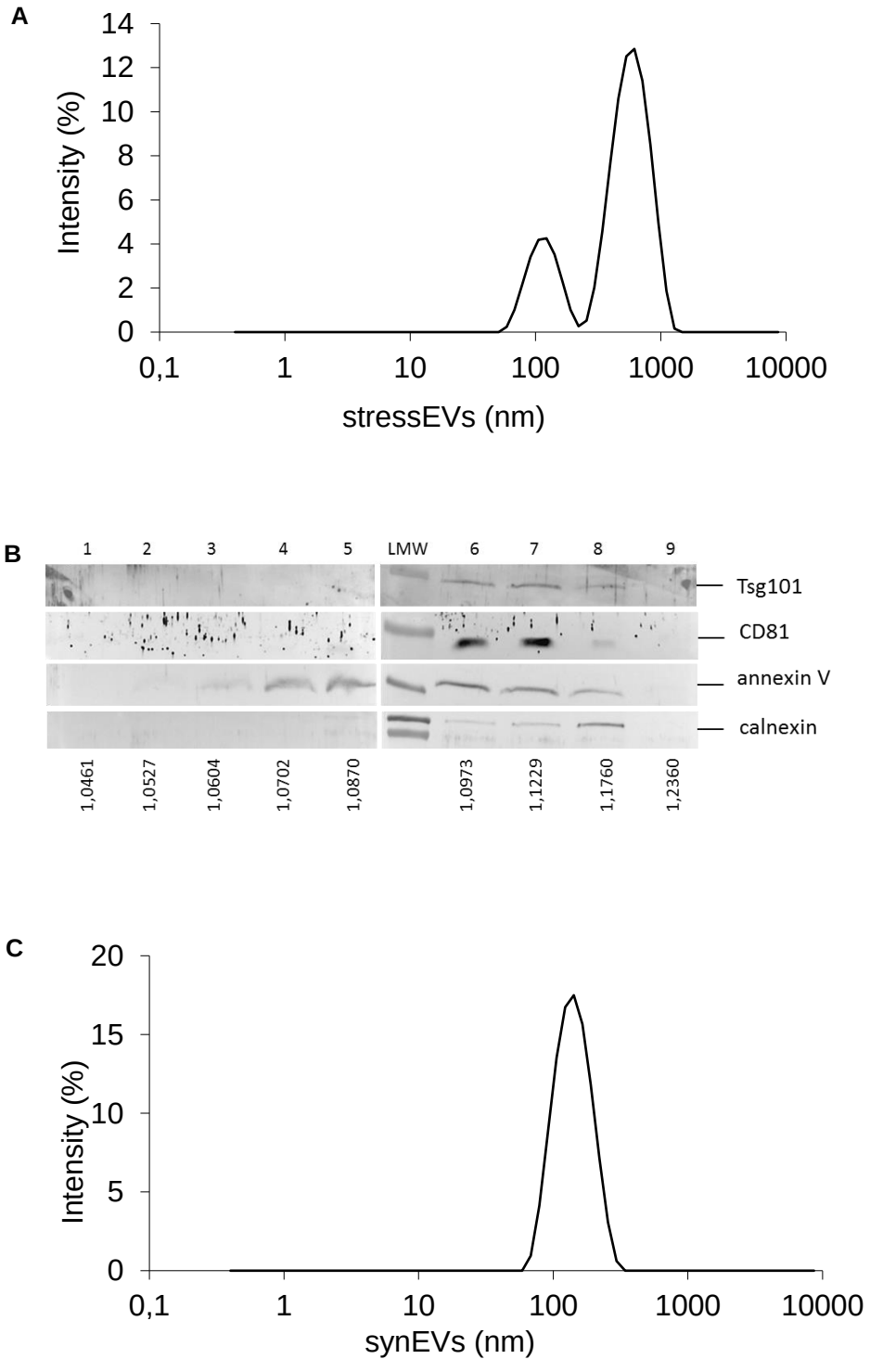

Fig. S1. Characterization of stressEVs and synEVs.

(*A* and *C*) Particle size distribution of stressEVs in (*A*) or synEVs in (*C*) were analyzed by DLS. (*B*) Western blot after density gradient ultracentrifugation of stressEVs. Tsg101, CD81, annexin V and calnexin were detected.

