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Supplemental Information

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Anti-inflammatory actions of acanthoic acid-related diterpenes involve activation of the PI3K p110-γ/δ subunits and inhibition of the NF-κB pathway

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Supplemental Data

Figure S1, related to Fig. 1B-C. ***Down-regulation of genes belonging to the Toll-like Receptor (TLR) pathway.*** Clustering of TLR pathway genes. Each branch of the dendrogram shows the inter-gene euclidian distance and the red/blue heatmap show the relative expression levels in each experimental condition (see legend). LPSR and DTP1+LPSR are biological replicates of LPS and DTP1+LPS, respectively (A). Peritoneal macrophages were pretreated with 10 µM of DTP1 or 5 and then activated with LPS (250 ng/ml) for 4h. The RNA levels of several genes present in the microarray were confirmed by qPCR and referred to the levels in untreated cells (considered as 1) (B). Results show mean±SD of the mRNA levels. *36B4* was used as house-keeping gene. *P<0.05; **P<0.01 *vs.* the corresponding value in the absence of DTP.

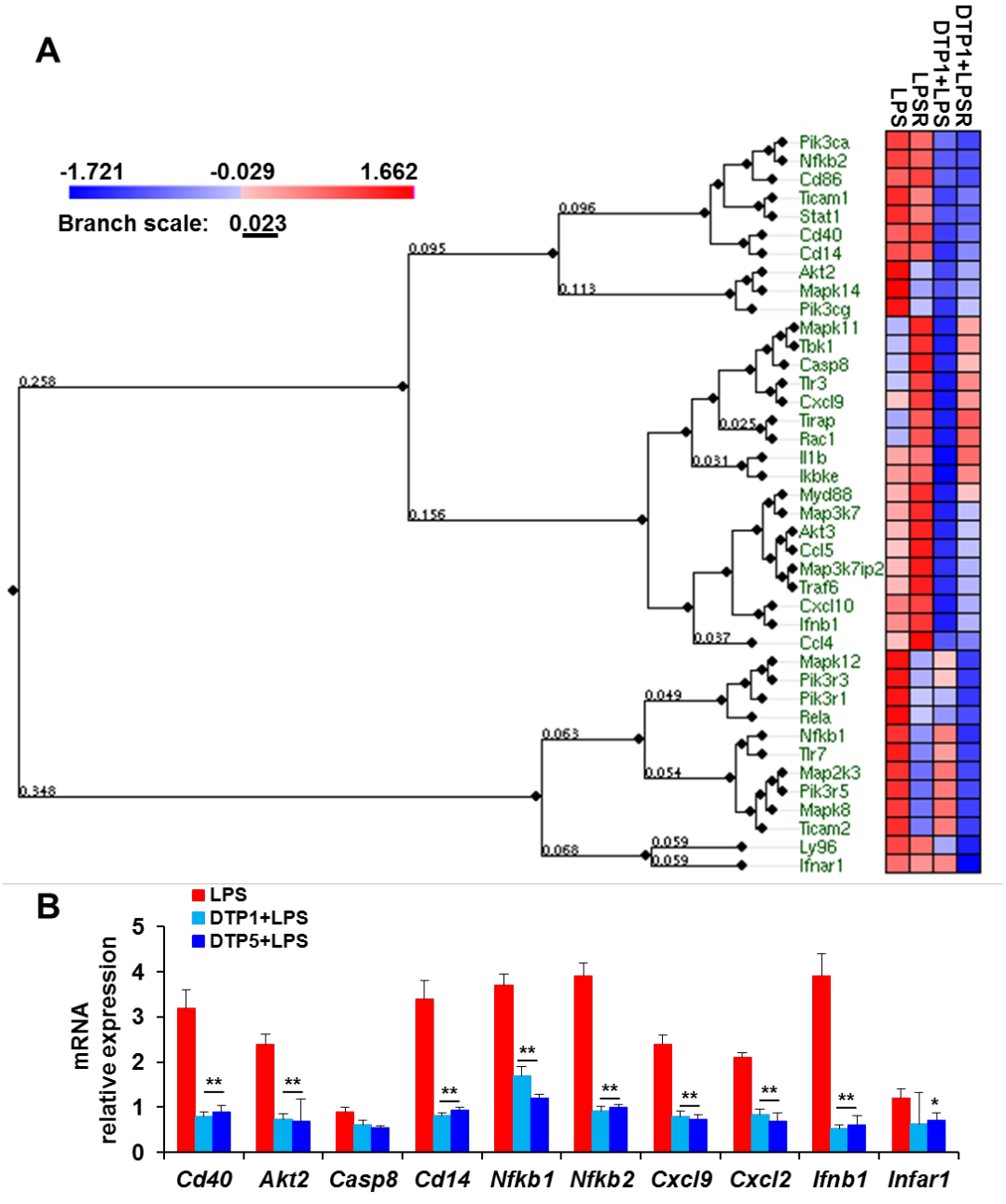
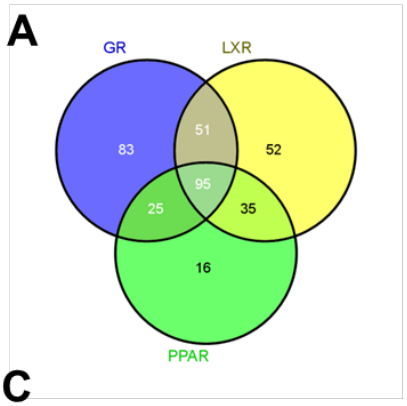


