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Supplemental Information

TAK1 Negatively Regulates NF-κB and p38 MAP Kinase Activation in Gr-1^{+}CD11b^{+} Neutrophils

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Inventory

Table 1: Blood cell counts from TAK1 WT and deficient mice

	Map3k7 $^{\Delta M/+}$ (n=9)	*Map3k7* $^{\Delta M/\Delta M}$ (n=12)
WBC* ($\times 10^6$)	7.978 ±0.795	11.290 ±0.987
Granulocytes*** ($\times 10^6$)	2.567 ±0.232	7.225 ±0.918
Platelets ($\times 10^6$)	747.0 ±82.1	541.9 ±75.3
Lymphocytes ($\times 10^6$)	4.644 ±0.498	3.350 ±0.391
Monocytes ($\times 10^6$)	0.767 ±0.088	0.717 ±0.096
RBC** ($\times 10^9$)	10.18 ±0.30	8.62 ±0.35
HGB** (g/dl)	15.28 ±0.43	13.25 ±0.47
HCT* (%)	44.86 ±1.30	39.470 ±1.52
RDW (%)	14.81 ±0.17	15.98 ±0.52

* P < 0.05; ** P < 0.01; *** P < 0.001.

Related to Figure 1 in the main text.

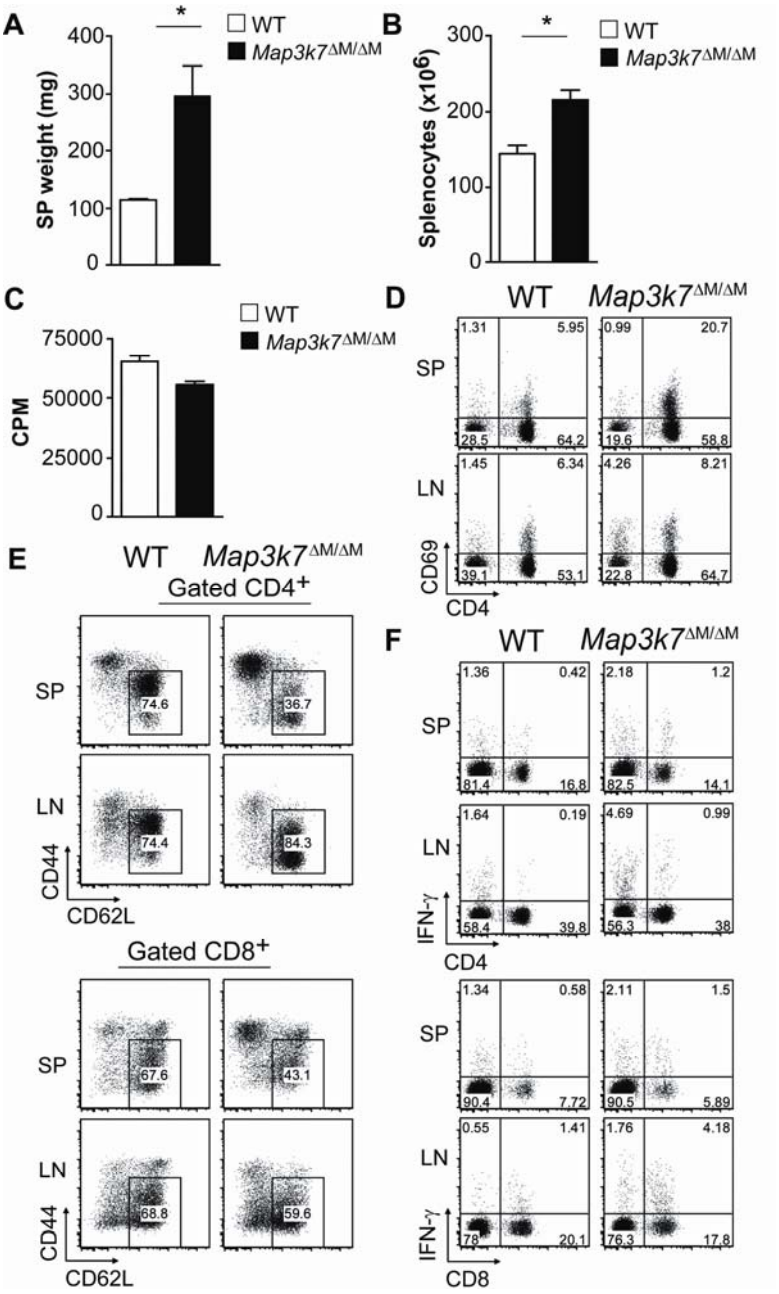

Figure S1. Spleen cell count and T cell activation are increased in TAK1-deficient mice. (A) Spleen weight and (B) total splenocyte number in WT and *Map3k7*$^{\Delta M/\Delta M}$ mice. (C) Proliferation of naïve T cells co-cultured with WT and TAK1 deficient neutrophils was measured by [^{3}H] thymidine assay. (D-F) Flow cytometric analysis of CD4 and/or CD8 T cells in spleens and lymph nodes of WT and *Map3k7*$^{\Delta M/\Delta M}$ mice using CD69 (D), CD44 and CD62L (E) and IFN-γ (F) markers. Results in (A-B) are plotted as mean ± s.d. * $P < 0.05$. Data shown are representative of at least four independent experiments. Related to Figures 1-2 in the main text.