Supplementary Fig. 2

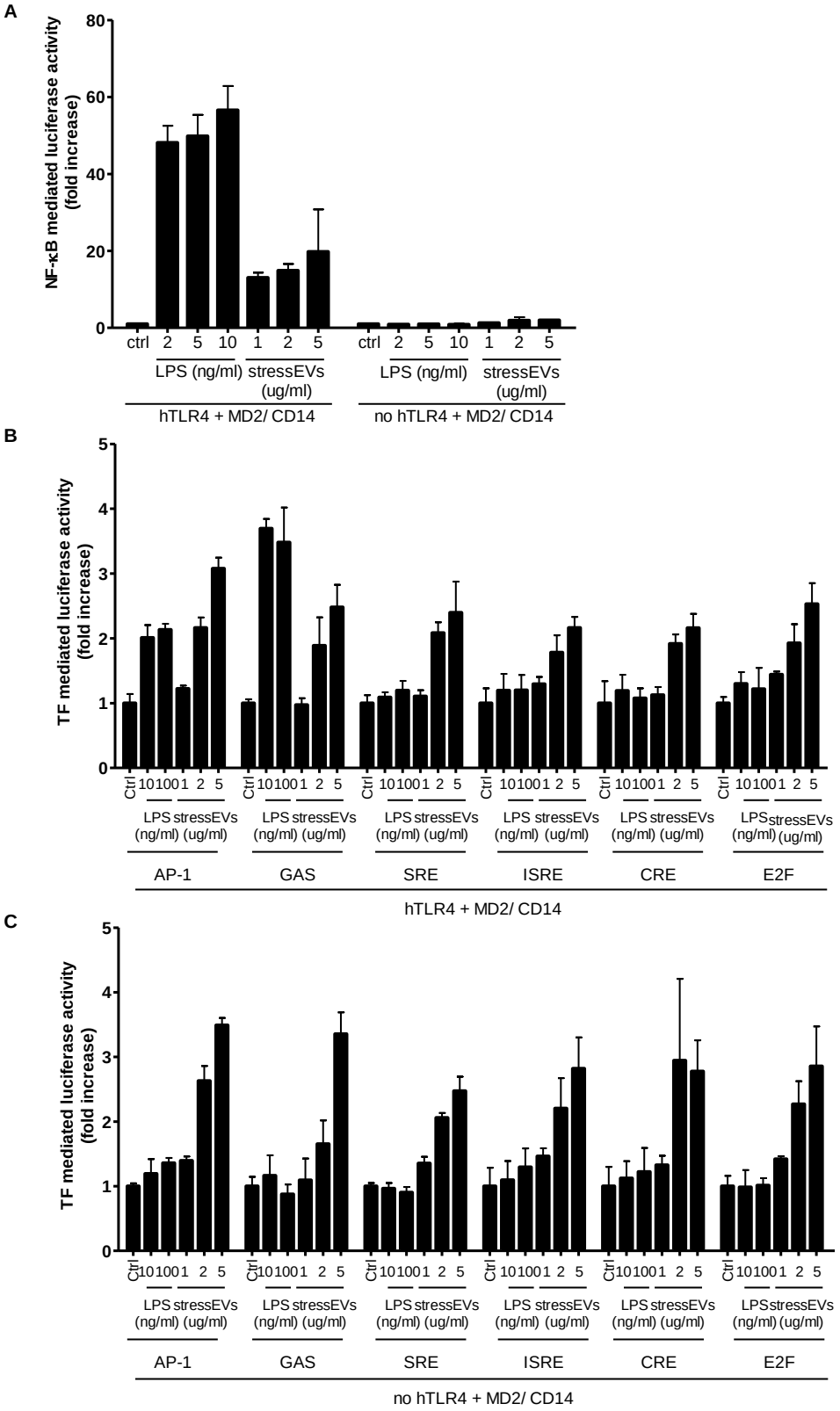

Fig. S2. LPS and stressEVs induce different transcriptional responses.

(*A, B* and *C*) HEK293T cells expressing hTLR4, hMD-2/CD14 in (*A* and *B*) or hMD-2/CD14 (A) or pcDNA alone in (*C*) and *Renilla* reporter plasmid for normalization and plasmids

expressing luciferase under NF-κB in (*A*) or AP-1, GAS, SRE, ISRE, CRE, E2F promotor in (*B* and *C*). HEK293T cells were incubated with stressEVs (1, 2 or 5 μg/ml) or LPS (2, 5 or 10 ng/ml) in (*A*) or LPS (10 or 100 ng/ml) in (*B* and *C*) for 24 h. Dual luciferase tests were performed. Negative controls are transfected but unstimulated cells. Data are representative of three (*A*) or two (*B*, *C*) independent experiments.