Figure S2, related to Fig. 1B-C. ***Transrepression profile by activated nuclear receptors and DTP1***. The transrepression activity of DTP1 was compared with that of activated GR, LXR and PPARγ using Gene Set Enrichment Analysis (GSEA) and the genesets defined by Ogawa et al. (Ogawa et al., 2005). Venn diagram showing the overlap of GR, LXR and PPARγ genesets; GSEA analysis was performed with both the total geneset (whole circles) and with subset that is unique to each nuclear receptor (A). GSEA results showing that target genes of GR, LXR and PPARγ (after activation with their respective ligands) resulted to be significantly enriched in the down-regulated part of the list (Negative Normalized Enriched Score, NES, and FDR q-val < 0.05) (B). GSEA enrichment plots of LXR, GR and PPARγ genesets (C,D,E).



B

Geneset	SIZE	ES	NES	NOM p-val	FDR q-val	FWER p-val
GR	148	−7E+07	−3E+07	0.000	0.000	0.000
LXR	127	−7E+06	−3E+07	0.000	0.000	0.000
PPAR	96	−6E+07	−3E+06	0.000	0.000	0.000
GR_ONLY	51	−7E+06	−3E+07	0.000	0.000	0.000
LXR_ONLY	25	−713336	−2E+07	0.000	0.000	0.000
PPAR_ONLY	7	−7E+06	−1E+07	0.091	0.034	0.082

