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Supplementary Materials for

Toll-like receptor 4 senses oxidative stress mediated by the oxidation of phospholipids in extracellular vesicles

Mateja Manček-Keber,* Mojca Frank-Bertoncelj, Iva Hafner-Bratkovič, Anže Smole, Mateja Zorko, Nina Pirher, Silvia Hayer, Veronika Kralj-Iglič, Blaž Rozman, Nejc Ilc, Simon Horvat, Roman Jerala*

*Corresponding author. E-mail: roman.jerala@ki.si (R.J.); mateja.mancek@ki.si (M.M.-K.)

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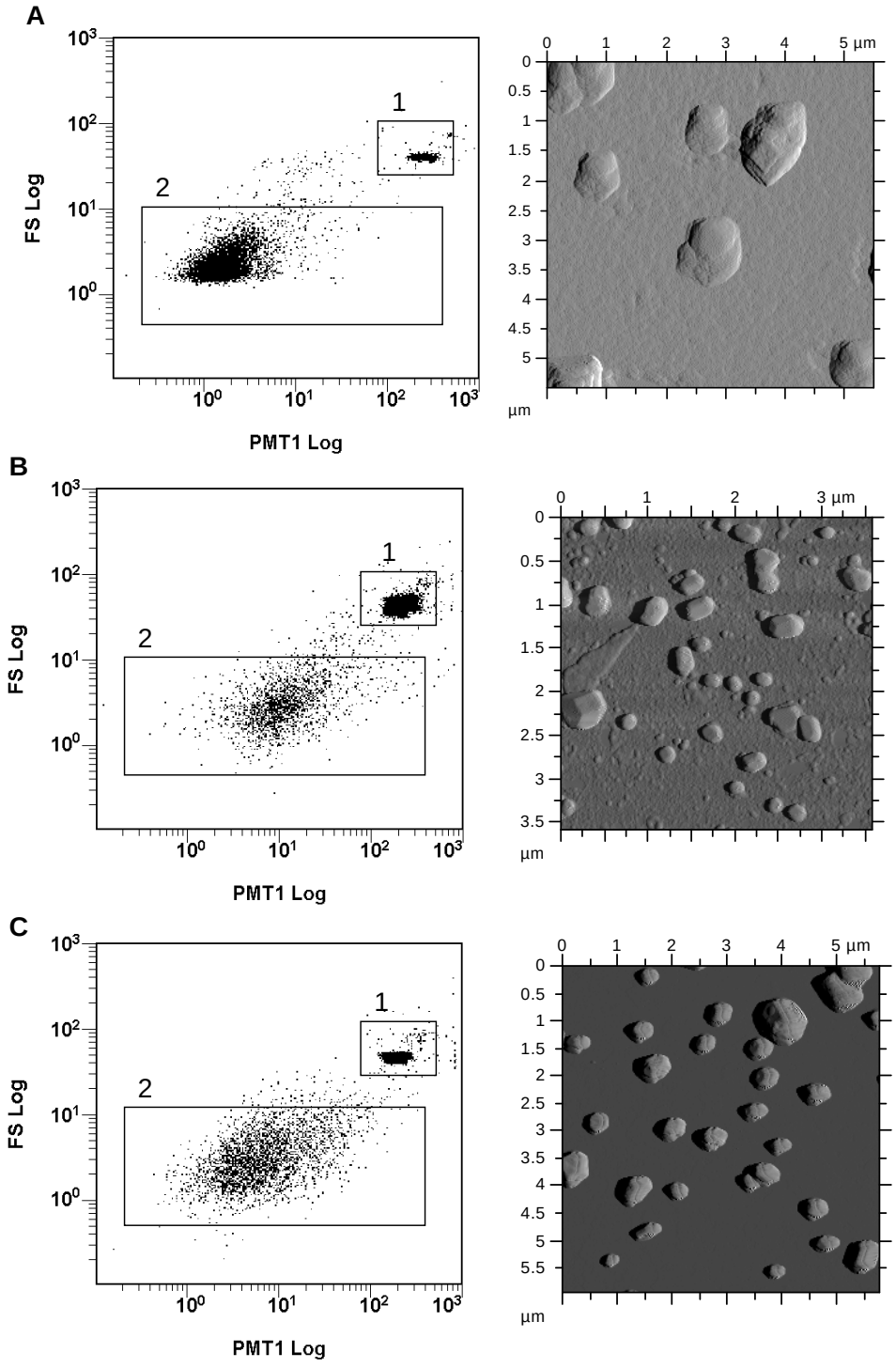


