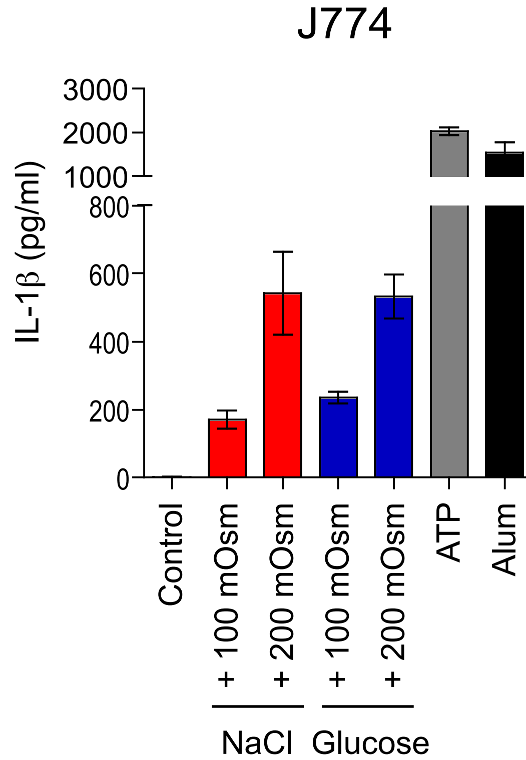
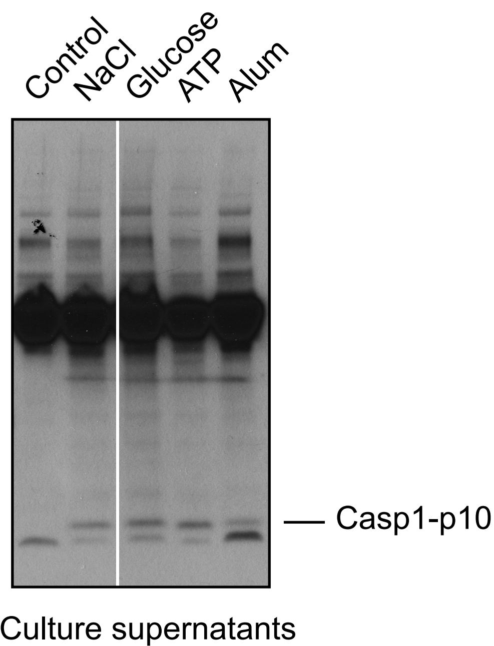
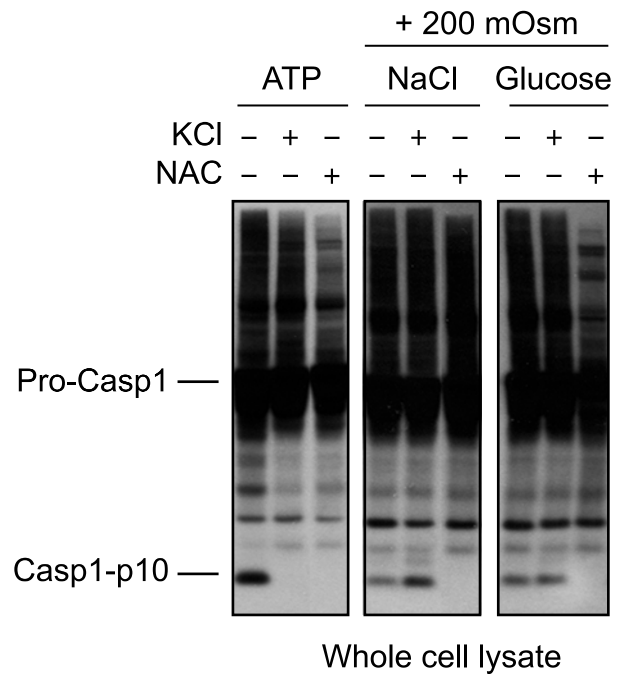


Supplementary Figure 1.

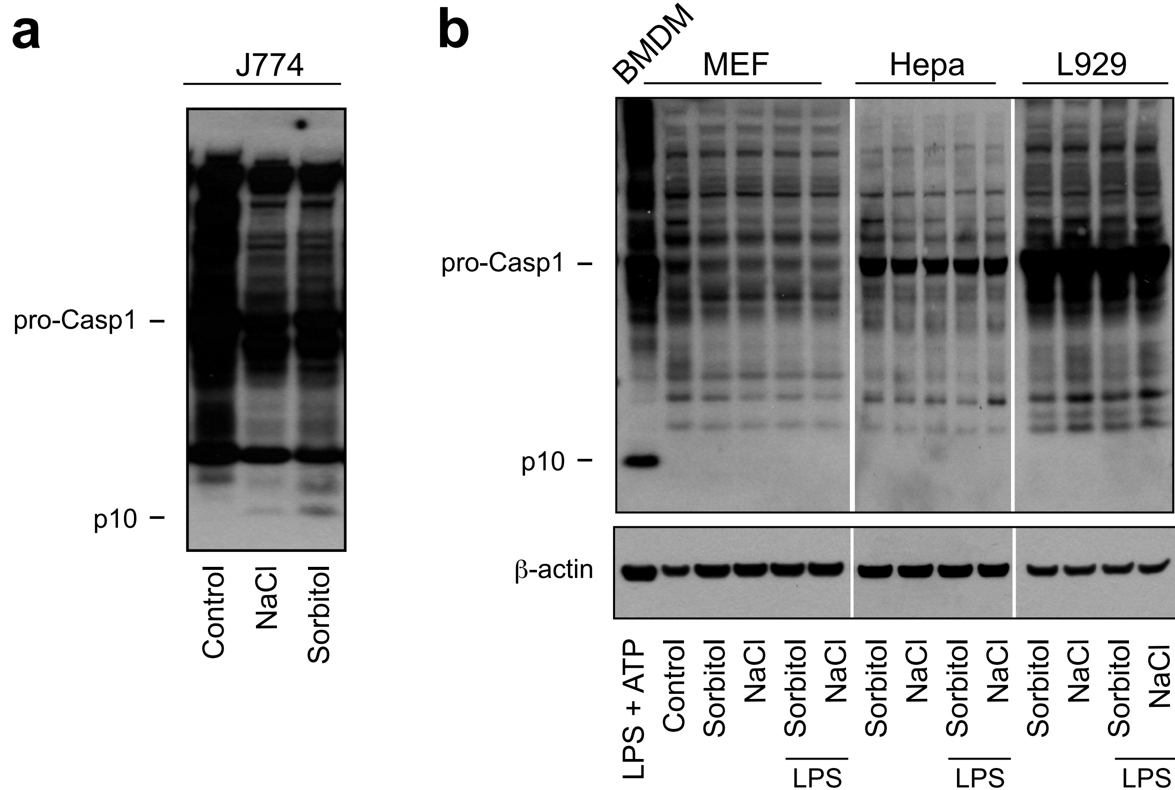
Hyperosmotic stress induces IL-1β secretion in macrophage cell lines. Macrophage cell line J774 were primed with LPS (100 ng/ml) for 3 h and incubated overnight in isotonic conditions (control) or hypertonic conditions as indicated by adding NaCl or glucose, or stimulated with ATP (5 mM) or alum (250 μg/ml) for 2 h or 6 h respectively. IL-1β in supernatants was measured by ELISA. Data indicate means ± s.d. of quadruplicates.