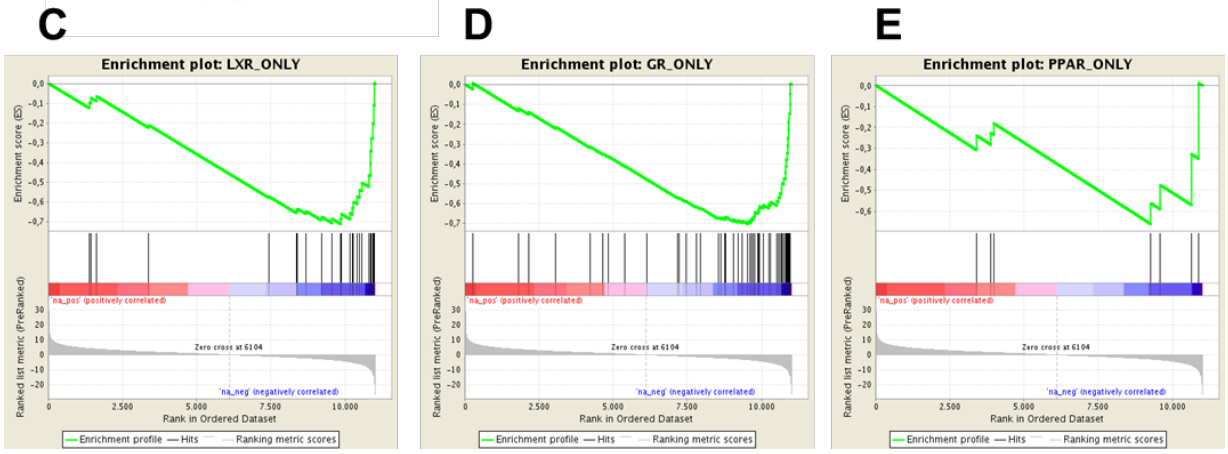


Figure S3, related to Fig. 3A-C and Fig. 6C. ***Dose-dependence and time course effect of DTPs on the response of macrophages to LPS.*** Peritoneal macrophages from LXRαβ$^{+/+}$ and LXRαβ$^{-/-}$ mice were pretreated with the indicated concentrations of DTPs and then challenged with LPS (250 ng/ml). The accumulation of NOx in the culture medium was determined at 18h (A). The time-course expression of NOS-2 and COX-2 was determined by immunoblot in LXRαβ$^{-/-}$ macrophages pretreated with 10 μM DTP1 and activated with 250 ng/ml of LPS (B). The effect of Akt inhibition (Akt inhibitor II, 10 μM) on the protein levels of NOS-2, COX-2, CXCL-1 and CXCL-10, was analyzed at 18h in macrophages from LXRαβ$^{-/-}$ mice treated with 10 μM DTP5 and 250 ng/ml LPS (C). Results show a representative experiment and the quantification of the bands *vs.* the LPS condition.