Fig. S1. Determination of the numbers of native EVs, stressEVs, and synEVs. (**A** to **C**) Left: The numbers of (A) natEVs, (B), stressEVs, and (C) synEVs were determined by flow cytometric analysis (region 2), based on known amounts of calibration beads (region 1) that were added to each sample. PMT1 refers to side scatter. FS, forward scatter. Right: AFM images of EVs, showing their structures and relative sizes in µm.

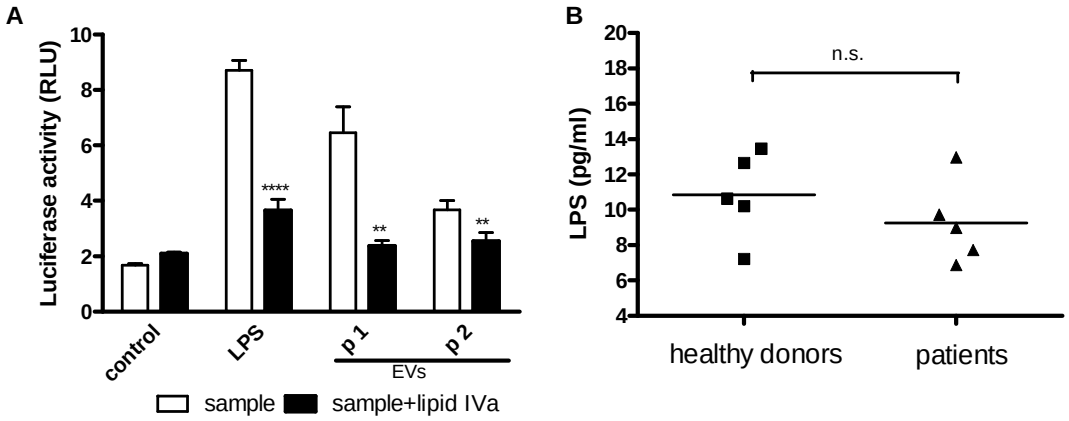


Fig. S2. Inhibition of LPS signaling also inhibits the activity of EVs. (**A**) HEK 293T cells transfected with luciferase and renilla reporter plasmids and plasmids encoding hTLR4, hMD-2, and hCD14 were left untreated or were pretreated with lipid IVa (1 μg/ml) before being treated with vehicle (control), LPS (100 ng/ml), or natEVs (2000 EVs/ml) from the indicated patients. Dual luciferase tests for NF-κB activity were then performed. Three technical replicates were performed per experiment. (**B**) Endotoxin neutralization capacity (ENC) assays were performed to compare plasma samples from RA patients and healthy donors. Plasma samples were incubated with LPS (200 ng/ml) for 1 hour, diluted with 0.85% NaCl, and analyzed for free LPS with the Limulus amebocyte lysate (LAL) assay. One technical replicate was performed per experiment. Each symbol represents a different donor. Data are representative of three (A) and two (B) independent experiments. ** $P < 0.05$; **** $P < 0.005$; n.s., not significant.