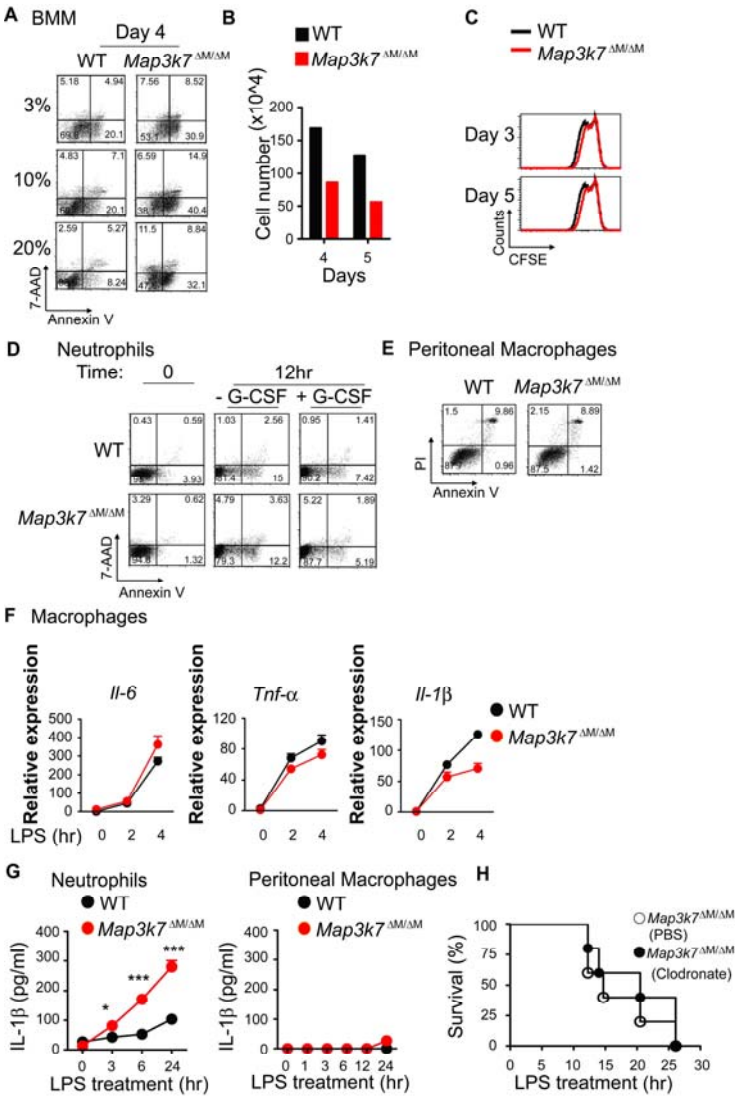

Figure S2. Apoptosis in *Map3k7*$^{\Delta M/\Delta M}$ bone marrow-derived macrophages (BMM) and pro-inflammatory cytokine gene expression. (A) Apoptosis was assayed using Annexin V/7-AAD staining and flow cytometric analysis of BMM cells cultured with different concentrations of M-CSF conditioned medium. F4/80$^+$ BMM cells were gated to determine percentage of apoptotic cells. (B) BMM cell number was quantified by trypan blue staining of BMM cells. (C) Cell proliferation was assessed in F4/80$^+$ BMM cells by CFSE staining and flow cytometry. (D and E) Annexin V/7-AAD staining was used to determine apoptosis in freshly isolated Gr-1$^+$ neutrophils treated with or without G-CSF (100 ng/ml) (D) and freshly isolated F4/80$^+$ peritoneal macrophages (E). (F) *Il-6, Tnf-α* and *Il-1β* gene expression in macrophages treated with LPS (100 ng/ml) for indicated time points determined by real-time PCR. (G) IL-1β secretion by neutrophils or macrophages treated with or without LPS (100 ng/ml) only for indicated time points was measured by ELISA. (H) Kaplan-Meier survival curve of *Map3k7*$^{\Delta M/\Delta M}$ treated with PBS- or clodronate-containing liposomes followed by treatment with high-dose LPS (30 mg/kg i.p.). Related to Figures 3, 4, 5 in the main text.