Supplementary Figure 2.

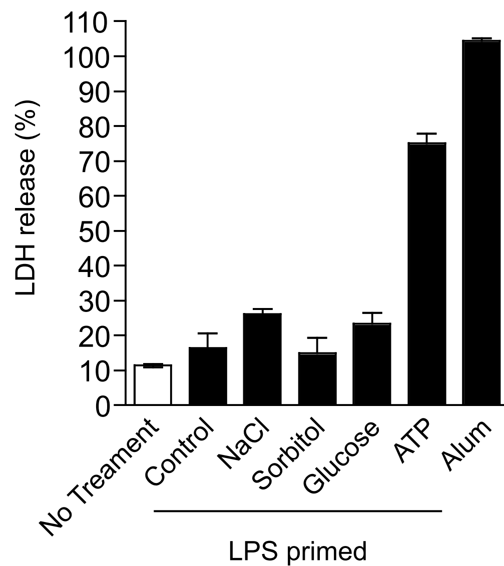
Caspase-1 activation by hyperosmotic stress in macrophages. (**a**,**b**) BMDMs primed with LPS were pretreated without (**a**) or with 10 mM N-acetyl-L-cysteine (NAC) for 30 min (**b**), and incubated in isotonic conditions (control) or hyperosmotic conditions (+ 200 mOsm) by adding NaCl or glucose for overnight (**a**) or 3 h (**b**), or stimulated with ATP (5 mM) for 2 h (**a**) or 30 min (**b**), or with alum (250 μg/ml) for 6 h (**a**), in the absence (**a**) or presence (**b**) of 100 mM extracellular KCl. The cleavage of caspase-1 to its active p10 subunit in BMDMs was detected by immunoblot analysis in media supernatants (**a**), see also Figure 3b, or cell lysates (**b**), see also Figure 5a.



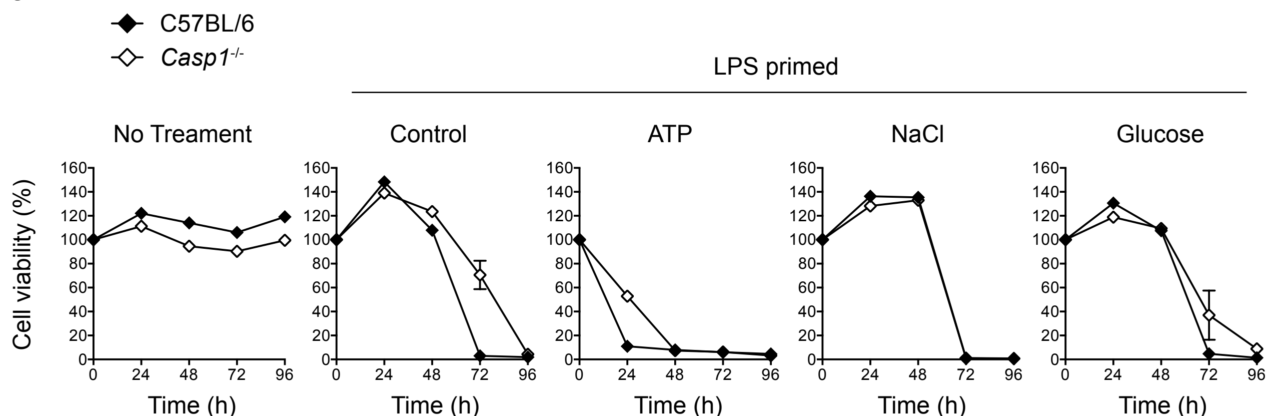
Supplementary Figure 3.

Caspase-1 activation upon hyperosmotic stress in macrophage and non-macrophage cell lines, and MEFs. (**a, b**) Macrophage cell line J774 cells (**a**), or non-macrophage cell line - Hepa cells or L929 cells, or MEFs (**b**) were primed with or without LPS (100 ng/ml) for 3 h and incubated for 4 h in isotonic conditions or hyperosmotic conditions (+ 200 mOsm) by adding NaCl or sorbitol. LPS-primed BMDMs stimulated with ATP for 30 min was used as a positive control (**b**). Cell lysates were analyzed by immunoblotting for caspase-1.