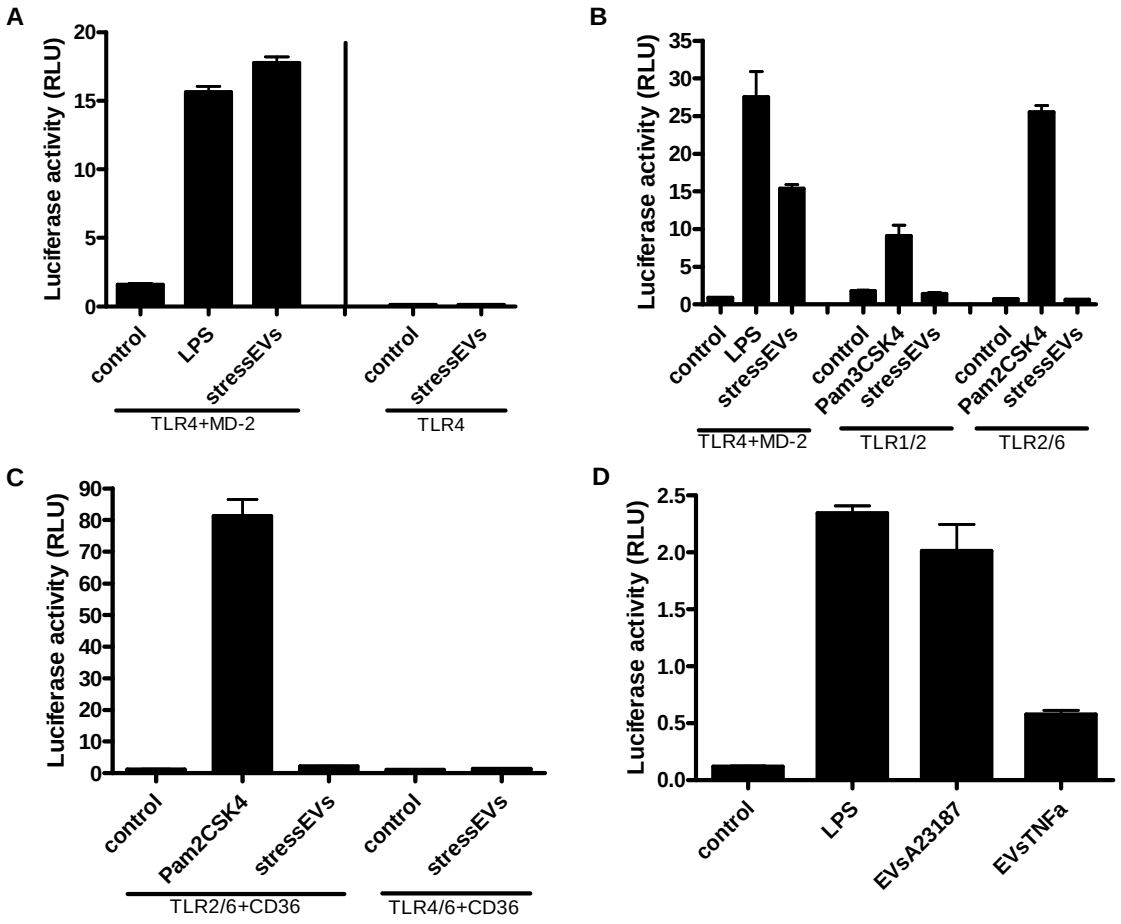
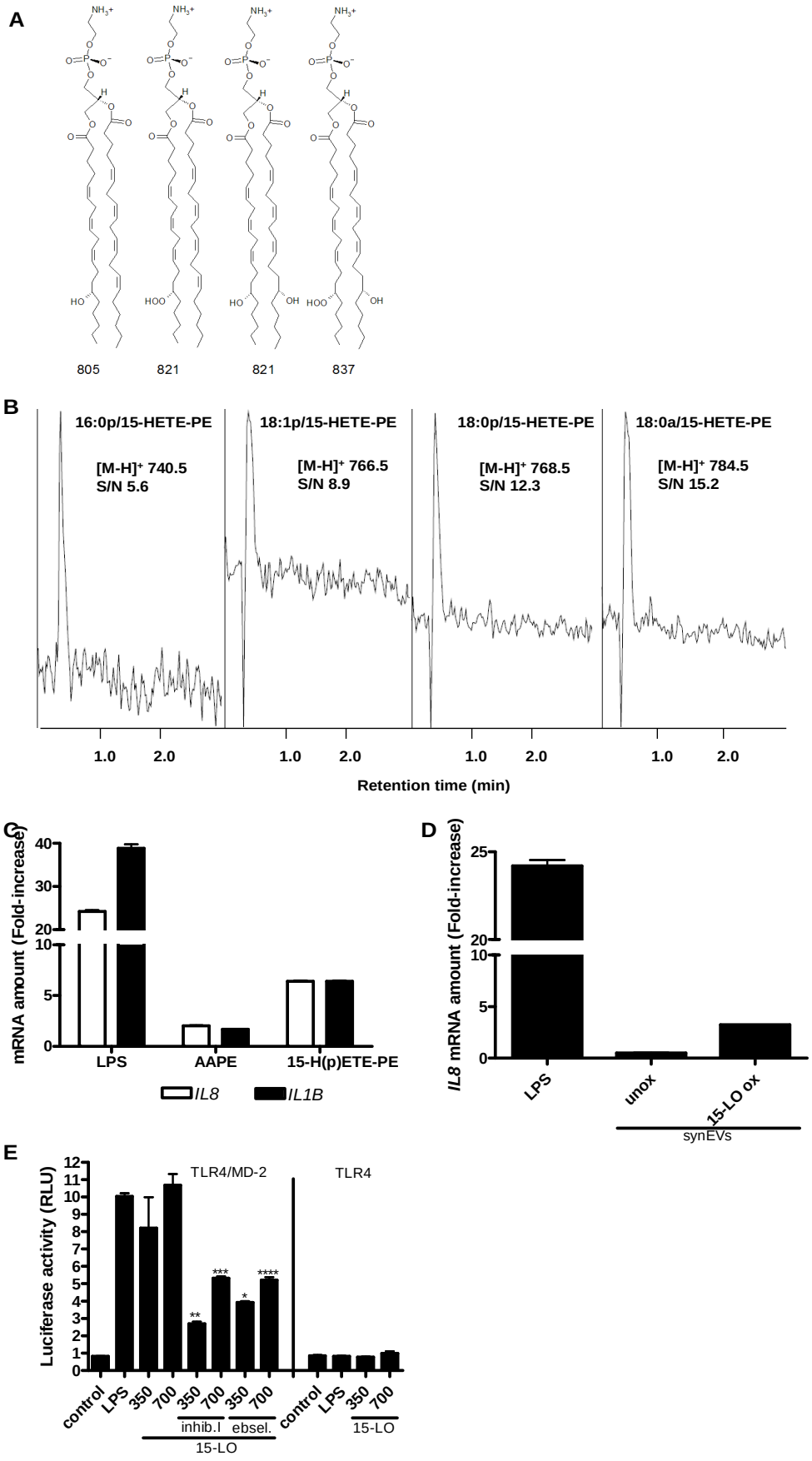


