Supplementary Information

TRPM2 links oxidative stress to the NLRP3 inflammasome activation

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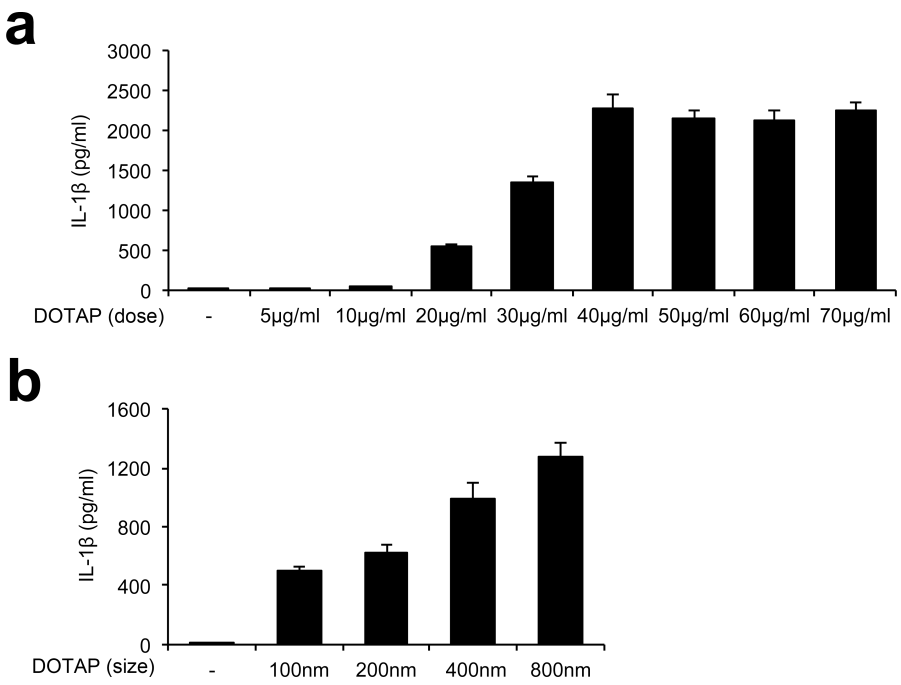
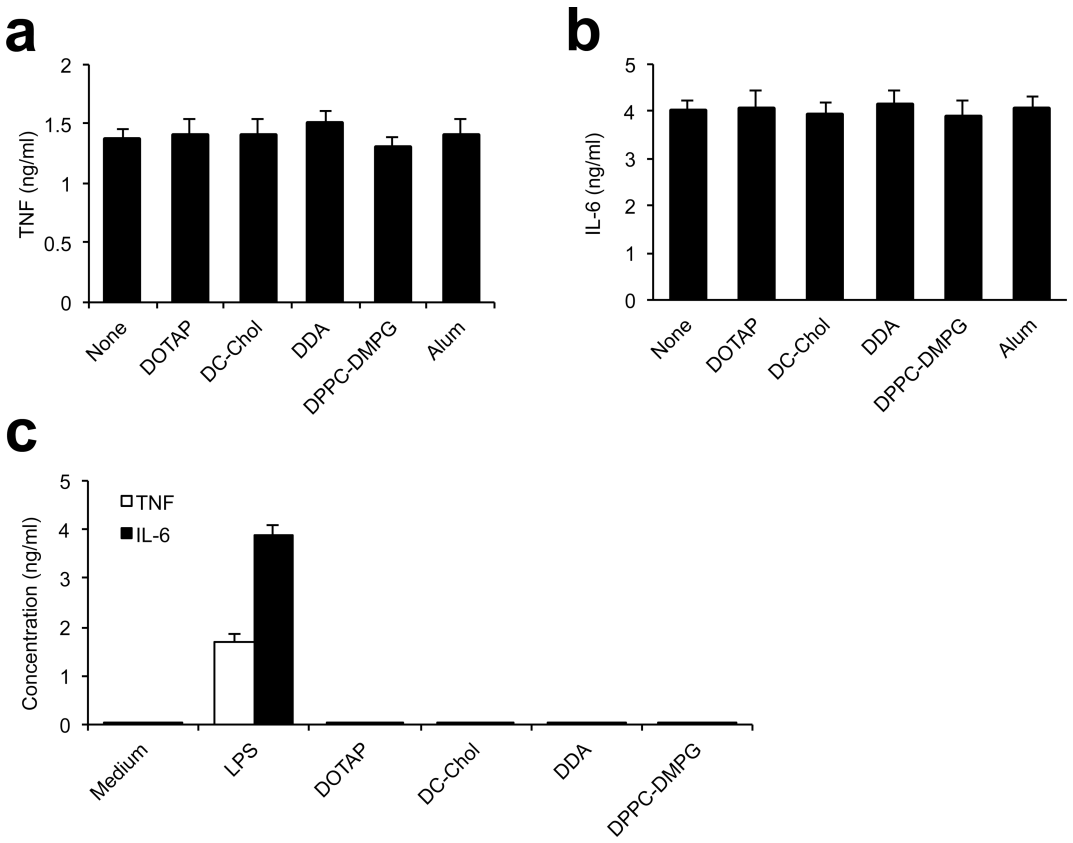
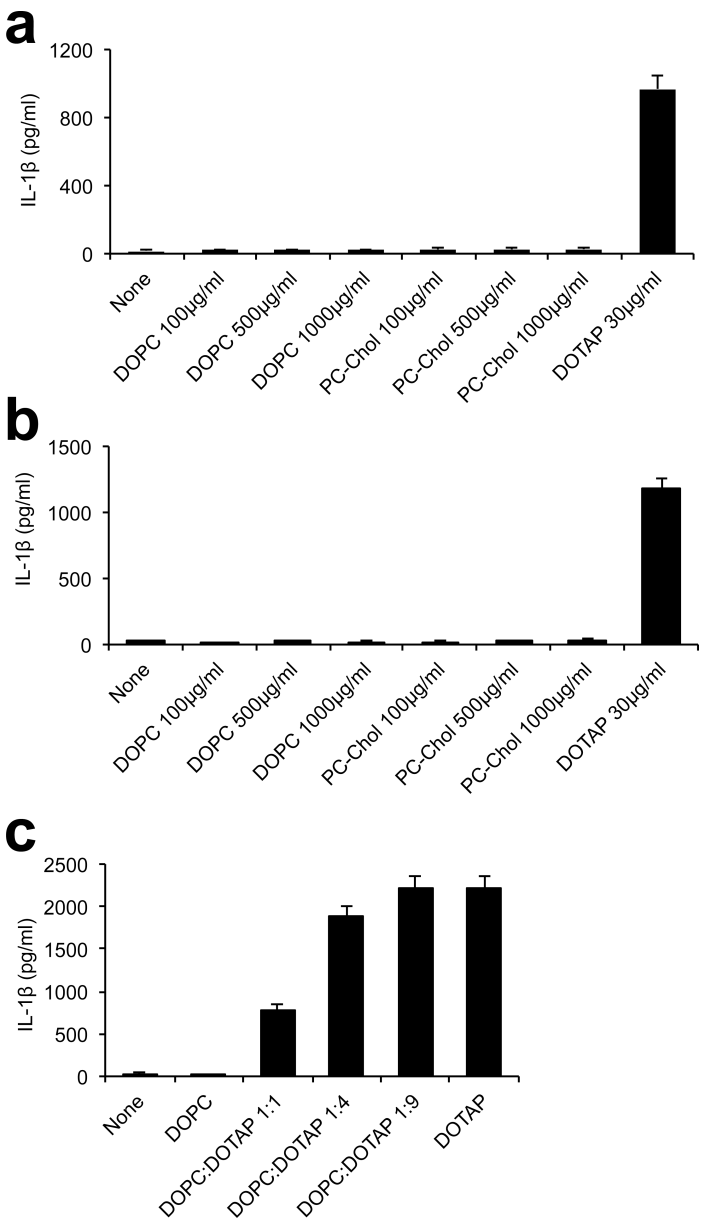