a

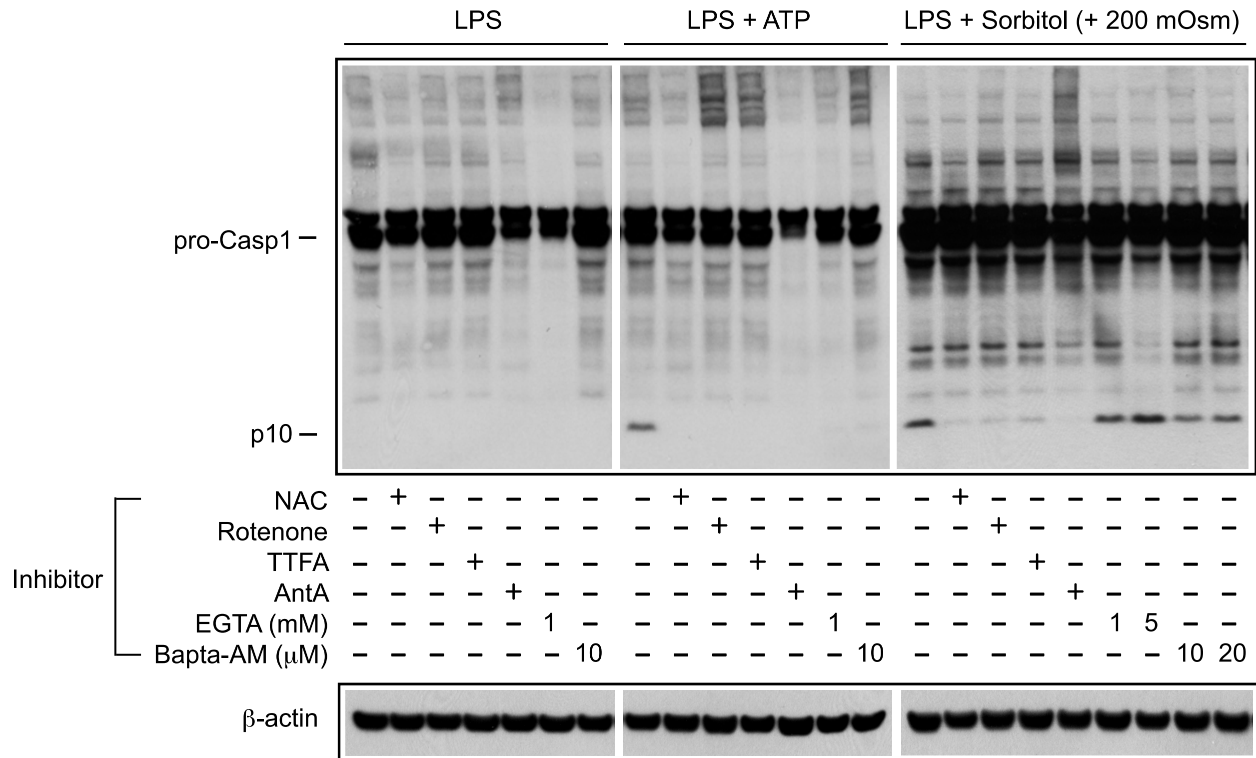


b



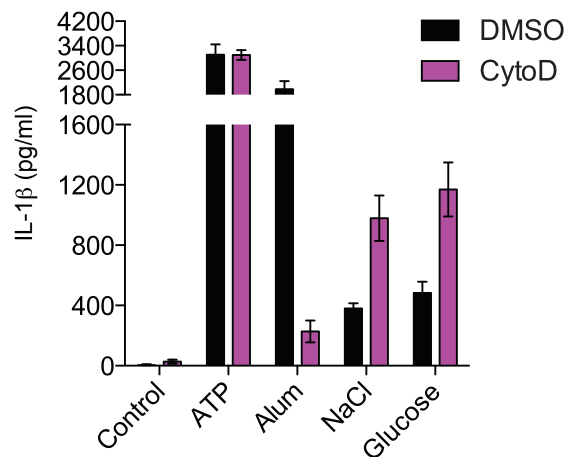
Supplementary Figure 4.

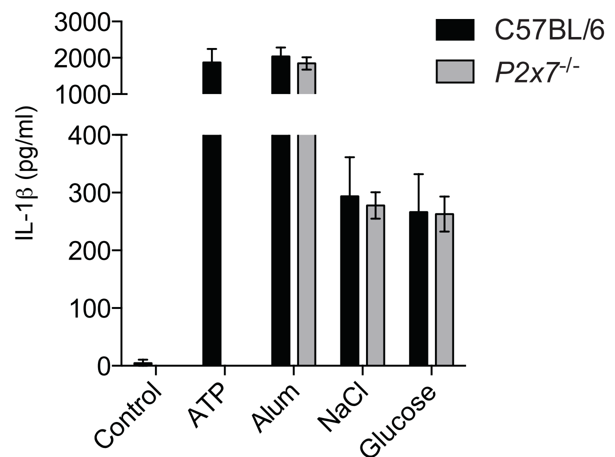
Lactate dehydrogenase release and cell viability of BMDMs upon hyperosmotic stress. (**a, b**) BMDMs generarted from wild-type (C57BL/6) or caspase-1-deficient (*Casp1*$^{-/-}$) mice and primed with LPS (100 ng/ml) were incubated in isotonic conditions (control) or hypertonic conditions (+ 200 mOsm) by adding NaCl, sorbitol or glucose, or stimulated with ATP (5 mM) or alum (250 μg/ml). Lactate dehydrogenase (LDH) release in supernatants after overnight incubation or stimulation (**a**) and cell viability of macrophages at the indicated times (**b**) were determined by CytoTox-ONE and CellTiter-Blue assays repectively as described in the Methods. Data indicate means ± s.d. of quadruplicates.



Supplementary Figure 5.

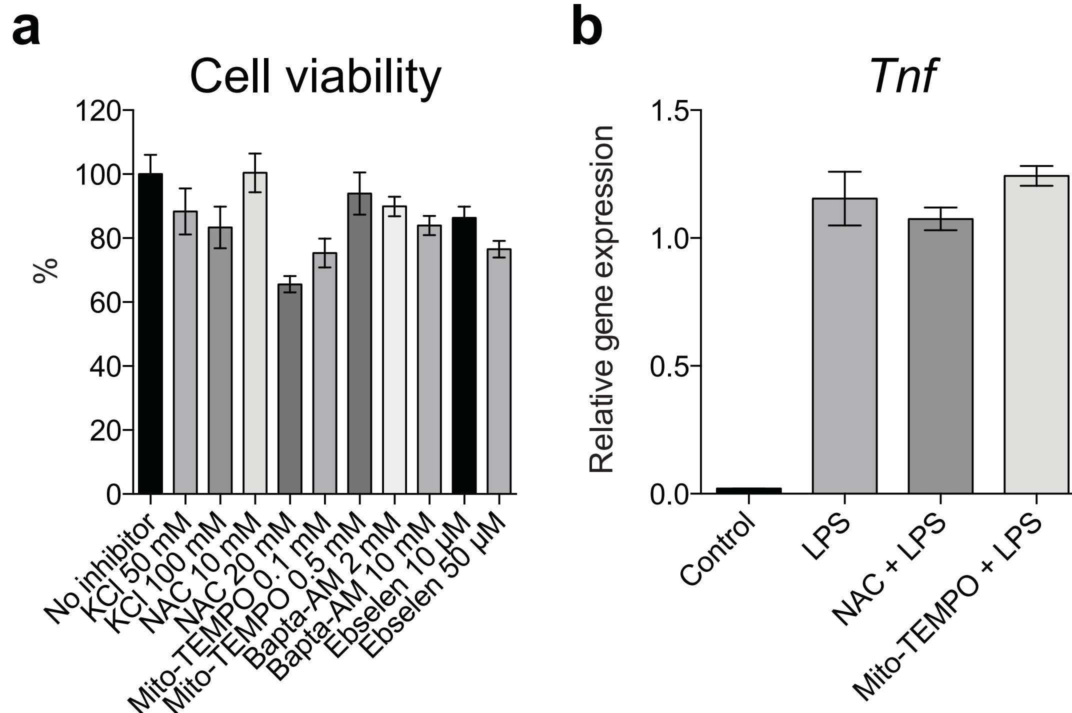
Caspase-1 activation by hyperosmotic stress is independent of Ca^{2+} influx. LPS-primed BMDMs generated from wild-type mice pretreated for 30 min without or with excellular or intracellular Ca^{2+} chelators - EGTA (1 or 5 mM) or Bapta-AM (10 or 20 μM), 10 mM NAC (an antioxidant), 1 μM Rotenone (aninhibitor of mitochondrial complex I), 100 μM TTFA (an inhibitor of mitochondrial complex II), or 100 μM antimycin A (AntA; an inhibitor of mitochondrial complex III) were incubated for 30 min with 5 mM ATP or for 3 h in hypertonic condtions (+ 200 mOsm) by adding sorbitol. Cell lysates were analyzed by immunoblotting for caspase-1.