Supplementary Fig. 3

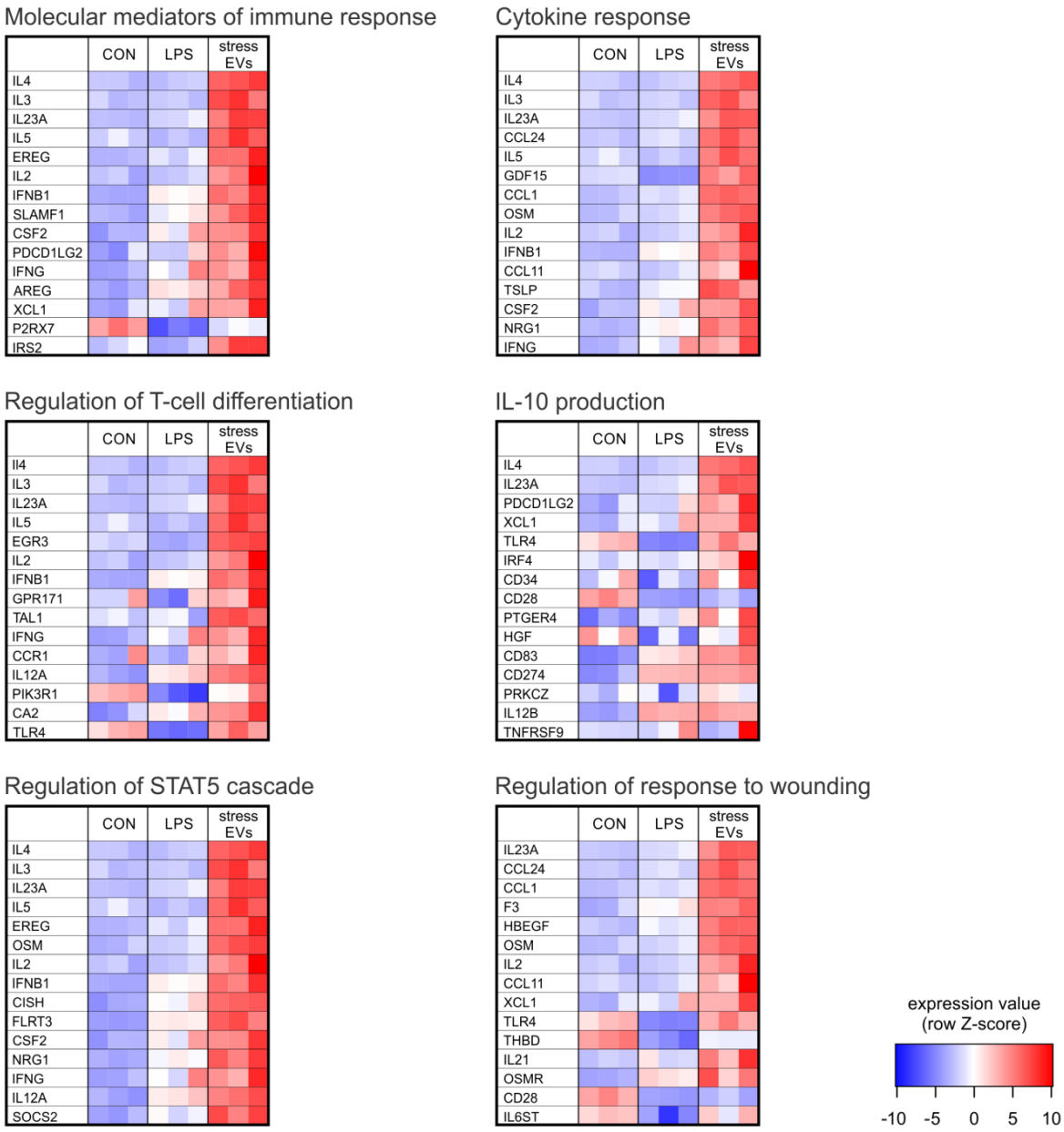

Fig. S3. The heat map of top 15 genes that were differently expressed by stressEVs in comparison to LPS.

Microarray data were analyzed by GSEA. Obtained gene sets were manually clustered into 6 groups of similar gene sets. Here, the heat maps show the normalized expression of 15 the most up-regulated genes from each cluster with respect to the contrast between stressEVs and LPS as measured by Signal-to-Noise metric.