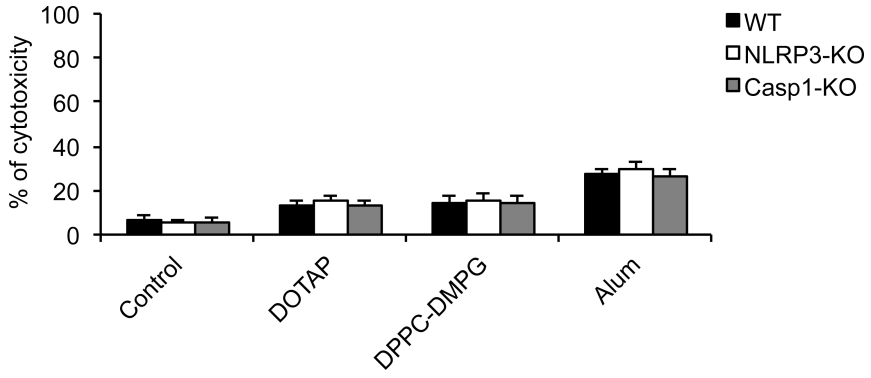
Supplementary Figure S1: Charged liposomes induce IL-1β secretion in a dose and size dependent manner. ELISA for IL-1β from supernatants of PMA-primed THP1 cells that were stimulated with DOTAP liposomes of different doses (**a**) or sizes (**b**, 100 nm, 200 nm, 400 nm or 800 nm in diameter, respectively) for 16 h. Data are shown as mean ± s.d., and are representative of three independent experiments.



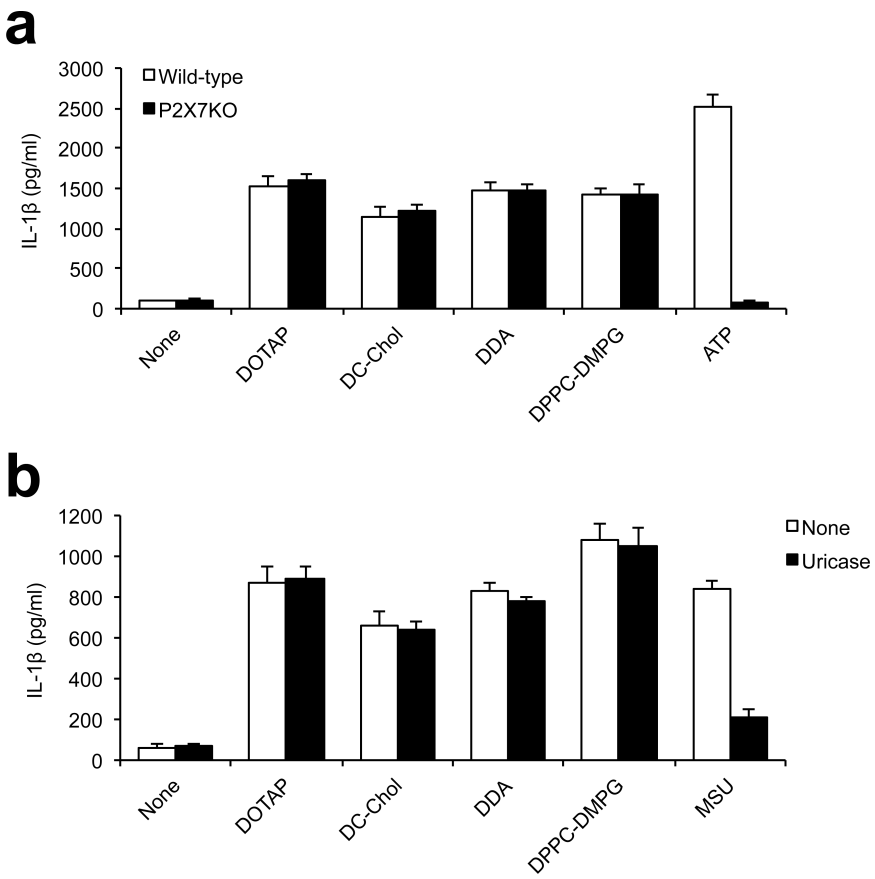
Supplementary Figure S2: Charged liposomes alone do not induce secretions of TNF and IL-6. ELISA for TNF (**a**) or IL-6 (**b**) from supernatants of LPS-primed wild-type mouse BMDMs that were stimulated with indicated liposomes. (**c**) TNF and IL-6 from supernatants of wild-type unprimed BMDMs treated with LPS (100 ng/ml) or indicated liposomes alone (30 μg/ml). Data are shown as mean ± s.d., and are representative of three independent experiments.