Fig. S3. StressEVs activate the TLR4–MD-2 complex. (**A**) StressEVs were isolated from HUVECs that were stimulated with 12 μM A23187 for 1 hour. HEK 293T cells transfected with luciferase and renilla reporter plasmids and plasmids encoding hTLR4 or hMD-2 were left untreated (control) or were treated with LPS (100 ng/ml) or stressEVs (1000 EVs/ml). Dual luciferase tests for NF-κB activity were then performed. Three technical replicates were performed per experiment. (**B** and **C**) HEK 293T cells transfected with luciferase and renilla reporter plasmids and combinations of plasmids encoding the indicated TLRs, MD-2, or the scavenger receptor CD36 were left untreated (control) or were treated with LPS (100 ng/ml), stressEVs (5000 EVs/ml), Pam2CSK4 (100 ng/ml), or Pam3CSK4 (100 ng/ml). Dual luciferase tests for NF-κB activity were then performed. Three technical replicates were performed per experiment. (**D**) StressEVs were isolated from HEK 293 cells that were treated with TNF-α (50 ng/ml) for 24 hours (EVsTNFa). HEK 293T cells transfected with luciferase and renilla reporter plasmids and plasmids encoding hTLR4 and hMD-2 were left untreated (control) or were treated with LPS (100 ng/ml) or EVs (5000 EVs/ml) isolated from HUVECs (EVsA23187) or HEK 293T cells (EVsTNFa). Dual luciferase tests for NF-κB activity were then performed. Three technical replicates were performed per experiment. Data are representative or two (for A, B and C) and three (D) independent experiments.