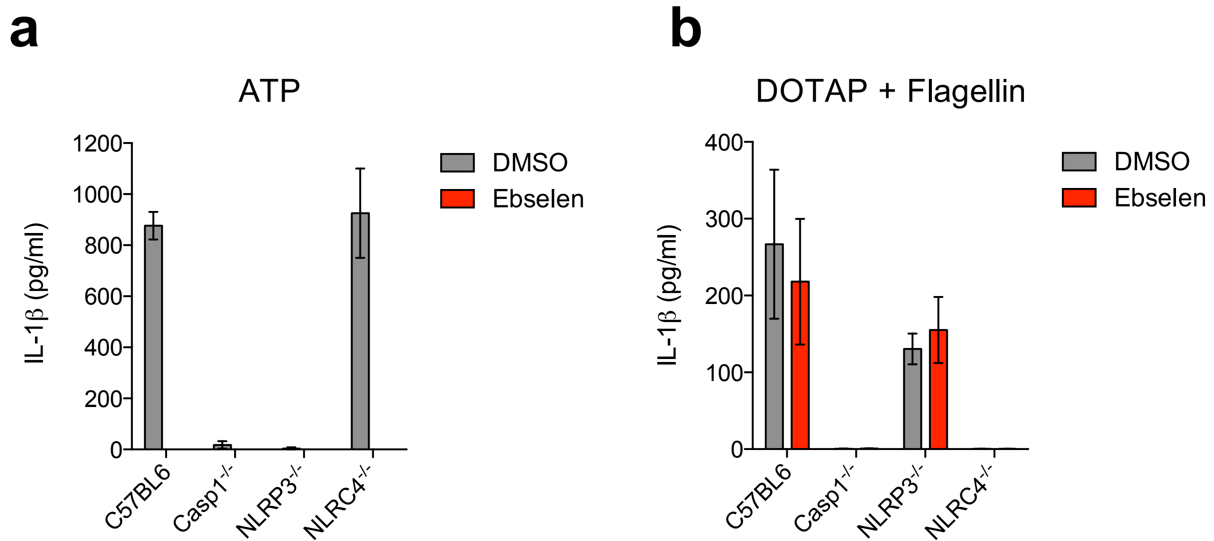
Supplementary Figure 6.

IL-1β secretion induced by hyperosmotic stress is independent of phagolysosomal membrane rupture via phagocytosis or ATP sensing via $P2X_7$ receptor. (**a**) After LPS (100 μg/ml) priming for 3 h, BMDMs generated from wild-type mice were pretreated for 30 min with cytochalasin D (CytoD)(10 μM) to block phagocytosis, and further incubated overnight in isontonic conditions (control) or hypertonic conditions (i.e. + 200 mOsm) by adding NaCl or glucose, or stimulated with ATP (5 mM) or alum (250 μg/ml) for 2 h or 6h respectively. (**b**) LPS-primed BMDMs generated from wild-type (C57BL6) or $P2X_7$ receptor-deficient (*$P2x7^{-/-}$*) mice were incubated as in **a**. IL-1β in media supernatatants was measured by ELISA. Data indicates mean ± s.d. of quadruplicates.



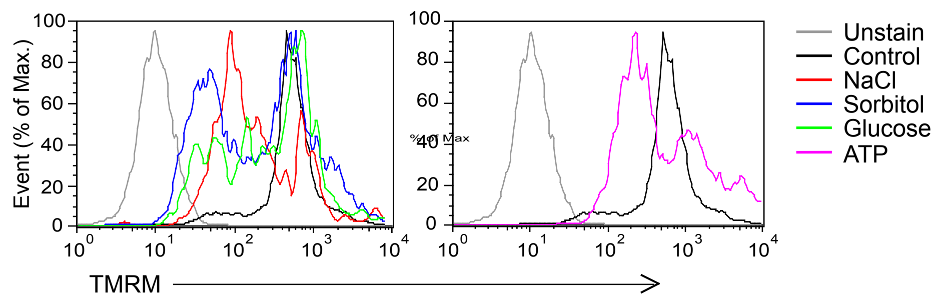
Supplementary Figure 7.

Assement of cytotoxic effects of the inhibitors used to block inflammasome activation. (**a**) BMDMs were incubated for 4 h in presence of KCl, NAC, Mito-TEMPO, Bapta-AM, or ebselen at the indicated concentrations. Cell viability of macrophages were determined by CellTiter-Blue as described in the Methods. (**b**) BMDMs were stimulated for 3 h with LPS (100 ng/ml) in the presence or absence of NAC (10 mM) or Mito-TEMPO (0.5 mM). Induction of TNF-α gene expression by LPS in macrophages was measured by qPCR. Data indicates mean ± s.d. of quadruplicates (**a**) or triplicate (**b**).



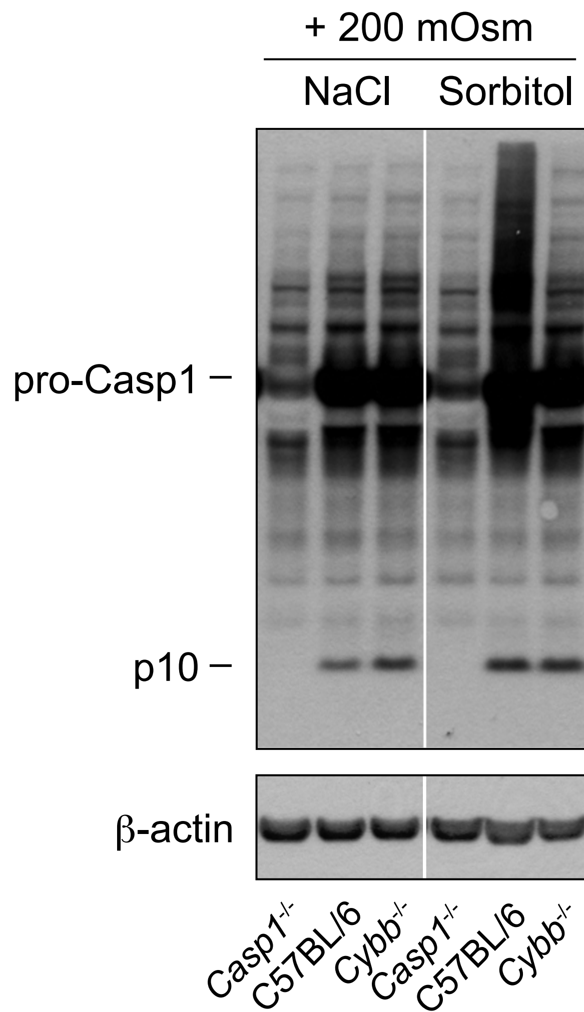
Supplementary Figure 8.