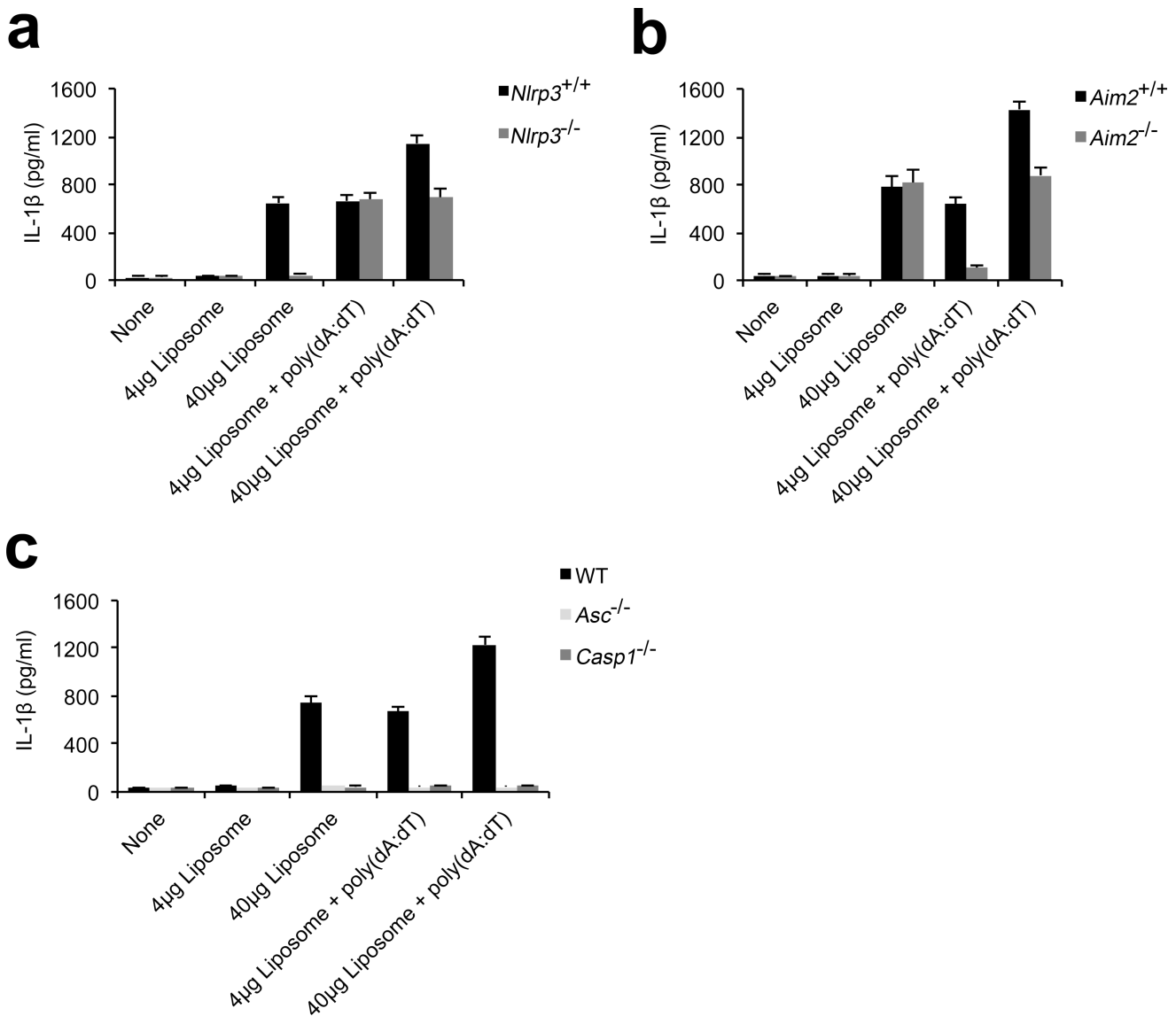
Supplementary Figure S3: Neutral liposomes fail to induce IL-1β secretion in macrophages. ELISA for IL-1β from supernatants of LPS-primed wild-type BMDMs (**a**) or PMA-primed THP1 cells (**b**) that were stimulated with indicated neutral liposomes for 24 h. (**c**) ELISA for IL-1β from supernatants of PMA-primed THP1 cells that were stimulated with pure DOPC or DOTAP liposomes, or chimeric liposomes (50 µg/ml) made with various molar ratio of DOPC versus DOTAP. Data are shown as mean ± s.d., and are representative of at least three independent experiments.



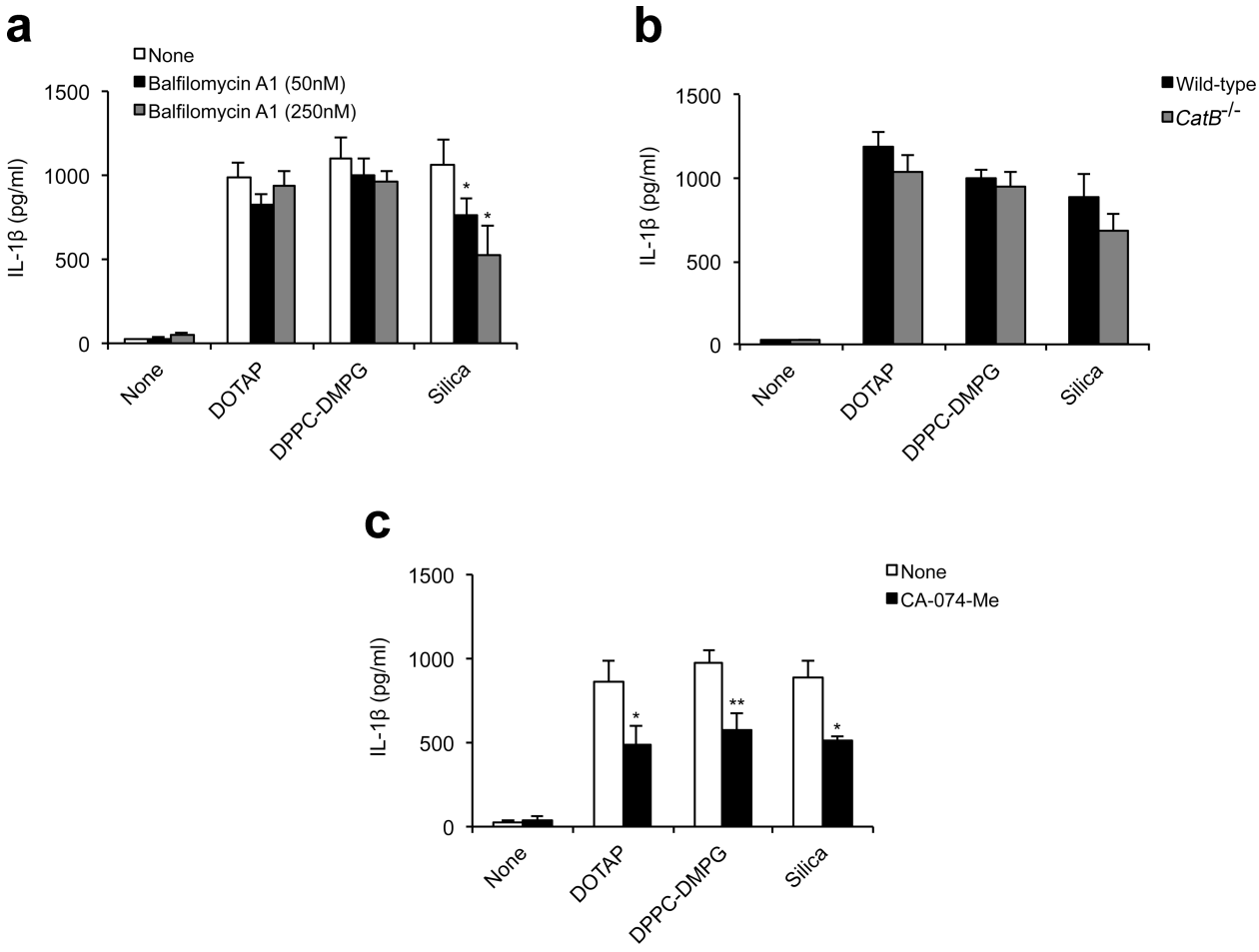
Supplementary Figure S4: Macrophage viabilities after treatment with charged liposomes or alum. The levels of released Lactate dehydrogenase (LDH) were measured from the supernatants of LPS-primed immortalized wild-type, *Nlrp3*$^{-/-}$ and *Caspase 1*$^{-/-}$ macrophages that were stimulated with the DOTAP or DPPC-DMPG liposomes (70 µg/ml) or alum (500 µg/ml) for 6 h. Measurements were determined in triplicate and are shown as mean ± s.d., and data are from one of three independent experiments.