Supplementary Fig. 4

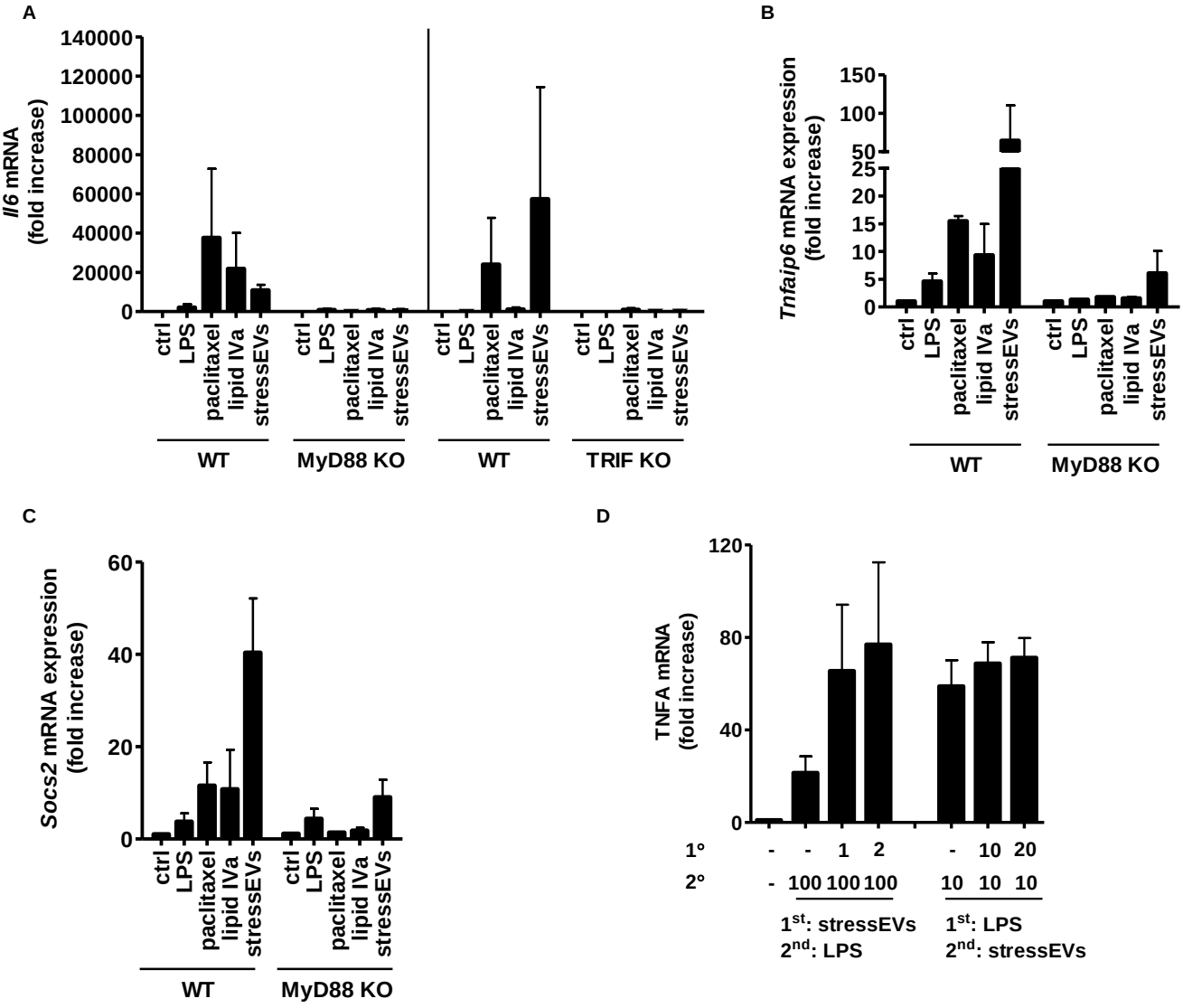

Fig. S4. LPS and stressEVs differ in stimulating the expression of different genes and no cross-tolerance is induced by LPS and stressEVs.

(*A-C*) WT or MyD88 KO macrophages as well as WT or TRIF KO BMDMs were stimulated for 6 h with paclitaxel (50 μM), lipid IVa (100 ng/ml), stressEVs (10 μg/ml) or with LPS (2 ng/ml) as a positive control. *Il6*, *Tnfaip6*, *Socs2* mRNA levels were determined using qPCR. (*D*) THP-1 cells were treated for 8 h with low concentrations of stressEVs (1 or 2 μg/ml) or LPS (10 or 20 ng/ml), followed by changing the media. Pretreated cells were subjected to switched treatment with LPS (100 ng/ml) or stressEVs (10 μg/ml) for 18 h. Negative control are unstimulated cells. *TNFα* levels were determined using qPCR. Data are pooled from two independent experiments.

Supplementary Fig. 5

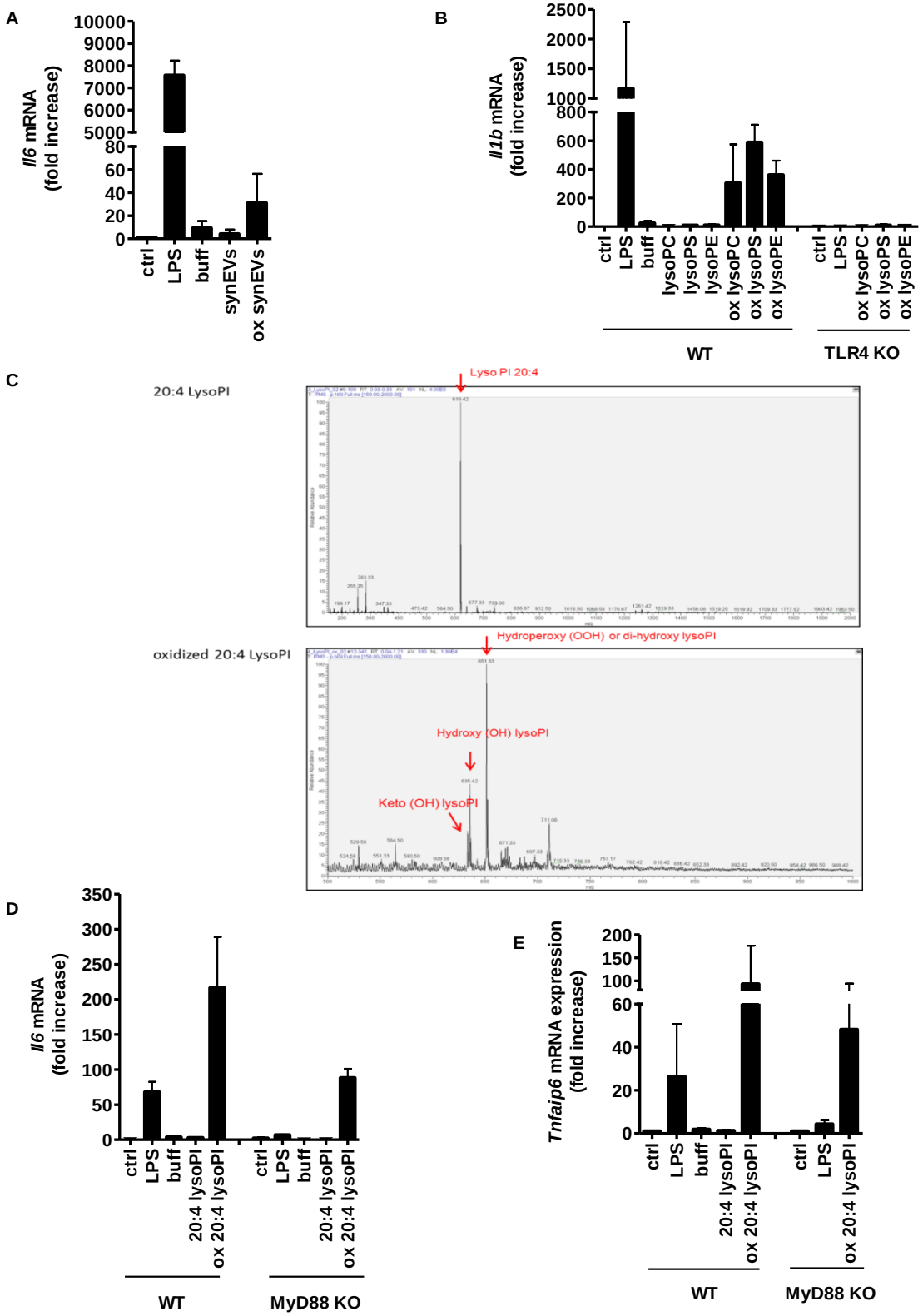

Fig. S5. 15-LO oxidized synEVs, lysoPLs or 20:4 lysoPI activate TLR4/MD-2.