A
805
821
821
837
B
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S/N 12.3
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[M-H]+ 784.5
S/N 15.2
1.0
2.0
Retention time (min)
C
mRNA amount (Fold-increase)
LPS
AAPE
15-H(p)ETE-PE
IL8
IL1B
D
IL8 mRNA amount (Fold-increase)
LPS
unox
15-LO ox
synEVs
E
Luciferase activity (RLU)
TLR4/MD-2
TLR4
control
LPS
350
700
inhib.I
ebsel.
15-LO

Fig. S4. 15-LO–hydroperoxylated PE compounds stimulate cytokine production, and 15-LO stimulates NF-κB signaling in a TLR4- and MD-2–dependent manner. (**A**) Possible 15-LO oxidized AAPE compounds and (**B**) their molecular weights. Lipids from stressEVs were extracted, dried, and resolved in the mobile phase, of which 2 μl were analyzed by MS. Several peroxidized phospholipids (15-HETE-PE) were detected and their S/N ratios were determined. (**C** and **D**) AAPE and synEVs were oxidized with 15-LO to produce 15-H(p)ETE-PE compounds. MonoMac6 cells were then treated with LPS (1 ng/ml), AAPE (10 μg/ml), 15-H(p)ETE-PE (10 μg/ml), or the indicated synEVs (7500 EVs/ml). Samples were then subjected to qPCR analysis to determine the relative abundances of *Il8* and *Il1b* mRNAs, which are expressed as fold-increases compared to the mRNA amounts in untreated cells. Three technical replicates were performed per experiment. (**E**) HEK 293T cells transfected with luciferase and renilla reporter plasmids and plasmids encoding hTLR4, hMD-2, or hCD14, as indicated, were incubated with 15-LO (350 or 700 U/ml). Dual luciferase tests for NF-κB activity were then performed. Three technical replicates were performed per experiment. Data in (C) to (E) are representative of three independent experiments.

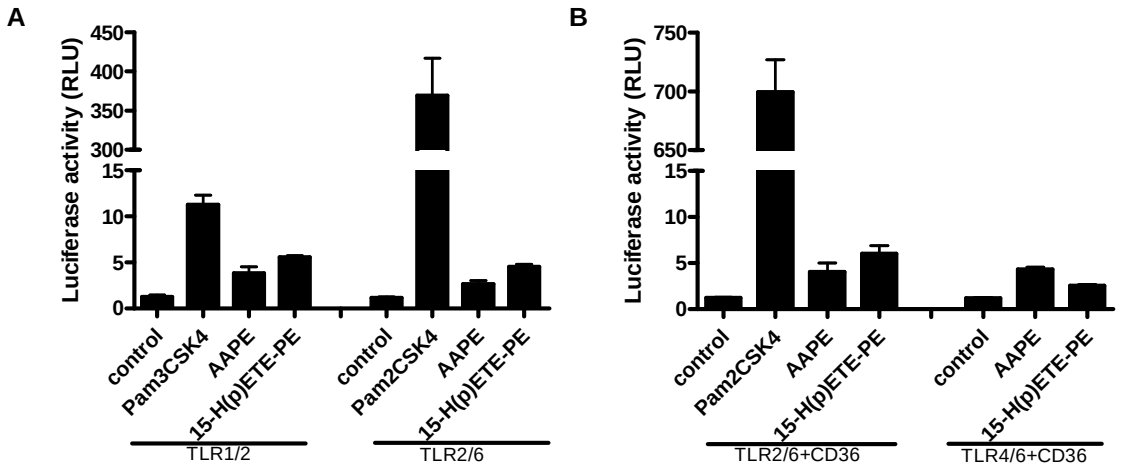

Fig. S5. 15-LO–hydroperoxylated PE compounds stimulate NF-κB activity in a TLR2-dependent manner. (**A** and **B**) HEK 293T cells transfected with luciferase and renilla reporter plasmids and plasmids encoding the indicated TLRs or CD36 were left untreated (control) or were treated with Pam3CSK4 (10 ng/ml), AAPE (20 μg/), 15-H(p)ETE-PE (20 μg/ml) or Pam2CSK4 (5 ng/ml). Dual luciferase tests for NF-κB activity were then performed. Three technical replicates were performed per experiment. Data are representative of two independent experiments.