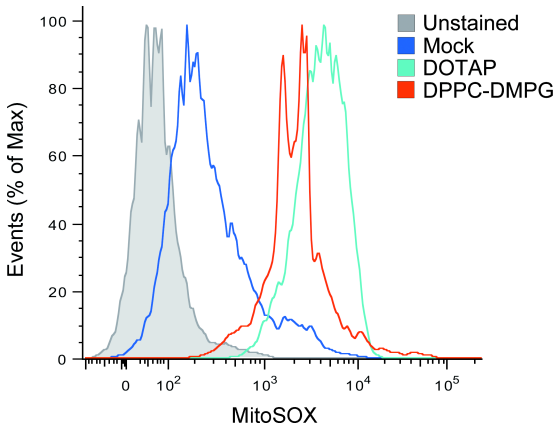
Supplementary Figure S5: Charged liposomes induce IL-1β release independent of ATP and uric acid crystals. ELISA for IL-1β from supernatants of (**a**) LPS-primed wild-type or *P2X*$_7^{-/-}$ BMDMs that were stimulated with ATP (2 mM, 30 min) or indicated liposomes (30 µg/ml, 6 h), or (**b**) LPS-primed wild-type BMDMs that were stimulated with monosodium urate crystals (MSU) or indicated liposomes for 6h in the presence uricase (8 U/ml). Data are shown as mean ± s.d., and are representative of three independent experiments.



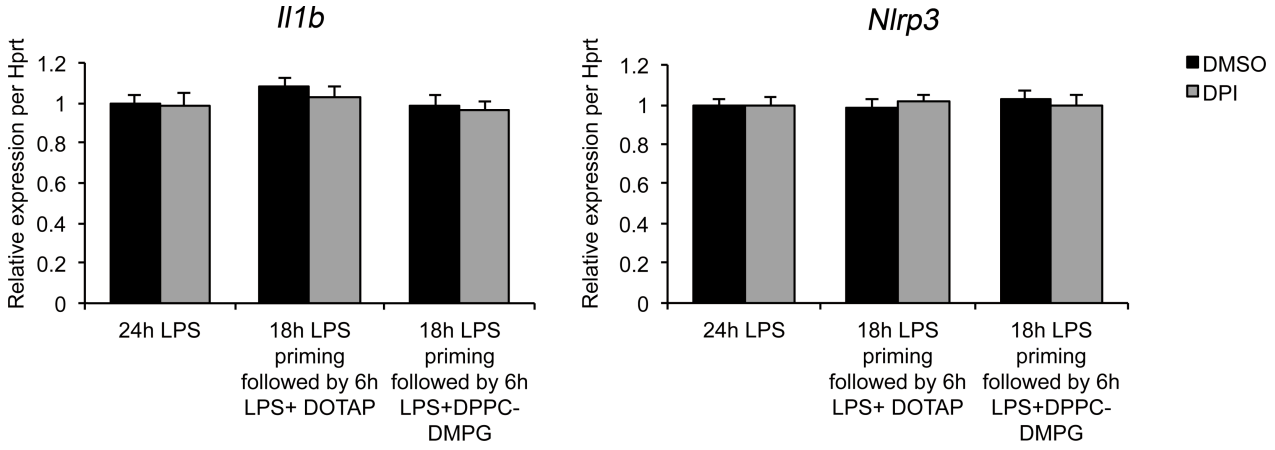
Supplementary Figure S6: Charged liposomes activate the NLRP3, but not AIM2, inflammasome. (**a**-**c**) ELISA for IL-1β from supernatants of LPS-primed immortalized murine macrophages from wild-type, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, *Caspase 1*$^{-/-}$ or *Aim2*$^{-/-}$ mice that were stimulated with indicated amount of empty cationic liposomes (Lipofectamine 2000) or poly(dA:dT)-loaded liposomes for 6 h. Data are shown as mean ± s.d., and are representative of three independent experiments.