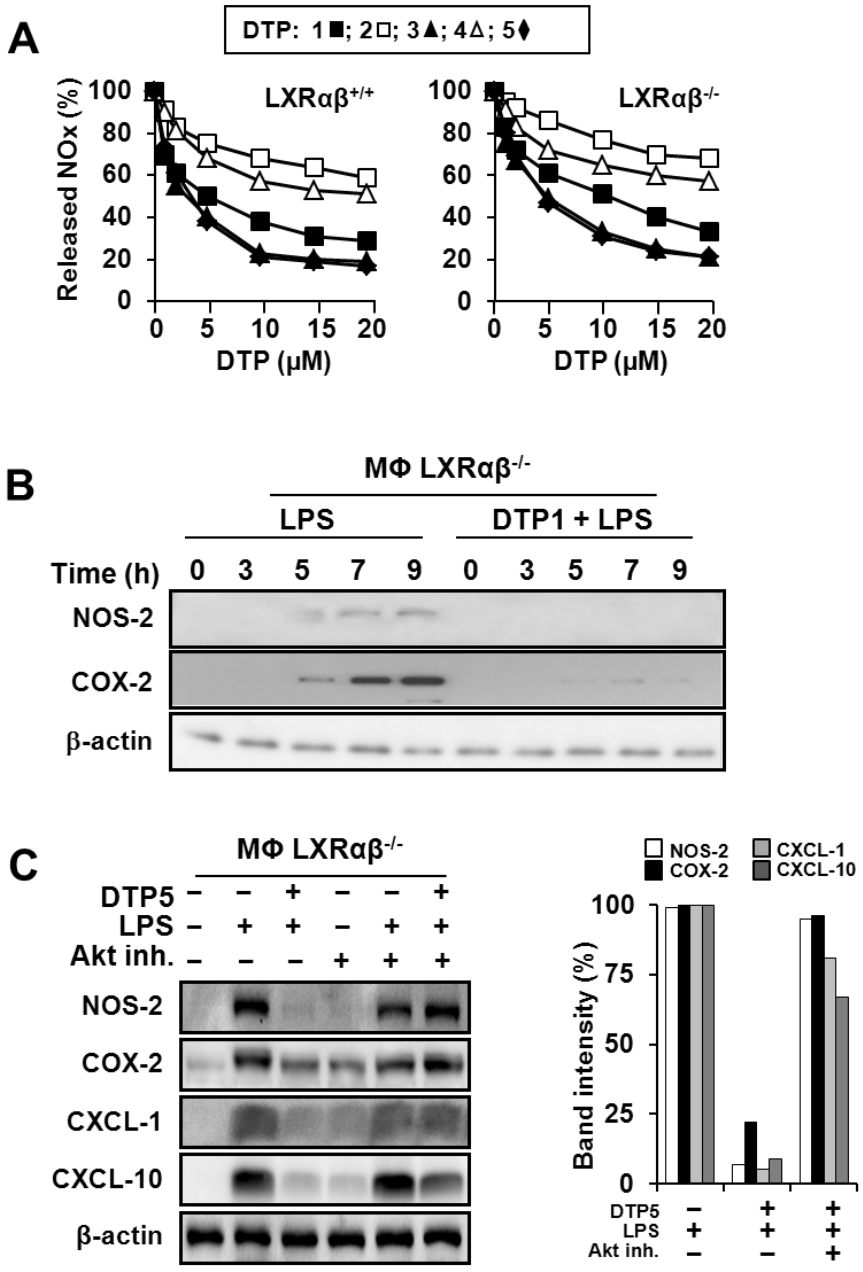
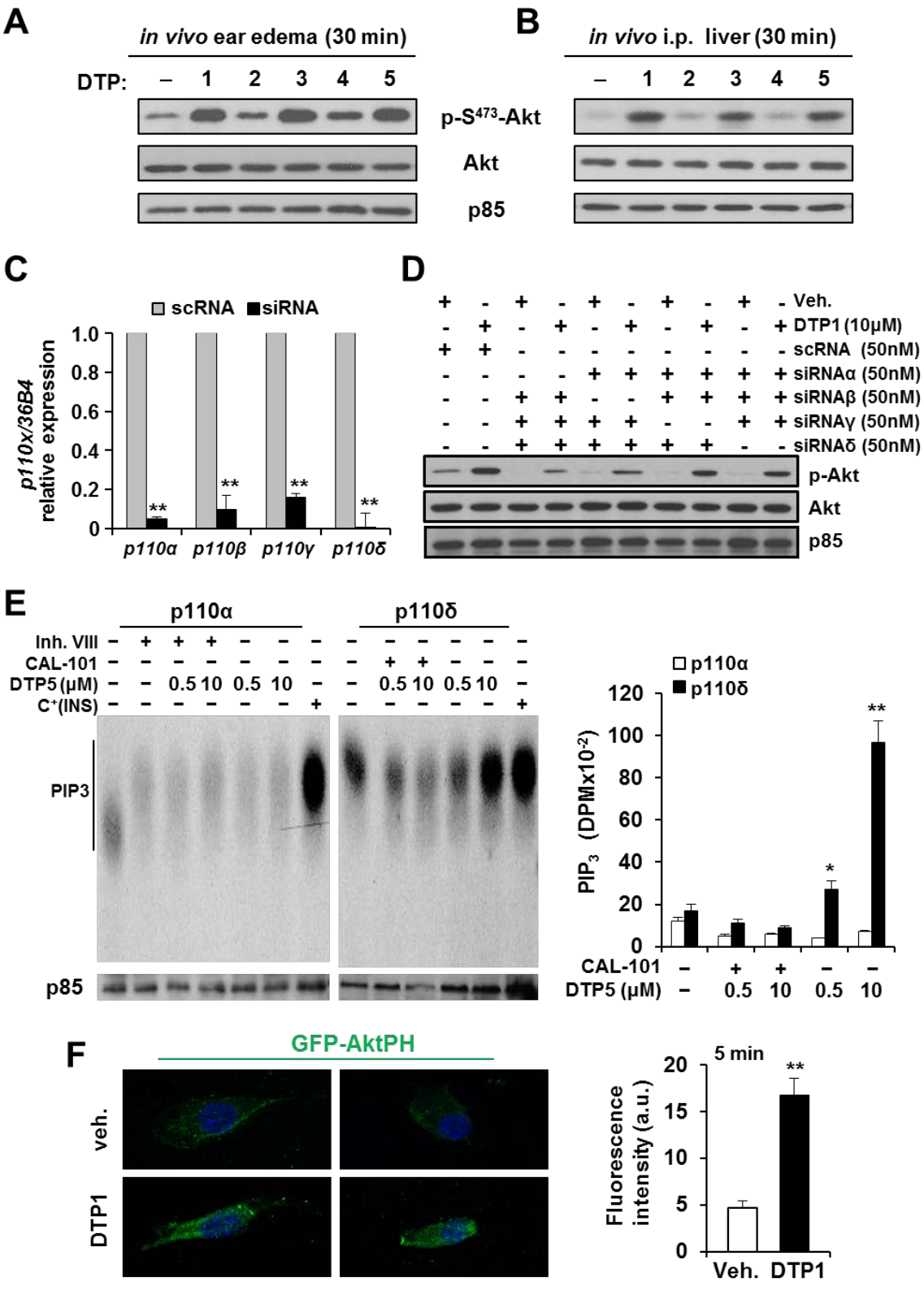