The requirement of ROS for inflammasome activation induced by ATP or cytosolic flagellin. (**a**,**b**) After LPS (100 μg/ml) priming for 3 h, BMDMs generated from wild-type(C57BL6), Casp1$^{-/-}$, NLRP3$^{-/-}$, or NLRC4$^{-/-}$ mice were stimulated with ATP (5 mM) for 2 h (**a**), or with a mixture of DOTAP and flagellin (1 μg/ml) for 12 h (**b**) in the absence (DMSO) or presence of 50 μM ebselen. IL-1β in media supernatatants was measured by ELISA. Data indicates mean ± s.d. of quadruplicates.



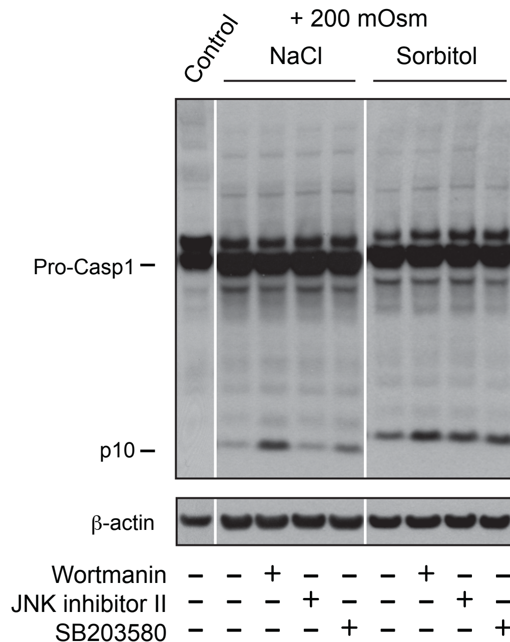
Supplementary Figure 9.

Loss of mitochondrial membrane potential upon hyperosmotic stress in BMDMs. LPS-primed BMDMs were incubated for 3 h in isontonic conditions (control) or hypertonic conditions (i.e. + 200 mOsm) by adding NaCl, sorbitol, or glucose, or stimulated for 15 min with ATP (5 mM). Macrophages were then labeled for 10 min with tetramethylrhodamine methyl ester (TMRM) (200 nM), a potentiometric dye that partitions into mitochondria on the basis of membrane potential.



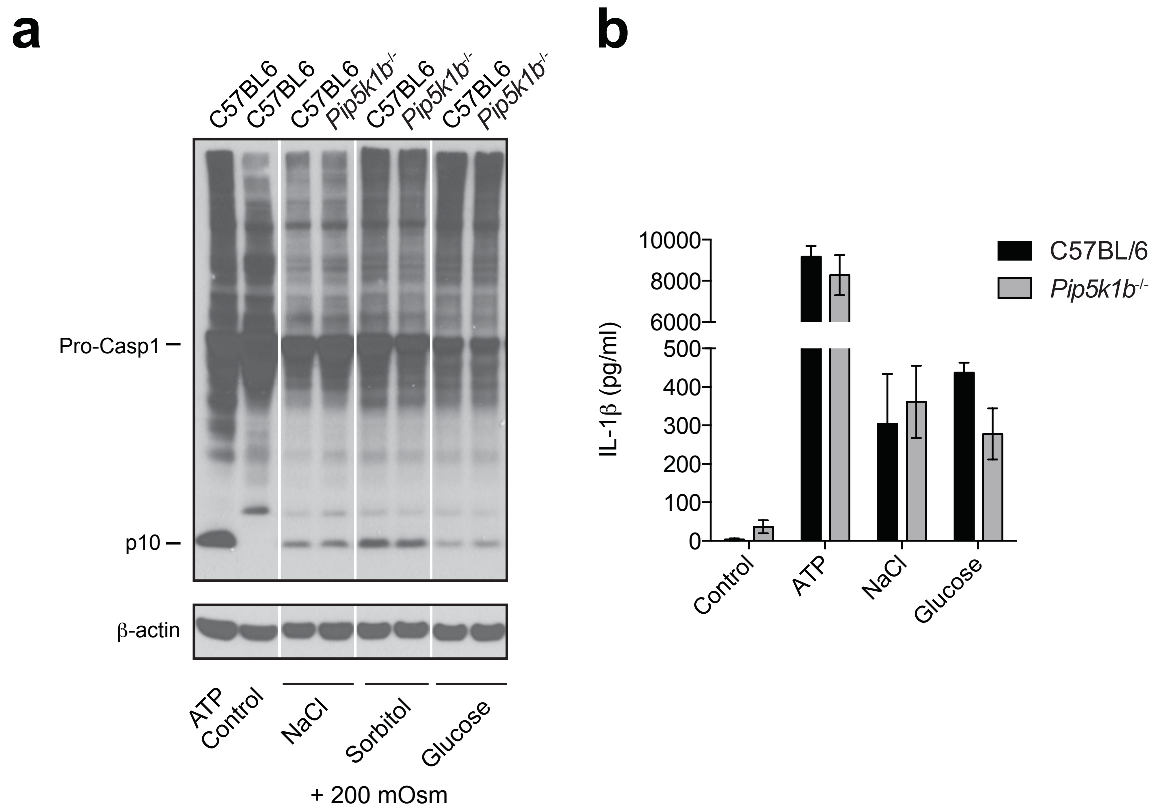
Supplementary Figure 10.

Caspase-1 activation by hyperosmotic stress is intact in BMDMs lacking gp91-phox. LPS-primed BMDMs generated from wild-type (C57BL6), caspase-1-deficient (*Casp1*$^{-/-}$) or gp91-phox-deficient (*Cybb*$^{-/-}$) mice were incubated for 3 h in hypertonic condtions (+ 200 mOsm) by adding NaCl or sorbitol. Cell lysates were analyzed by immunoblotting for caspase-1.