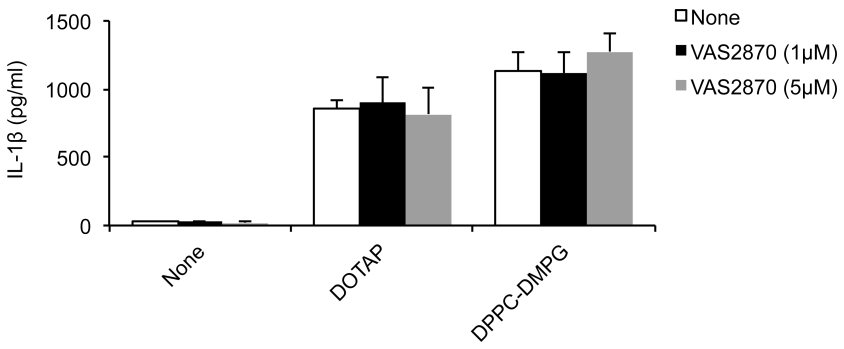
Supplementary Figure S7: Lysosomal cathepsin-B is dispensable for liposome-induced NLRP3 inflammasome activation. (**a, c**) ELISA for IL-1β from supernatants of LPS-primed wild-type BMDMs that were pretreated with balfilomycin A1 (50 nM and 250 nM in **a**) or CA-074-Me (10 μM in **c**) followed by stimulations with DOTAP or DPPC-DMPG liposomes (30 μg/ml) or silica (500 μg/ml). (**b**) ELISA for IL-1β from supernatants of LPS-primed immortalized murine wild-type or cathepsin B deficient (*CatB*$^{-/-}$) macrophages that were stimulated with DOTAP or DPPC-DMPG liposomes (70μg/ml). Data are shown as mean ± s.d., and all data are representative of at least three independent experiments. *, $p<0.05$, and **, $p<0.01$ versus controls. Statistical significance was determined by the standard two-tailed Student's *t-test*.



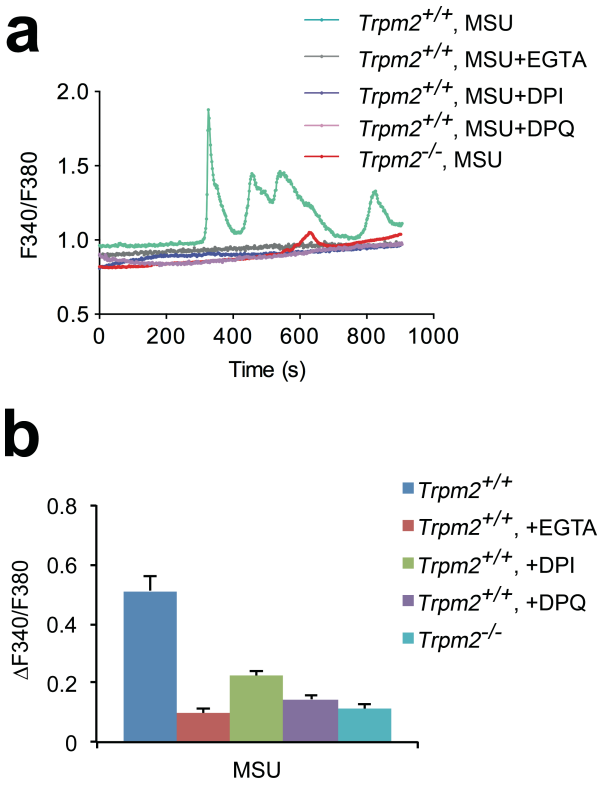
Supplementary Figure S8: Induction of mitochondrial ROS by charged liposomes without LPS priming. Mitochondrial ROS was measured by flow cytometry in unprimed BMDMs that were stimulated with indicated liposomes (30 µg/ml) and labeled with MitoSOX. Data are representative of three independent experiments.



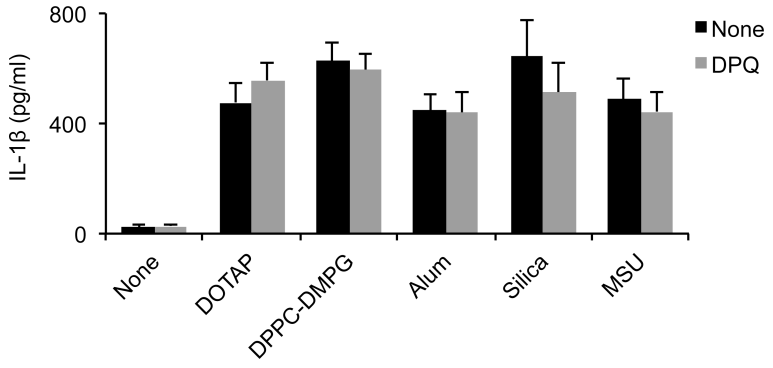
Supplementary Figure S9: DPI treatment does not affect *Il1b* and *Nlrp3* mRNAs after prolonged LPS priming.
The mRNA levels of *Il1b* (left) and *Nlrp3* (right) were quantified by real-time RT-PCR in wild-type BMDMs that were stimulated as indicated. DPI (25 μM) was added after 18 h LPS priming but 45min before addition of liposomes. Gene expression data are presented as relative expression to HPRT1, and the gene relative expression level in 24 h LPS treated wild-type macrophages (DMSO group) were designated as 1. Data are representative of three independent experiments.



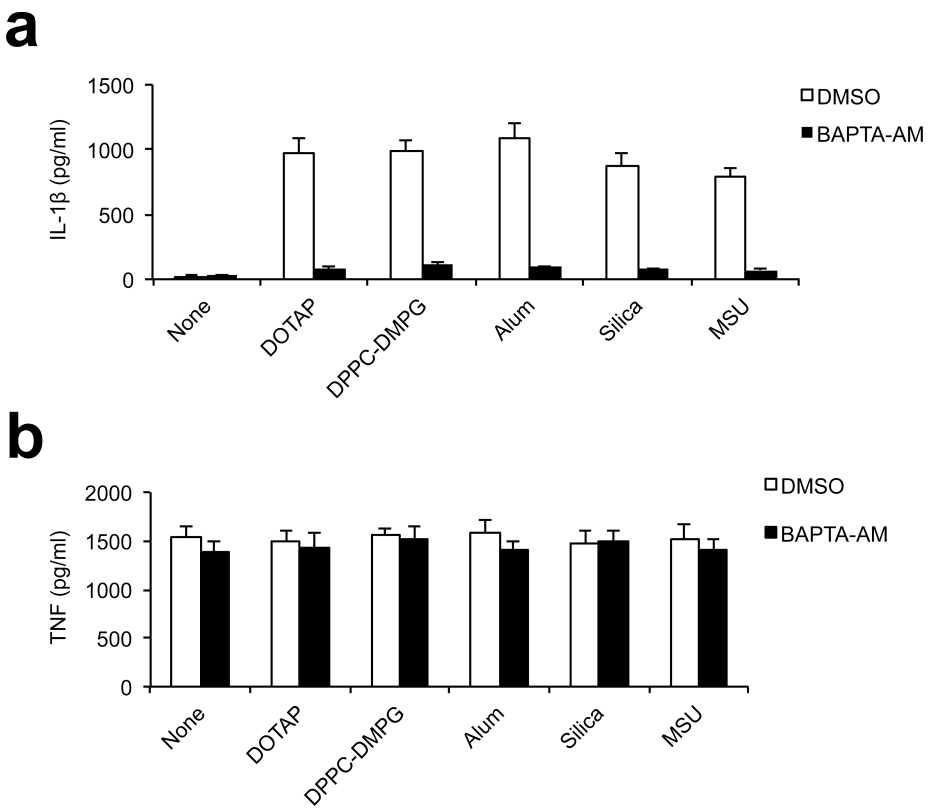
Supplementary Figure S10: Inhibition of NADPH oxidase does not affect liposome-induced IL-1β secretion. The levels of IL-1β were measured by ELISA from supernatants of LPS-primed wild-type BMDMs that were pretreated with VAS2870 for 30 min followed by stimulations with DOTAP or DPPC-DMPG liposomes (30 μg/ml) for 6 h. Data are shown as mean ± s.d., and are representative of three independent experiments.