Figure S4, related to Fig. 6A (panels A-B) and Fig. 7 (panels C-F). ***DTP1 and DTP5 promote PI3K-dependent Akt phosphorylation in vivo and in macrophages ex vivo through activation of p110γ and p110δ.*** DTP1, DTP3 and DTP5, but not DTP2 or DTP4 promote the phosphorylation of Akt in a TPA-induced ear edema in mice (30 min of treatment) in similar experiments to that described in Fig. 2A (A). *In vivo* phosphorylation of Akt in the liver was analyzed by Western blot after i.p. administration (30 mg/kg body weight; 30 min) of DTPs (B). Peritoneal macrophages were transfected with specific p110 siRNAs for 48h. The efficiency of the gene knockdown was evaluated by qPCR. The graph represents the relative mRNA amount of each catalytic subunit compared to the corresponding scRNA treated cells (C). Cells were transfected with the indicated specific p110 siRNAs (as in C) and then treated in the presence or absence of DTP1. The protein levels of Akt phosphorylation were determined at 30 min (D). The activity of *in vitro* transcribed and translated *p110α* and *p110δ* was determined after immunoprecipitation (IP) of p85 (included in the translation reaction) as in Fig 6E, using PIP_2 and [γ-^{32}P]-ATP as substrate (10 min reaction time) in the presence of selective inhibitors as described in Fig. 7. Macrophages treated with insulin (5 min) were used as positive control. The TLC and the corresponding radioactivity measurement are shown (E). NIH3T3 cells were transfected with a GFP-tagged-Akt pleckstrin homology domain (*GFP-AktPH*) vector that was used as a molecular sensor of PI3K activation as previously described (Toettcher et al., 2011). The cells were treated for 5 min with 10 μM DTP1 and the distribution of the GFP fluorescence was quantified by confocal microscopy (F). Results show a representative blot or image (A, B, D, E, F) or the mean±SD of three experiments. *P<0.05; **P<0.01 *vs.* the corresponding scRNA (C), and basal/vehicle condition without DTP (E, F). The content of p85 in the IP was determined by Western blot (E).