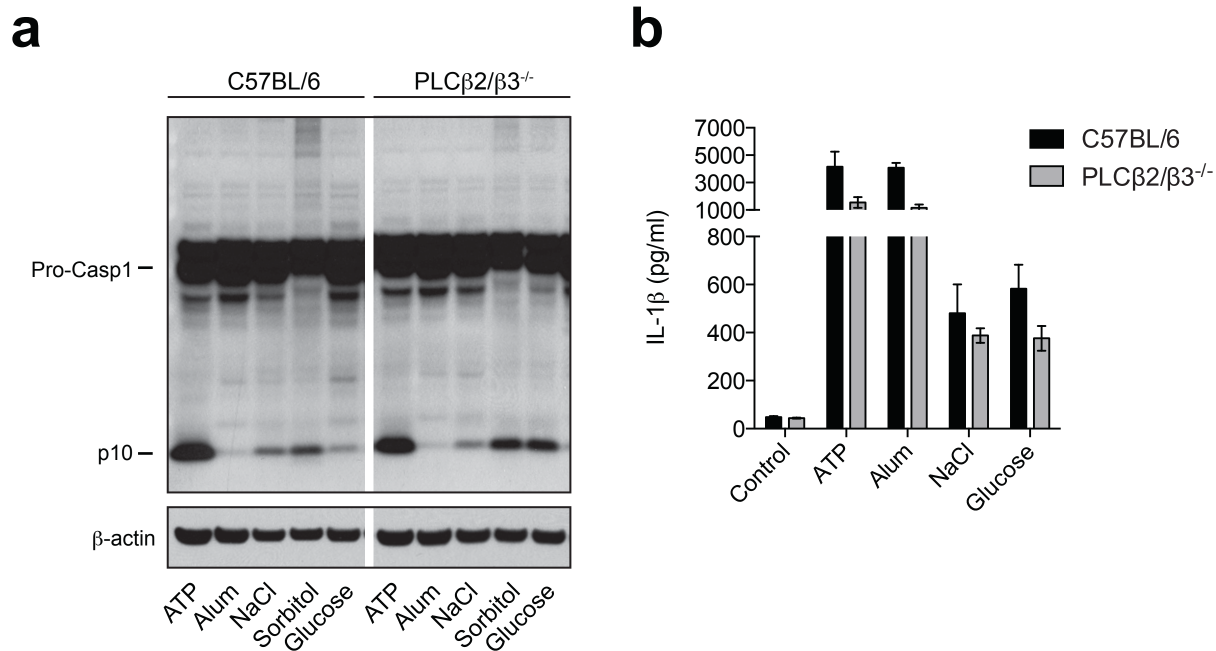
Supplementary Figure 11.

Caspase-1 activation by hyperosmotic stress is not inhibited by blocking signaling pathway via phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), c-Jun N-terminal kinases (JNKs) or p38 mitogen-activated protein kinases (MAPKs). LPS-primed BMDMs generated from wild-type mice were pretreated for 45 min with Wortmanin (100 nM), JNK Inhibitor II (500 nM) or SB203580 (2.5 nM) and incubated for further 3 h in hypertonic condtions (+ 200 mOsm) by adding NaCl or sorbitol. Cell lysates were analyzed by immunoblotting for caspase-1.



Supplementary Figure 12

Caspase-1 activation by hyperosmotic stress is independent of activating type I phosphatidylinositol 4-phosphate 5-kinase β isoform (PIP5KIβ). (**a**.**b**) BMDMs generated from wild-type (C57BL6) or PIP5KIβ-deficient (*Pip5k1b*$^{-/-}$) mice were primed with LPS (100 ng/ml) for 3 h and incubated for further 3 h (**a**) or overnight (**b**) in isontonic conditions (control) or hypertonic conditions (+ 200 mOsm) as indicated by adding NaCl, sorbitol, or glucose, or stimulated with ATP (5 mM) for 30 min (**a**) or 2 h (**b**). Cell lysates were analyzed by immunoblotting for caspase-1 (**a**). IL-1β in media supernatatants was measured by ELISA (**b**). Data indicates mean ± s.d. of quadruplicates (**b**).

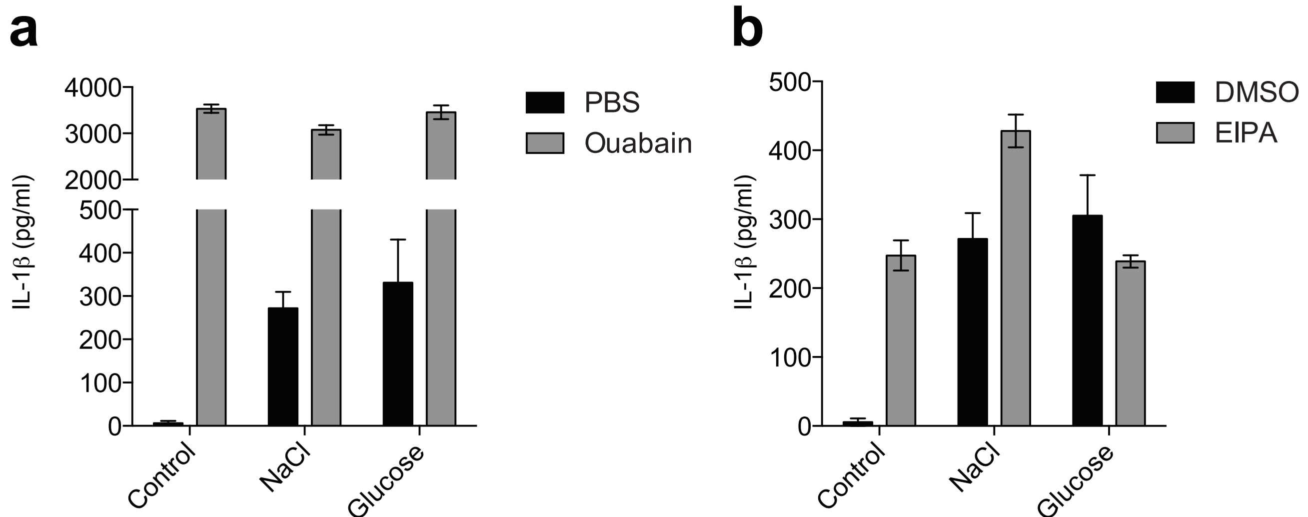


Supplementary Figure 13.

Caspase-1 activation by hyperosmotic stress is independent of phospholipase C (PLC)-β2 and PLC-β3. (**a**.**b**) BMDMs generated from wild-type (C57BL6) or PLC-β2 and PLC-β3 double-deficient (PLCβ2/β3$^{-/-}$) mice were primed with LPS (100 ng/ml) for 3 h, and incubated for 3 h (**a**) or overnight (**b**) in isontonic conditions (control) or hypertonic conditions (+ 200 mOsm) as indicated by adding NaCl, sorbitol, or glucose, or stimulated with ATP (5 mM) for 30 min (**a**) or 2 h (**b**) or with alum (250 μg/ml) for 3 h (**a**) or 6 h (**b**). Cell lysates were analyzed by immunoblotting for caspase-1 (**a**). IL-1β in media supernatatants was measured by ELISA (**b**). Data indicates mean ± s.d. of quadruplicates (**b**).