SynEVs composed of PLs and lysoPLs were prepared. (*A*, *B*, *D* and *E*) WT or MyD88 KO macrophages as well as WT or TLR4 KO BMDMs were stimulated for 6 h with synEVs or

oxidized synEVs (10 μg/ml) in (*A*), single lysoPLs or oxidized lysoPLs (10 μg/ml) in (*B*), 20:4 lysoPI or oxidized 20:4 lysoPI (5 μg/ml) in (*D* and *E*), or LPS (2 ng/ml) as a positive control. *Il6*, *Il1b* and *Tnfaip6* mRNA levels were determined using qPCR. Negative control are unstimulated cells. (*C*) 20:4 lysoPI and oxidized 20:4 lysoPI were analyzed by MS. Hydroxy, hydroperoxy or keto products were detected. Data are pooled from two (*A*, *B*, *D* and *E*) independent experiments.

Supplementary Fig. 6

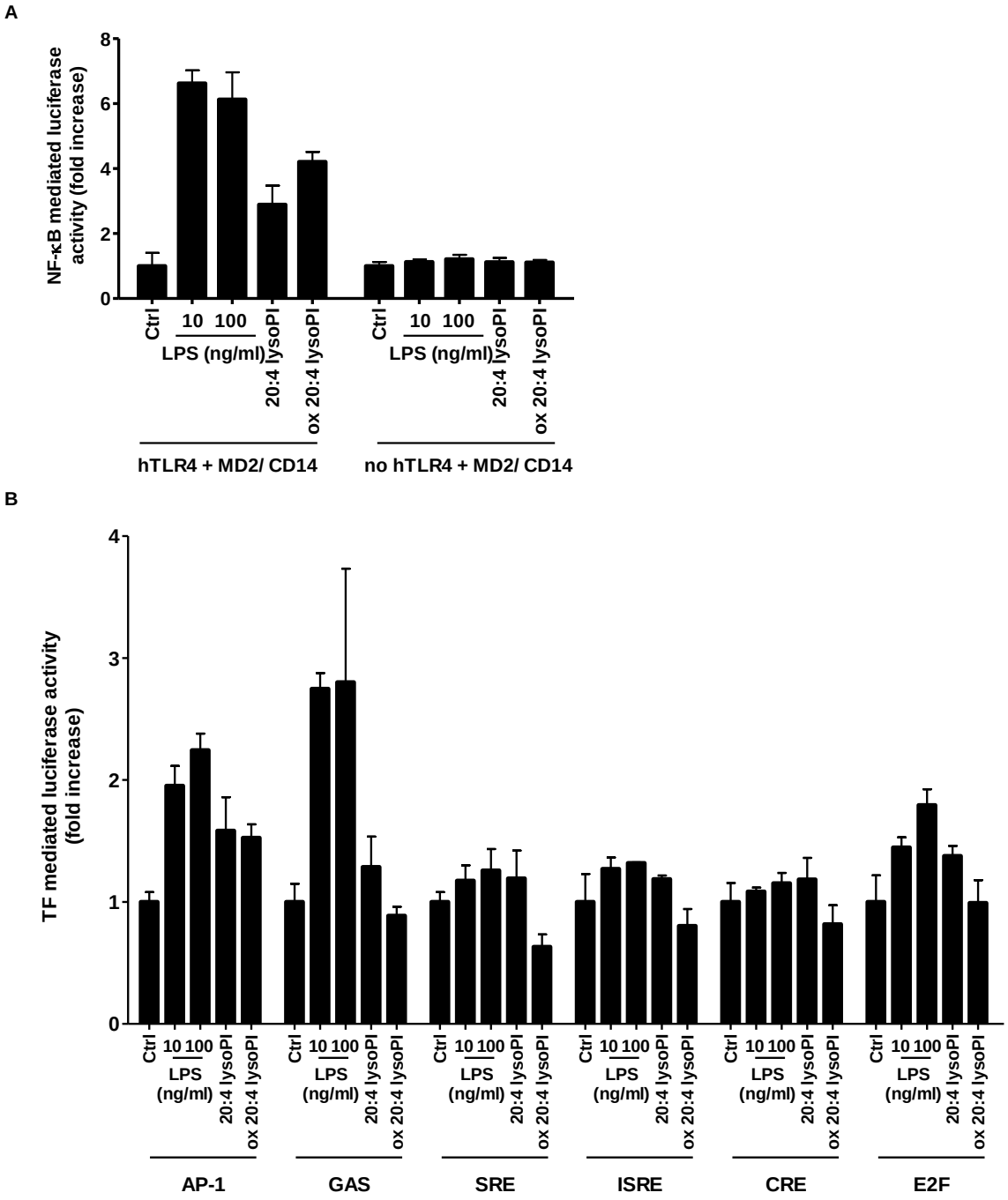

Fig. S6. LPS and ox 20:4 lysoPI induce similar transcriptional responses.