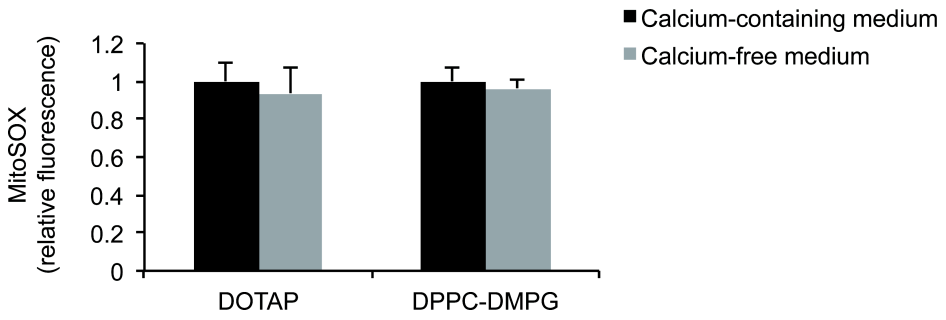
Supplementary Figure S11: MSU crystals induce a TRPM2-mediated Ca^{2+} influx in macrophages. (**a**) Time-dependent change of $[Ca^{2+}]_i$, represented by F340/F380, was shown in LPS-primed *Trpm2*$^{+/+}$ BMDMs cultured in calcium-containing solution that were pretreated with DPI (25 μM) or DPQ (200 μM) for 45min for before the addition of MSU crystals (250 μg/ml). The same doses of MSU crystals were used to stimulate the LPS-primed *Trpm2*$^{-/-}$ BMDMs cultured in calcium-containing solution. The $[Ca^{2+}]_i$ over time after additions of these stimuli into LPS-primed *Trpm2*$^{+/+}$ BMDMs cultured in calcium-free 0.5 mM EGTA-containing solution are also shown. MSU crystals were added 1 min after the starting of calcium recording. (**b**) The maximum $[Ca^{2+}]_i$ elevations, represented by ΔF340/F380, were shown in LPS-primed BMDMs in response to the stimulations described in **a**. Data are representative of two independent experiments and are shown as mean ± s.e.m. in **b** (n=21-38).



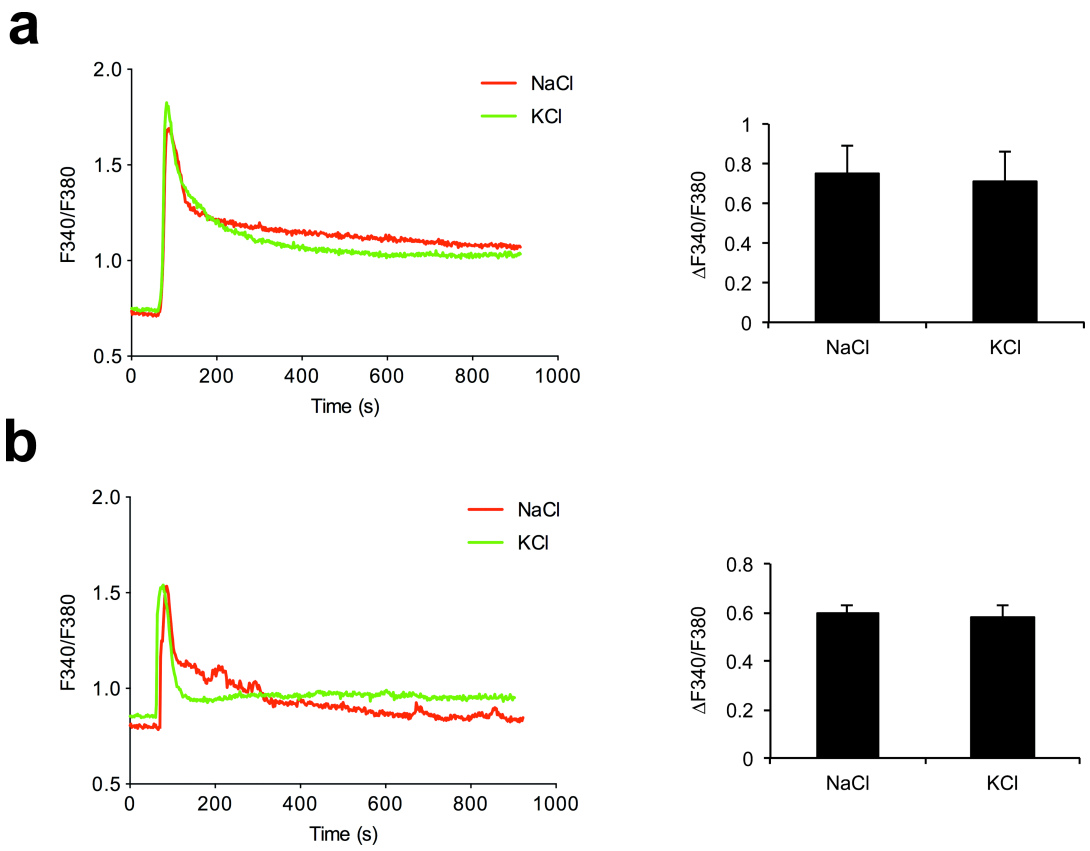
Supplementary Figure S12: DPQ does not further reduce particles-induced IL-1β release in *Trpm2*$^{-/-}$ BMDMs. ELISA for IL-1β from supernatants of LPS-primed *Trpm2*$^{-/-}$ BMDMs that were pretreated with DPQ (200 μM) followed by stimulations with indicated liposomes or crystals. Data are shown as mean ± s.d., and are representative of three independent experiments.