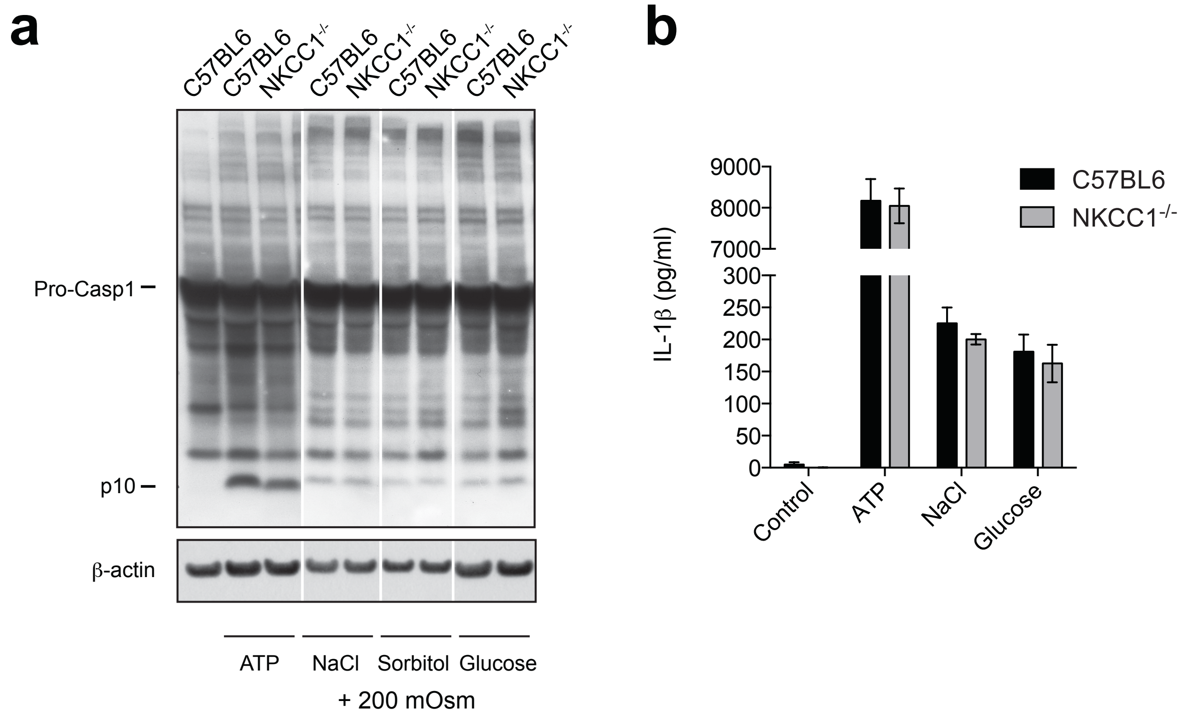
Supplementary Figure 14.

IL-1β secretion induced by hyperosmotic stress is independent of signaling through Rho GTPase pathway. LPS-primed BMDMs generated from wild-type mice were pretreated for 45 min wtih *Clostridium difficile* toxin B (25 ng/ml) and incubated overnight in isontonic conditions (control) or hypertonic conditions (+ 200 mOsm) as indicated by adding NaCl or glucose, or stumulated for further 2 h with ATP (5 mM). IL-1β in media supernatatants was measured by ELISA. Data indicates mean ± s.d. of quadruplicates.



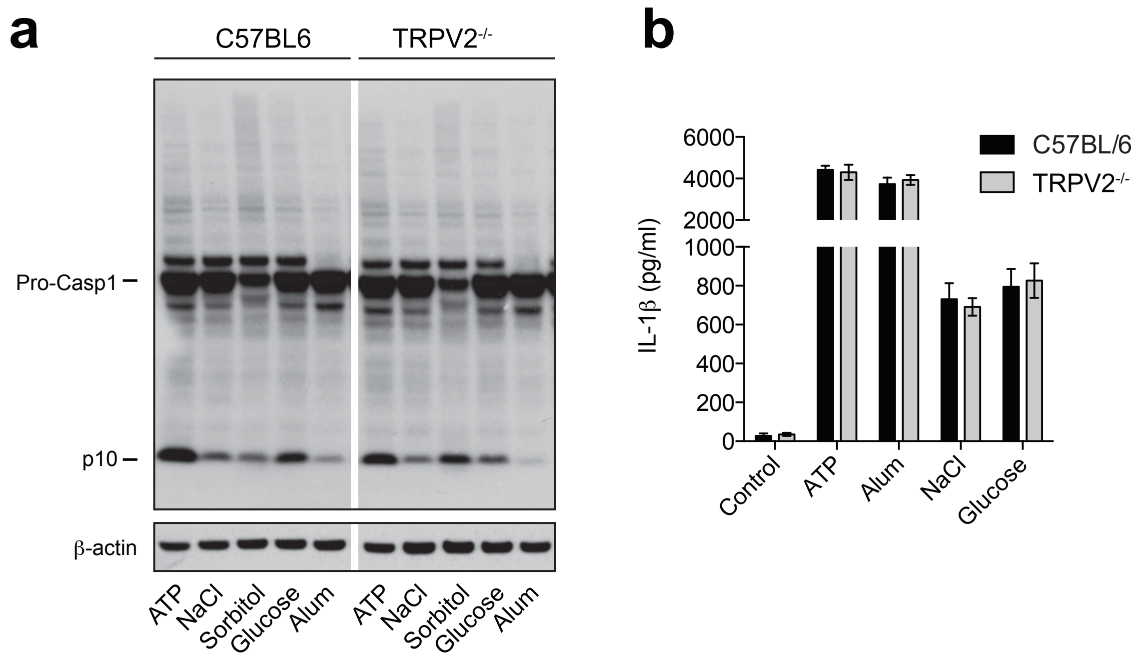
Supplementary Figure 15.

IL-1β secretion induced by hyperosmotic stress is independent of ion channel sodium-potassium adenosine triphosphatase (Na^{+}/K^{+}-ATPase) or sodium-hydrogen antiporter (NHE). (**a**.**b**) LPS-primed BMDMs generated from wild-type mice were pretreated for 45 min with ouabain (1 mM), an inhibitor of Na^{+}/K^{+}-ATPase (**a**), or 5-(N-ethyl-N-isopropyl)amiloride (EIPA) (100 μM), an inhibitor of NHE (**b**), and incubated overnight in isontonic conditions (control) or hypertonic conditions (i.e. + 200 mOsm) as indicated by adding NaCl or glucose. IL-1β in media supernatatants was measured by ELISA. Data indicates mean ± s.d. of quadruplicates.