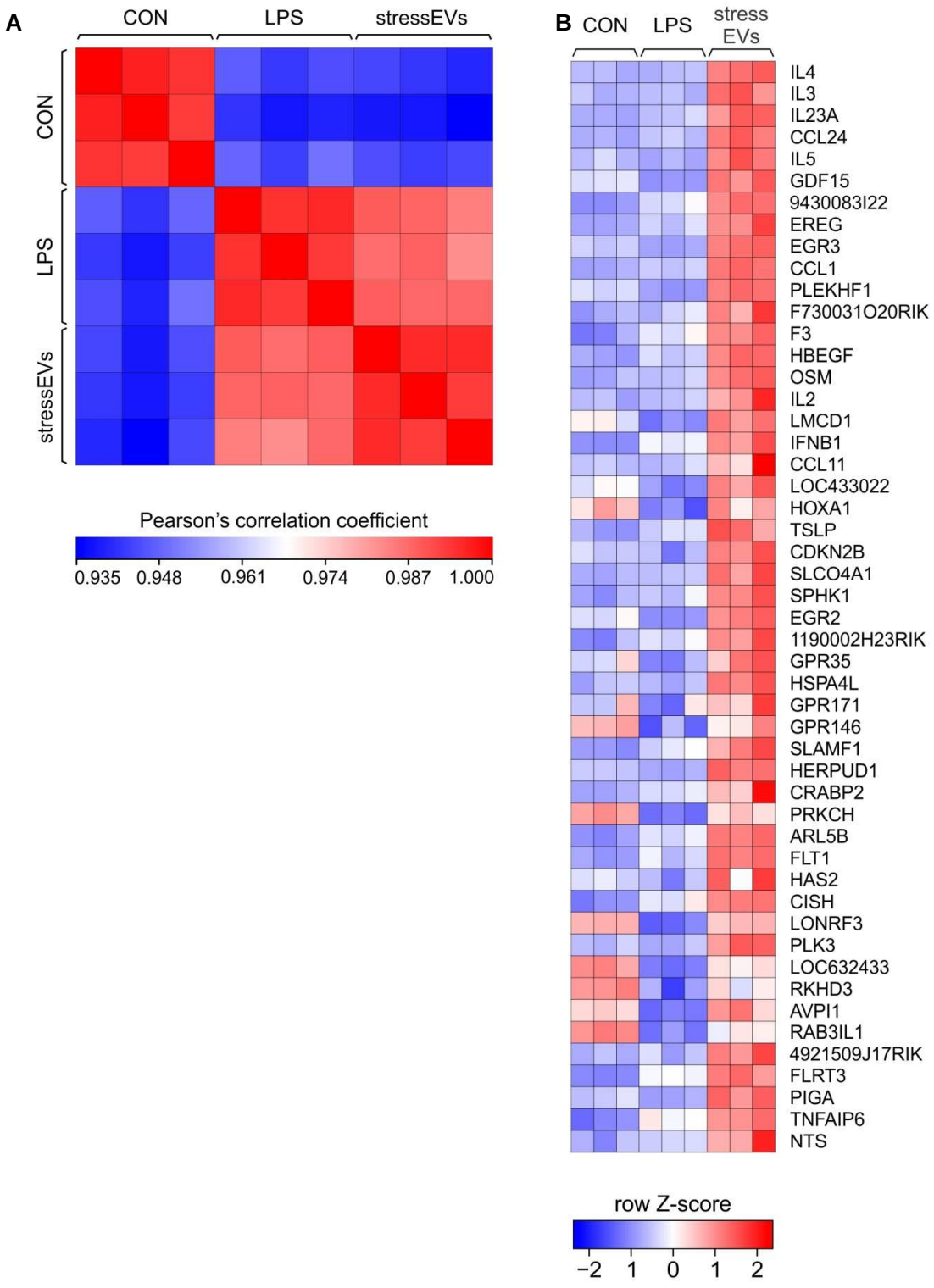

Fig. S6. Heat maps of the pairwise signal comparison (Pearson's correlation) and the 50 genes whose expression was most affected by stressEVs. (**A**) Signal concordance was evaluated by calculating the Pearson's correlation coefficient (r) values to compare the signal values obtained in two arrays. The r values were converted into a color scale, which is

indicated below the figure. The pairwise signal comparison of all 12 arrays demonstrates a high concordance within treatment arrays. (**B**) Microarray data were analyzed by GSEA. The heat map shows the 50 genes whose expression was most statistically significantly different transcribed between stressEVs and LPS. Calculated Z-scores values were converted into a color scale.