(*A* and *B*) HEK293T cells expressing hTLR4, hMD-2/CD14 or only hMD-2/CD14 and *Renilla* reporter plasmid for normalization and plasmids expressing luciferase under NF-κB in (*A*) or AP-1, GAS, SRE, ISRE, CRE, E2F promotor in (*B*). HEK293T cells were incubated with stressEVs (1, 2 or 5 μg/ml) or LPS (10 or 100 ng/ml) for 24 h. Dual luciferase tests were performed. Negative controls are transfected but unstimulated cells. Data are representative of two independent experiments.

Supplementary Fig. 7

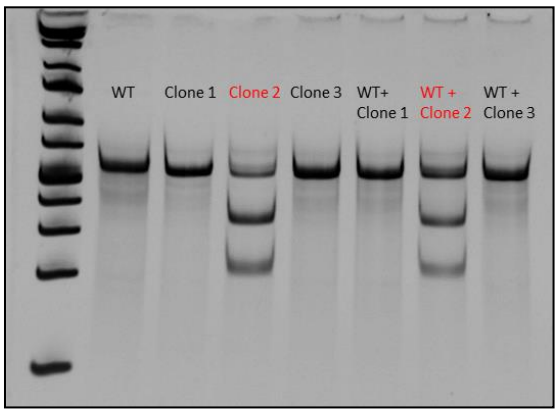

Fig. S7. Determination of indel mutations using T7 endonuclease I assay**.**

HEK293 cells were transfected with pX330 constructs expressing 15-LO sgRNA. Genomic DNA was extracted from different single clones. Target fragment was amplified by PCR and a T7E1 assay for indel mutation detection was performed. The total size of the PCR product (600 bp) was cleaved into two fragments (214 and 386 bp in size) by T7 endonuclease I and analyzed on 10 % native PAGE gel. Clone 2 was used for further experiments.

Supplementary Fig. 8

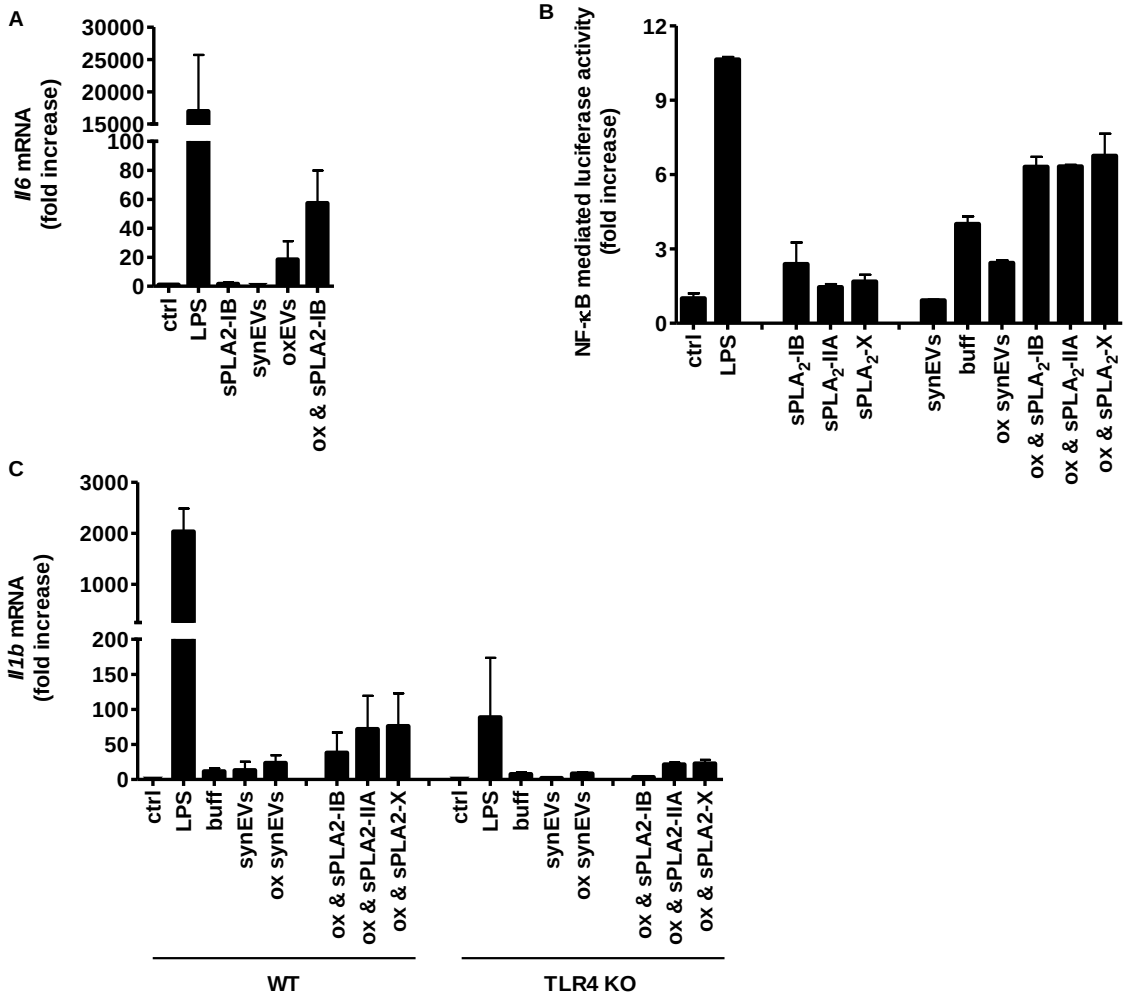

Fig. S8. sPLA$_2$s contribute to the activation of TLR4/MD-2.