A
in vivo ear edema (30 min)
DTP: – 1 2 3 4 5
p-S473-Akt
Akt
p85
B
in vivo i.p. liver (30 min)
– 1 2 3 4 5
C
scRNA siRNA
p110x/36B4 relative expression
1.0
0.8
0.6
0.4
0.2
0
**
p110α p110β p110γ p110δ
D
Veh.
DTP1 (10μM)
scRNA (50nM)
siRNAα (50nM)
siRNAβ (50nM)
siRNAγ (50nM)
siRNAδ (50nM)
p-Akt
Akt
p85
E
p110α
p110δ
Inh. VIII
CAL-101
DTP5 (μM) – – 0.5 10 0.5 10 – – 0.5 10 0.5 10 –
C+(INS)
PIP3
p85
p110α
p110δ
PIP3 (DPMx10-2)
120
100
80
60
40
20
0
*
**
CAL-101 – + + – –
DTP5 (μM) – 0.5 10 0.5 10
F
GFP-AktPH
veh.
DTP1
5 min
Fluorescence intensity (a.u.)
20
15
10
5
0
**
Veh. DTP1

Supplemental Experimental Procedures

Peritoneal macrophage isolation. Animals were used aged 8-12 weeks as follows: Four days prior to the assay, mice were intraperitoneally (i.p.) injected 2.5 ml of thioglycollate broth. Elicited peritoneal macrophages were prepared from mice killed by cervical dislocation (4-8 animals per condition), as previously described (Rodriguez-Prados et al., 2010). Cells were seeded at $2.5\times10^6/cm^2$ in RPMI 1640 medium supplemented with 10% of FCS and antibiotics. Then, the medium was changed to RPMI 1640 with 2% FCS and cells were stimulated for the indicated periods of time.

Quantitative PCR (qPCR). 1 µg of total RNA, extracted with TRI Reagent® (Ambion, Life Technologies) according to the manufacturer's instructions, was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit for RT-PCR following the indications of the manufacturer (Roche). qRT-PCR was conducted with SYBR® Green (Roche) on a MyiQ Real-Time PCR System (Bio-Rad). PCR thermocycling parameters were 95°C for 10 min, 40 cycles of 95°C for 15s, and 60°C for 1 min. Each sample was run in duplicate and normalized to *36B4* (house-keeping gene). For specific p110 isoforms, we used TaqMan real-time PCR (Life Technologies) combining cDNA with FastStart Universal Probe Master (Rox) (Roche). The replicates were then averaged, and fold induction (FI) or relative quantity was determined in a ΔΔCt based fold-change calculations. Primer sequences are available on request.

Characterization of proteins by immunoblot. Protein extracts were boiled in loading buffer (250 mM Tris-HCl; pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol) and size-separated by 10-15% SDS-PAGE gels. The gels were blotted onto a Hybond-P membrane (GE) and incubated with anti-NOS-2, anti-COX-2, anti-IκBα, anti-IκBβ and anti-phospho-Akt (Thr308) (Santa Cruz Biotech.), anti-phospho-IKKαβ, anti-IKKβ, anti-phospho-Akt (Ser473) and anti-Akt (Cell Signaling), anti-CXCL-1 (R&D Systems), anti-CXCL-10 (PeproTech), anti-p85 (Millipore) and anti-β-actin (Sigma). The levels of phosphorylated and total p38, JNK and ERKs p44/p42 were determined by Western blot using total extracts and specific commercial Abs (Cell Signaling). In experiments using anti-phospho-S32-IκBα (Cell Signaling), the blot incubation solution contained 100 ng/ml purified-IκBα (Ser32) (Santa Cruz Biotech.). The blots were submitted to sequential reprobing with Abs after treatment with 100 mM β-mercaptoethanol and 2% SDS in Tris-buffered saline and heated at 60ºC for 30 min. The blots were revealed by ECL Advance (GE). Different exposure times of the blots were acquired with a Charged Coupling Device camera in a luminescent image analyzer (Molecular Imager, Bio-Rad) ensuring that bands were not saturated. Quantification of the images was performed by using Quantity One software (Bio-Rad).