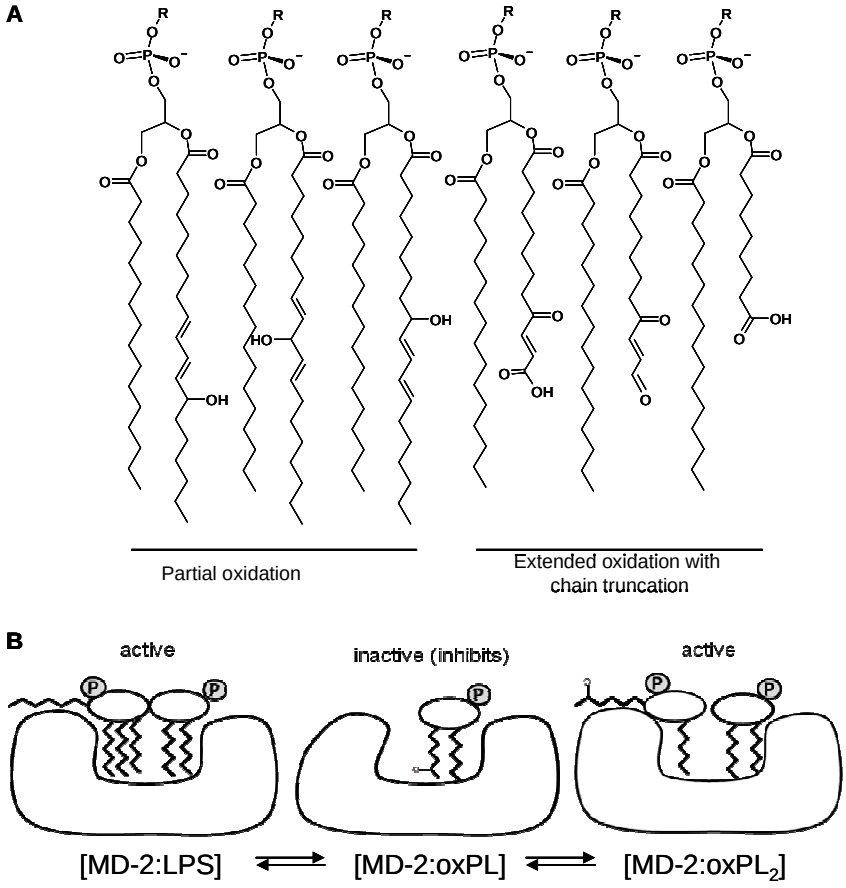

Fig. S7. Schematic representation of different phospholipid oxidation compounds, molecular complexes, and equilibria between MD-2 and different molecular species. (**A**) Left: Oxidation compounds of phospholipids resulting from the insertion of oxygen. Right: Representative oxidation compounds resulting in truncation of the acyl chain and insertion of several oxygen atoms. (**B**) The occupation state of MD-2 determines its ability to activate TLR4. Left: The occupation of MD-2 by lipid A causes a conformational change within MD-2 and exposure of the acyl chain that activates TLR4. Middle: The partial occupation of MD-2 with a single hydroperoxylated phospholipid (oxPL) does not result in TLR4 activation, and competes with the binding of LPS to MD-2. Right: Full occupation of the MD-2 pocket by two phospholipid molecules ($oxPL_2$) causes a conformational change in MD-2 that is similar to that caused by LPS, charge arrangement, and exposure of the acyl chain, resulting in TLR4 activation. Proposed equilibria between MD-2, LPS, and oxidized phospholipids are presented.