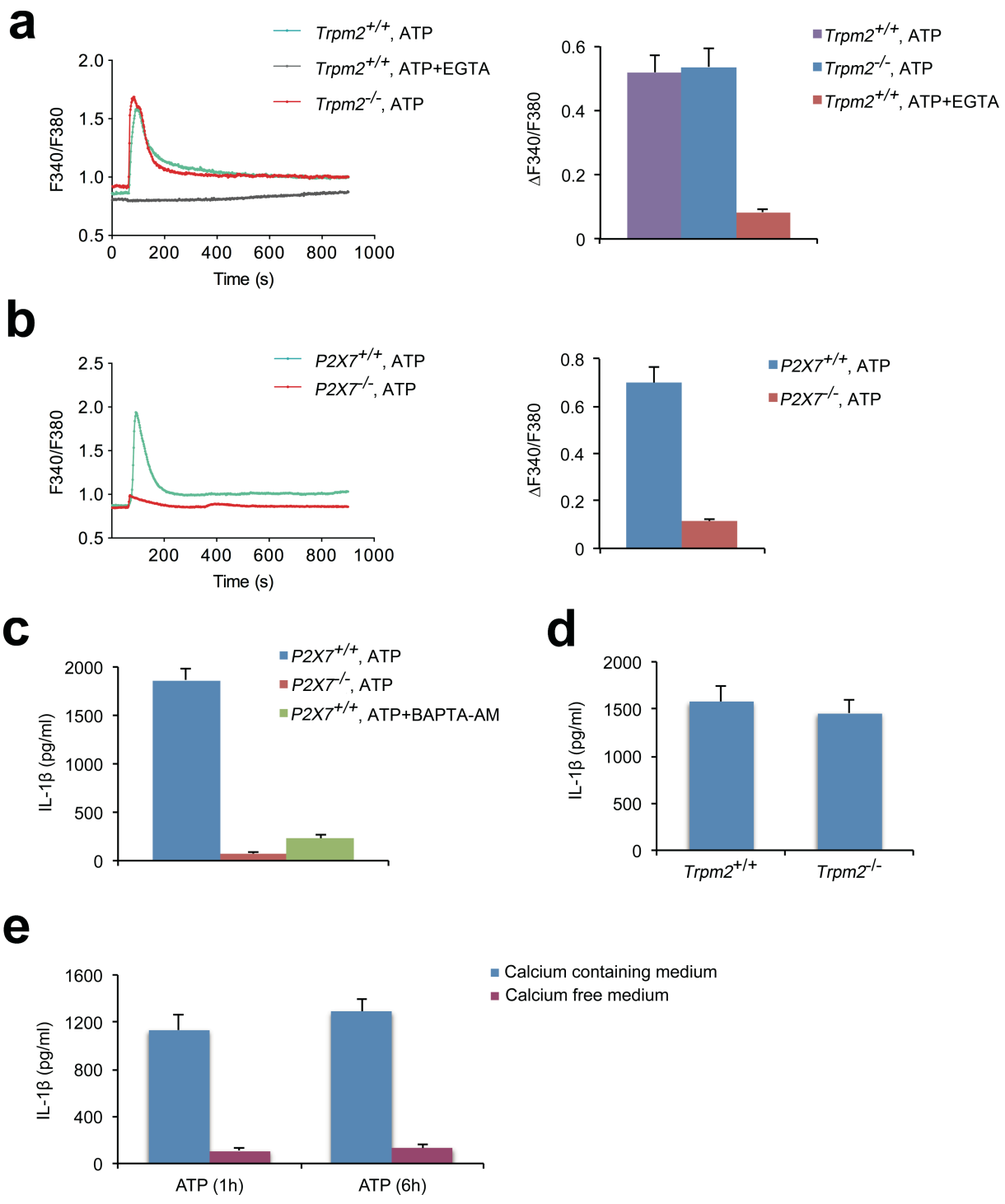
Supplementary Figure S13: Blocking $[Ca^{2+}]_i$ elevation abolishes the NLRP3 inflammasome activation by particulates. ELISA for IL-1β (**a**) or TNF (**b**) from supernatants of LPS-primed wild-type BMDMs that were pretreated with BAPTA-AM (25 μM) followed by stimulations with liposomes or crystals. Data are shown as mean ± s.d., and are representative of three independent experiments.



Supplementary Figure S14: Removal of extracellular calcium does not impair mitochondrial ROS production. LPS-primed wild-type BMDMs were treated with indicated liposomes (30 µg/ml) for 6 h in the presence of calcium containing or calcium free medium. Then the cells were stained with MitoSOX and the levels mitochondrial ROS were measured by flow cytometry. Data are representative for two independent experiments and are normalized to untreated controls (n=3).

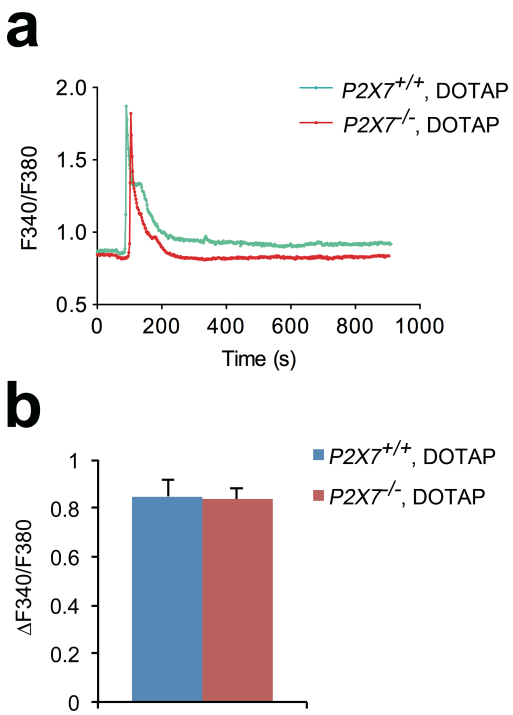


Supplementary Figure S15: Blockade of K^+ efflux does not affect Ca^{2+} influx induced by liposomes or ATP. Time-dependent change of $[Ca^{2+}]_i$, represented by F340/F380, was shown in LPS-primed wild-type BMDMs, cultured in calcium-containing solution supplemented with either 150 mM KCl or NaCl, followed by stimulation with DOTAP liposomes (**a**, left panel) or ATP (**b**, left panel). The maximum $[Ca^{2+}]_i$ elevations (right panels of **a** and **b**), represented by ΔF340/F380, were shown in LPS-primed BMDMs in response to the indicated stimulations. DOTAP liposomes or ATP were added 1 min after the starting of calcium recording. Data are representative of two independent experiments, and are shown as mean ± s.e.m. in right panels of **a** and **b** (n=24-31).