SynEVs composed of 30% of AAPE and 70% of POPC were prepared. WT macrophages (*A*), WT or TLR4 KO BMDMs (*C*) were stimulated for 6 h with indicated sPLA$_2$ (30 ng/ml) or synEVs (10 μg/ml), oxidized synEVs (10 μg/ml), indicated oxidized & hydrolyzed synEVs (10 μg/ml) or LPS (2 ng/ml) as a positive control. *Il6* and *Il1b* mRNA levels were determined using qPCR. (*B*) HEK293T cells expressing hTLR4, hMD-2/CD14 as well as luciferase under NF-κB promotor and *Renilla* luciferase for normalization were stimulated with indicated sPLA$_2$ (30 ng/ml), synEVs (10 μg/ml), oxidized synEVs (10 μg/ml), indicated oxidized & hydrolyzed synEVs (10 μg/ml) or LPS (10 ng/ml) as a positive control for 24 h. Negative controls are transfected but unstimulated cells. Dual luciferase tests for NF-κB activity were performed. Data are pooled from two (*A*, and *C*) independent experiments. (*B*) Data are representative of two independent experiments.

Supplementary Fig. 9

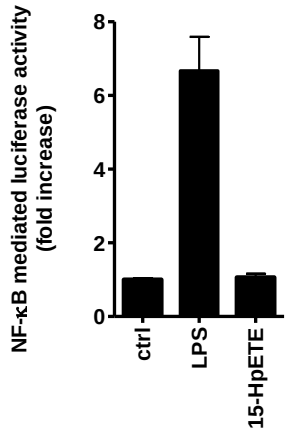

Fig. S9. 15-HpETE does not activate TLR4/MD-2 signaling.

HEK293T cells expressing hTLR4, hMD-2/CD14 as well as luciferase under NF-κB promotor and *Renilla* luciferase for normalization were stimulated with 15(S)-HpETE (48 μM) or LPS (10 ng/ml) as a positive control for 24 h. Dual luciferase test was performed. Negative control are transfected but unstimulated cells. Data are representative of three independent experiments.