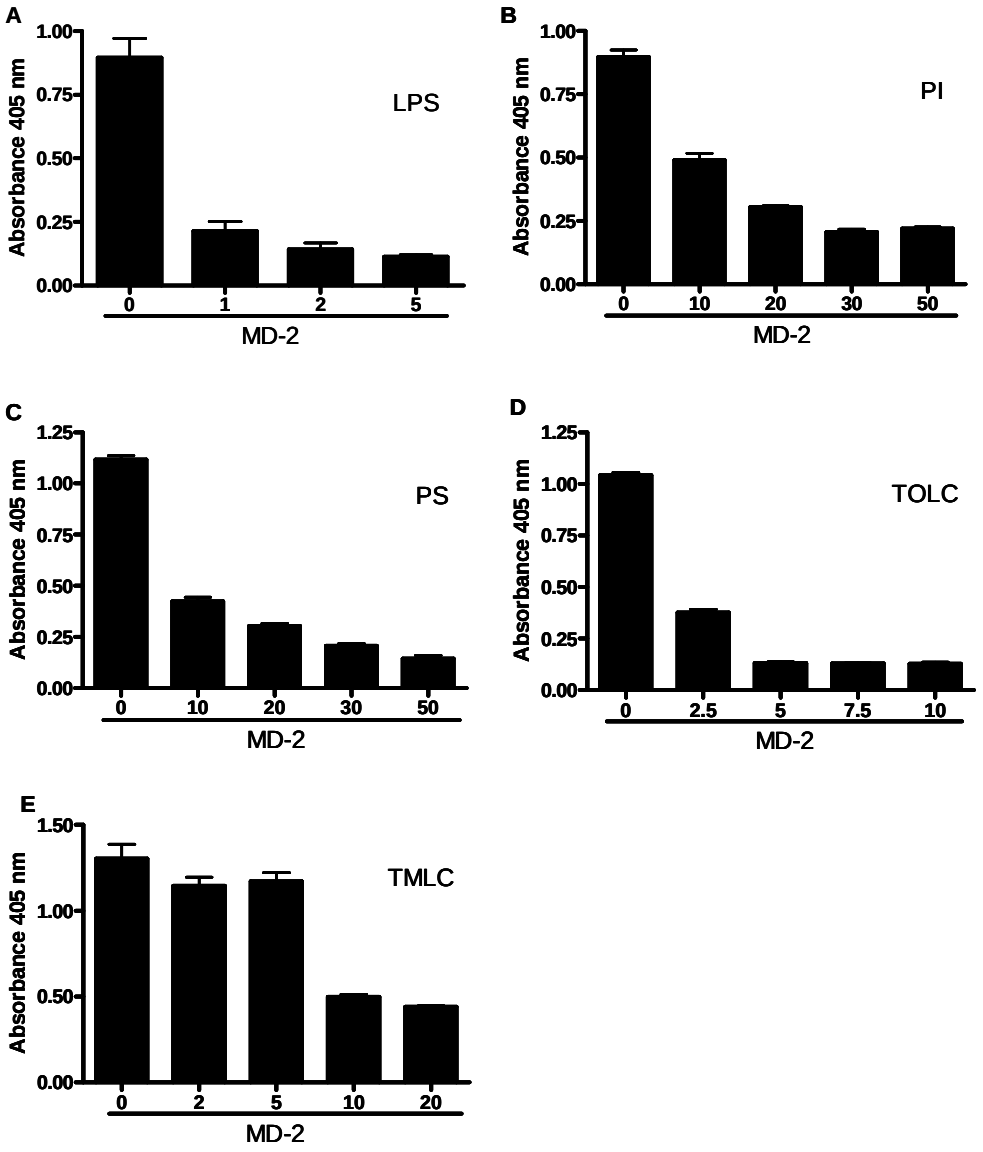

Fig. S8. Phospholipids bind to MD-2 at a region that overlaps with its LPS-binding site. (**A** to **E**) Sandwich ELISA assays showing the binding of (A) LPS, phosphatidylinositol (, B), phosphatidylserine (PS, C), tetraoleylcardiolipin (TOCL, D), and tetramyristoylcardiolipin (TMCL, E) to MD-2. Recombinant WT hMD-2 protein and the indicated compounds (in μM) were preincubated to enable the formation of complexes. Compounds bound to the xxx pocket of MD-2 prevented binding of the 9B4 antibody in a concentration-dependent manner. Three technical replicates were performed per experiment. Data are representative of two independent experiments.