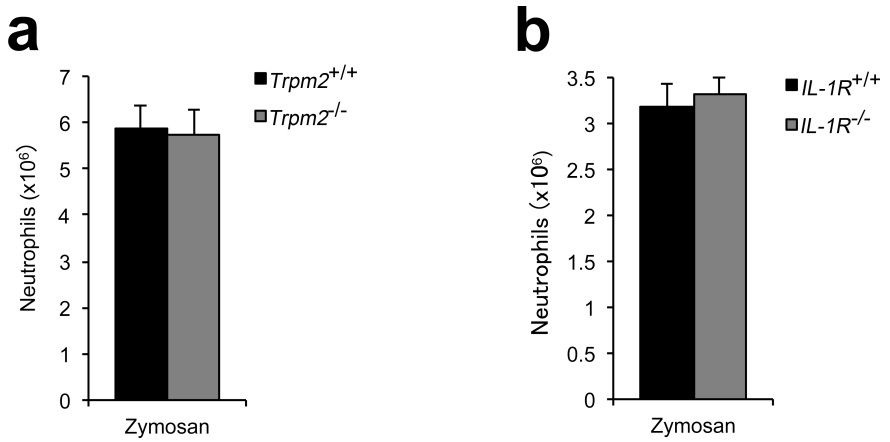


Supplementary Figure S16: ATP activates the NLRP3 inflammasome by inducing Ca^{2+} influx via $P2X_7$ channel. Time-dependent change of $[Ca^{2+}]_i$, represented by F340/F380, was shown in LPS-primed *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ BMDMs (**a,** left pannel) or *P2X*$_7^{+/+}$ and *P2X*$_7^{-/-}$ BMDMs (**b,** left pannel) cultured in Ca^{2+}-containing solution that were stimulated with ATP (2 mM). The calcium flux over time after ATP stimulation in LPS-primed *Trpm2*$^{+/+}$ BMDMs cultured in calcium-free 0.5 mM EGTA-containing solution was also shown. The maximum $[Ca^{2+}]_i$ elevations (right panels of **a** and **b**), represented by ΔF340/F380, were shown in in LPS-primed BMDMs in response to the ATP stimulation. ATP was added 1 min after the

starting of calcium recording. Data are representative of four independent experiments and are shown as mean ± s.e.m. in **a** (n=20-36) and **b** (n=22-28). (**c**) ELISA for IL-1β from supernatants of LPS-primed *P2X*$_7^{-/-}$ or wild-type (*P2X*$_7^{+/+}$) BMDMs that were pretreated with BAPTA-AM (25 μM) followed by stimulations with ATP. (**d**) IL-1β from supernatants of LPS-primed *Trpm2*$^{+/+}$ or *Trpm2*$^{-/-}$ BMDMs that were treated with ATP. (**e**) IL-1β from supernatants of LPS-primed wild-type BMDMs that were stimulated with ATP in calcium-containing or calcium-free medium. Data are shown as mean ± s.d. in **c**-**e**, and are representative of three independent experiments.



Supplementary Figure S17: Deficiency in $P2X_7$ does not affect liposome-induced $[Ca^{2+}]_i$ elevation. (**a**) Time-dependent change of $[Ca^{2+}]_i$, represented by F340/F380, was shown in LPS-primed $P2X_7^{+/+}$ and $P2X_7^{-/-}$ BMDMs cultured in calcium-containing solution that were stimulated with DOTAP liposomes (30 μg/ml). DOTAP liposomes were added 1 min after the starting of calcium recording. (**b**) The maximum $[Ca^{2+}]_i$ elevations, represented by ΔF340/F380, were shown in LPS-primed BMDMs in response to the stimulation as described in **a**. Data are representative of two independent experiments, and are shown as mean ± s.e.m. in **b** (n=25-36).



Supplementary Figure S18: Deficiency in TRPM2 or IL-1R does not affect zymosan-induced peritonitis. IL-1β concentrations (**a**) and neutrophils influx (**b**) were quantified in peritoneal lavage fluid from wild-type (*Il1rl*$^{+/+}$ or *Trpm2*$^{+/+}$), *Il1rl*$^{-/-}$ or *Trpm2*$^{-/-}$ mice 6h after intraperitoneal injection of PBS (0.5 ml) supplemented with zymosan (0.2 mg). Data are representative of two independent experiments (mean and s.e.m. of five mice per group).

Supplementary Table S1: Primers sequences for real-time PCR analysis.

Mouse gene	Forward	Reverse
Il1b	CTGCAGCTGGAGAGTGTGG	GGGGAACTCTGCAGACTCAA
Nlrp3	ATGGCTGTGTGGATCTTTGC	CACGTGTCATTCCACTCTGG
Hprt1	CTGGTGAAAAGGACCTCTCG	TGAAGTACTCATTATAGTCAAGGGCA

Supplementary Table S2: Liposome formulations

Lipid composition	Lipid Molar percentage	Size (nm)	Concentration (mg/ml)
DOTAP	100	100	10
DOTAP	100	200	10
DOTAP	100	400	10
DOTAP	100	800	10
DOPC	100	100	10
DOPC	100	200	10
DOPC	100	400	10
DOPC	100	800	10
DOTAP:DOPC	50:50	100	20
DOTAP:DOPC	80:20	100	10
DOTAP:DOPC	90:10	100	10
DPPC:DMPG	70:30	100	10
DC-Cholesterol	100	100	10
DDA	100	100	10
L-α-PC: Cholesterol	70:30	2000	23
DOTAP-OVA (OVA encapsulated)	100	400	10 mg/ml of lipid and 2 mg/ml of encapsulated ovalbumin