Supplementary Fig. 10

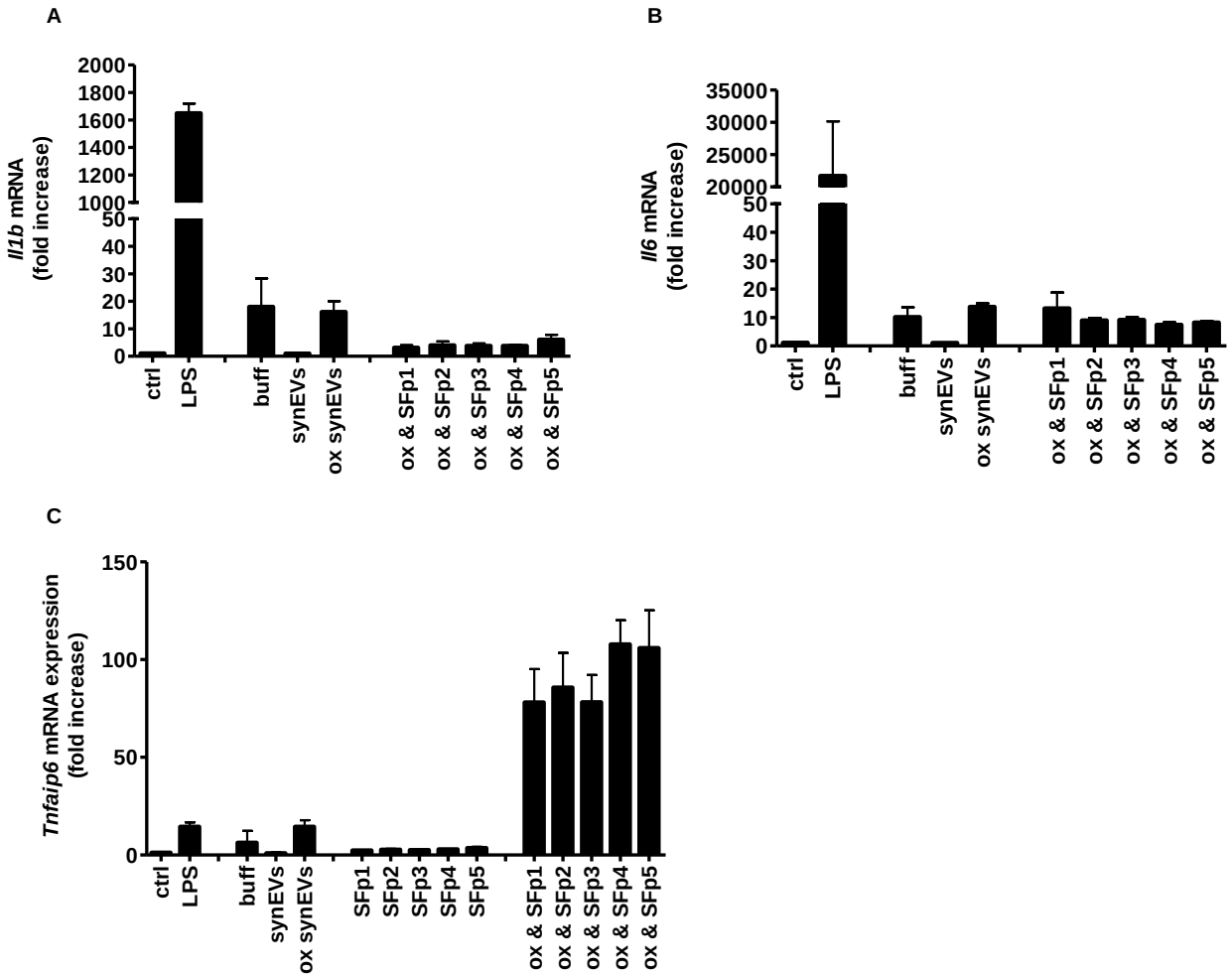

Fig. S10. Synovial fluid from RA patients contributes to agonist formation from synEVs.

SynEVs composed of 30% of AAPE and 70% of POPC were prepared. WT macrophages were stimulated for 6 h with indicated SF (14,7 μl), synEVs (10 μg/ml), oxidized synEVs (10 μg/ml), indicated oxidized & hydrolyzed synEVs (10 μg/ml) or LPS (2 ng/ml) as a positive control. *Il1b*, *Il6* and *Tnfaip6* mRNA levels were determined using qPCR. Data are pooled from two independent experiments.

References

1. Slaymaker IM, Gao, L, Zetsche, B, Scott, DA, Yan, WX, Zhang, F (2016) Rationally engineered Cas9 nucleases with improved specificity. *Science* 351(6268):84-88.
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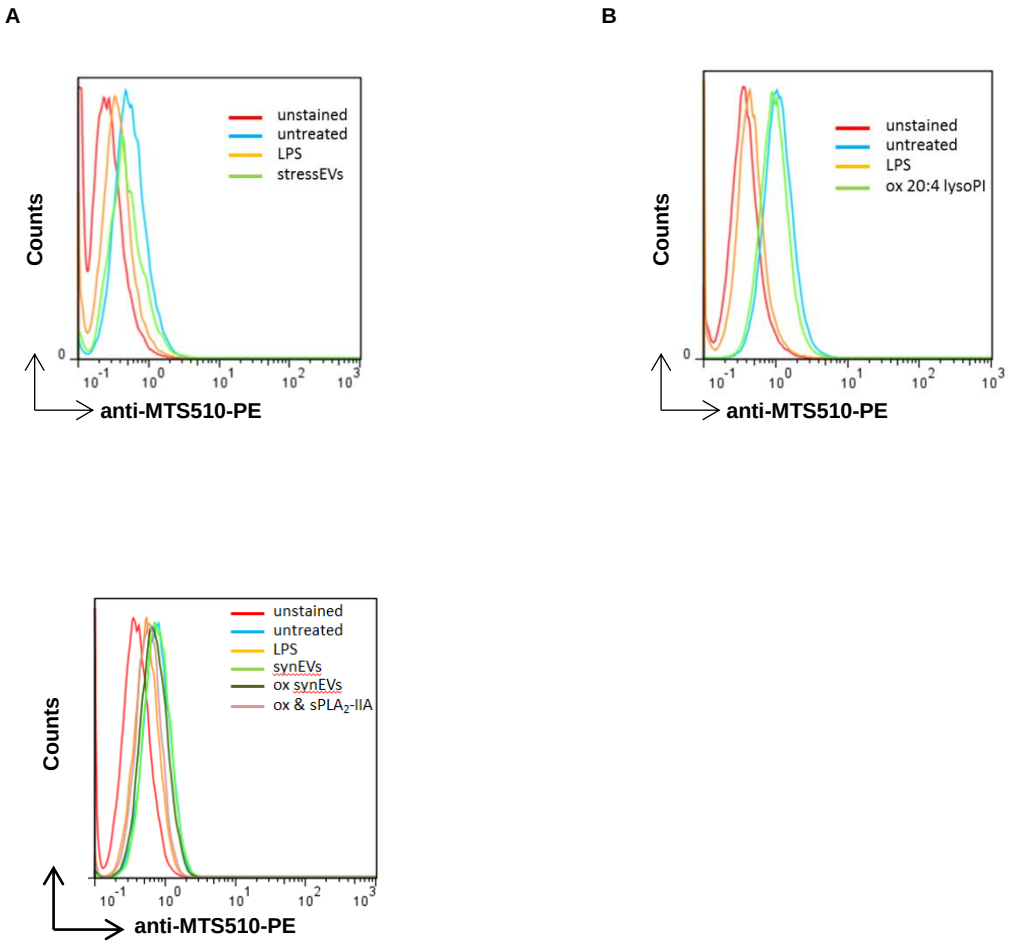
3. Weerheim AM, Kolb AM, Sturk A, & Nieuwland R (2002) Phospholipid composition of cell-derived microparticles determined by one-dimensional high-performance thin-layer chromatography. *Anal Biochem* 302(2):191-198.

SI Appendix Additional data



Additional independent experiments for Fig. 2*I* (*A*), Fig. 4*B* (*B*) and Fig. 5*D* (*C*) are shown.