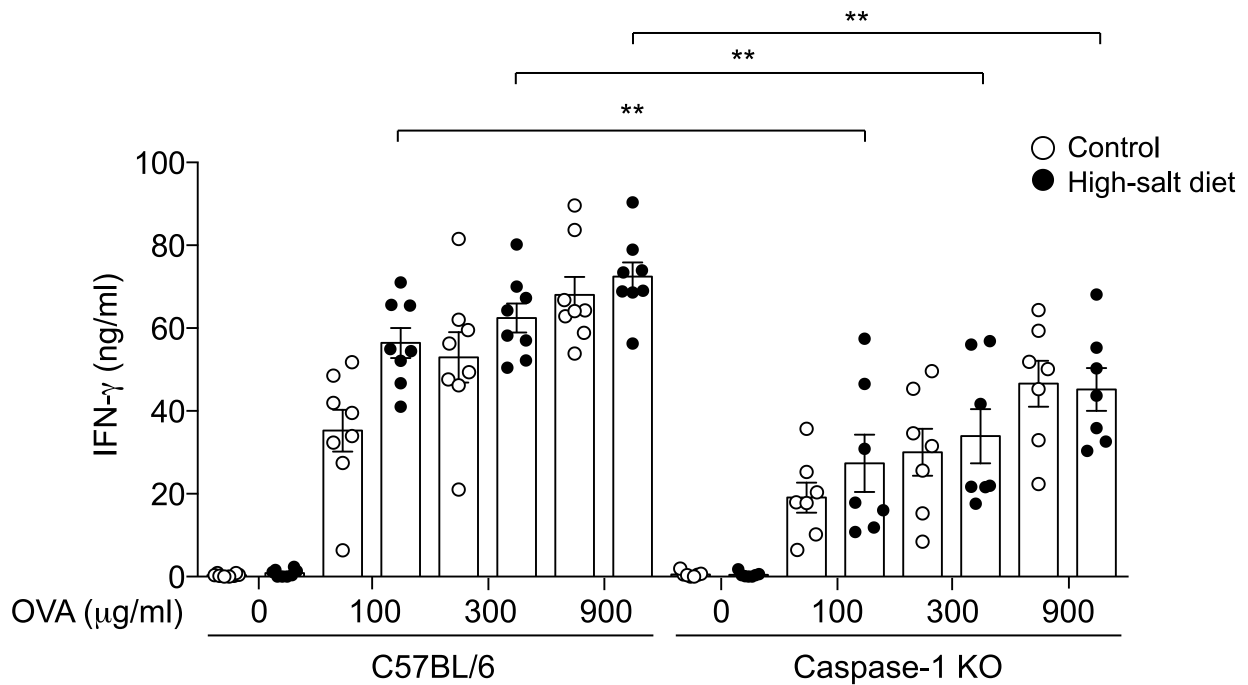
Supplementary Figure 16.

Caspase-1 activation by hyperosmotic stress is independent of ion channel Na-K-Cl cotransporter 1 (NKCC1). (**a**.**b**) BMDMs generated from wild-type (C57BL6) or NKCC1-deficient (NKCC1$^{-/-}$) mice were primed with LPS (100 ng/ml) for 3 h, and incubated for further 3 h (**a**) or overnight (**b**) in isontonic conditions (control) or hypertonic conditions (+ 200 mOsm) as indicated by adding NaCl, sorbitol, or glucose, or stimulated with ATP (5 mM) for 30 min (**a**) or 2 h (**b**). Cell lysates were analyzed by immunoblotting for caspase-1 (**a**). IL-1β in culture supernatatants was measured by ELISA (**b**). Data indicates mean ± s.d. of quadruplicates (**b**).



Supplementary Figure 17.

Caspase-1 activation by hyperosmotic stress is independent of transient receptor potential (TRP) channel TRPV2. (**a**.**b**) BMDMs generated from wild-type (C57BL6) or TRPV2-deficient (TRPV2$^{-/-}$) mice were primed with LPS (100 ng/ml) for 3 h, and incubated for further 3 h (**a**) or overnight (**b**) in isontonic conditions (control) or hypertonic conditions (i.e. + 200 mOsm) as indicated by adding NaCl, sorbitol, or glucose, or stimulated with ATP (5 mM) for 30 min (**a**) or 2 h (**b**) or with alum (250 μg/ml) for 3 h (**a**) or 6 h (**b**). Cell lysates were analyzed by immunoblotting for caspase-1 (**a**). IL-1β in culture supernatatants was measured by ELISA (**b**). Data indicates mean ± s.d. of quadruplicates (**b**).



Supplementary Figure 18.

High-salt diet has no effect on Th1 response upon immunization with OVA peptide/LPS. Draining lymph node cells from high-salt diet or control diet animals (wild-type (C57BL/6) or Caspase-1 KO mice) immunized with OVA peptide/LPS were restimulated *in-vitro* with the indicated concentrations of OVA for 48 h. IFN-γ secretion in media supernatants were determined by ELISA. Student's *t*-test: **, $P < 0.01$.

Signaling Pathway or Ion Channel	References[1] for Hyperosmotic Stress Sensing	Reagent Used for In-Vitro Testing	Results
p38	1, 2	Inhibitor - SB203580	Supp. Fig. 11
JNK	2	Inhibitior - JNK inhibitor II	Supp. Fig. 11
PI3K	3	Inhibitor - Wortmannin	Supp. Fig. 11
$PI(4,5)P_2$	4	PIP5KIβ$^{-/-}$ BMDM	Supp. Fig. 12
PLC	5	PLCβ2/β3$^{-/-}$ BMDM	Supp. Fig. 13
Rho GTPase	6	Inhibitor - *Clostridium difficile* Toxin B	Supp. Fig. 14
Na^+/K^+-ATPase	7	Inhibitor - Ouabain	Supp. Fig. 15
NHE	7	Inhibitor - Ethylisopropyl amiloride (EIPA)	Supp. Fig. 15
NKCC1	7	NKCC1$^{-/-}$ BMDM	Supp. Fig. 16
TRPV2	8	TRPV2$^{-/-}$ BMDM	Supp. Fig. 17

Supplementary Table 1.

Hyperosmotic stress sensing pathways or ion channels tested *in vitro* for their contribution to inflammasome activation by hyperosmotic stress in macrophages. [1]See Supplementary References.

Gene	Forward Primer	Reverse Primer
Gapdh	GGTGCTGAGTATGTCGTGGA	CGGAGATGATGACCCTTTTG
Ifng	ATGAACGCTACACACTGCATCTTGGCTT	CCTCAAACTTGGCAATACTCATGAATGC
Il17a	TCCAGAAGGCCCTCAGACTA	TCAGGACCAGGATCTCTTGC

Supplementary Table 2.

Real-time QPCR primer sequences from 5’ to 3’.

Supplementary References

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