EMSAs. The sequence 5'-TGCTAGGGGGATTTTCCCTCTCTCTGT-3', corresponding to the consensus NF-κB binding site (nucleotides -978 to -952) of the murine *NOS-2* promoter (Castrillo et al., 2001a) was used. Oligonucleotides were annealed with their complementary sequence by incubation for 5 min at 85°C in 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT. Aliquots of 50 ng of these annealed oligonucleotides were end-labeled with Klenow enzyme fragment

in the presence of 50 µCi of [α-^{32}P]dCTP and the other unlabeled dNTPs in a final volume of 50 µl. A total of 5×10^4 dpm of the DNA probe was used for each binding assay of nuclear extracts as follows. 3 µg of nuclear protein were incubated for 15 min at 4°C with the DNA and 2 µg of polyI:C, 5% glycerol, 1 mM EDTA, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 10 mM Tris-HCl (pH 7.8) in a final volume of 20 µl. The DNA-protein complexes were separated on native 6% polyacrylamide gels in 0.5% Tris-borate-EDTA buffer (Castrillo et al., 2001a). Supershift assays were carried out after incubation of the nuclear extracts with 2 µg of Abs (anti-p50, anti-c-Rel, anti-p65) (Santa Cruz Biotech.) for 1h at 4°C, followed by EMSAs (not shown).

Measurement of IKKβ activity. Cells (10^7) were homogenized in cytosolic buffer and centrifuged at 13,000***g*** for 15 min. The supernatant (1 ml) was pre-cleared, and IKKβ was immunoprecipitated with 1 µg of anti-IKKβ (Castrillo et al., 2001b). After extensive washing of the immunoprecipitate with cytosolic buffer, the pellet was resuspended in kinase buffer (modified cytosolic buffer containing 0.1 mM EDTA, 5 mM $MgCl_2$ and 10 nM okadaic acid). Kinase activity was assayed in 100 µl of kinase buffer containing 100 ng of immunoprecipitate, 1 mM ATP and 100 ng of purified IκBα(Ser32) as substrate. Aliquots of the reaction mixture were stopped at various times in 1 ml of ice-cold cytosolic buffer supplemented with 5 mM EDTA, followed by Western blotting using anti-phospho-S32-IκBα Ab. The linearity of the kinase reaction was confirmed over a period of 30 min. *In vitro* IKKβ activity was measured by homogeneous time-resolved fluorescence (HTRF) assay, using cloned and expressed IKKβ and biotinylated-IκBα (aminoacids 28 to 40) as substrate. Fluorescence (excitation at 330 nm and emission at 615 and 665 nm) was recorded after addition of europium cryptate phospho-Ab recognizing the S32/S36 phosphorylation peptide and streptavidin-XL665. Inhibition of IKKβ by staurosporine was used as control.

In vivo evaluation of Akt phosphorylation by DTPs in liver. Mice were i.p. injected with the indicated DTPs at 30 mg/kg in 0.5 ml of Solutol HS15 (BASF, Ludwigshafen, Germany), and after 30 min samples of liver were collected to determine the phosphorylation status of Akt.

In vivo administration of DTP and ex vivo whole blood assay. C57BL/6 mice were starved overnight and administrated orally with 25 mg/kg of DTP1 and DTP5 prepared in 40% Solutol HS15. 90 min later, whole blood was collected into heparinized tubes through cardiac puncture, pooled (n=4) and mixed 1:3 with pre-warmed complete RPMI 1640 medium. 0.9 ml/well of the diluted whole blood was dispensed into 24-well tissue culture plate and stimulated with LPS (10 and 100 ng/ml) diluted in RPMI-1640 basal medium. The 24-well plate was then incubated for 4h with occasional mixing. Finally, samples were transferred to 1.5 ml tubes and centrifuged at 14,000***g*** for 30s at RT. Supernatants were assayed for TNF-α levels by the Cytoset ELISA kits.

Supplemental References

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