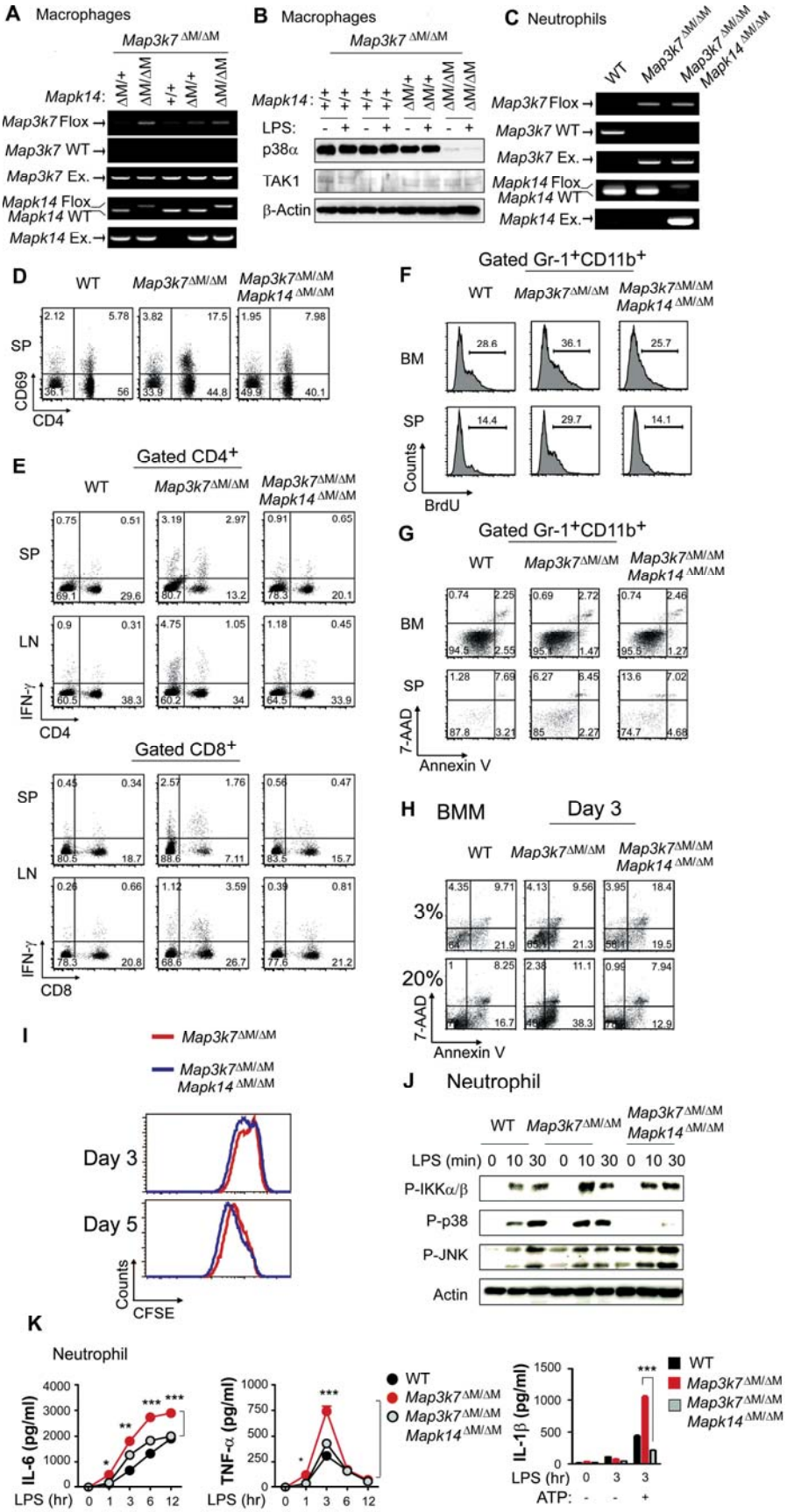
A Macrophages
Map3k7 ΔM/ΔM
Mapk14: ΔM/+ ΔM/ΔM +/+ ΔM/+ ΔM/ΔM
Map3k7 Flox
Map3k7 WT
Map3k7 Ex.
Mapk14 Flox
Mapk14 WT
Mapk14 Ex.
B Macrophages
Map3k7 ΔM/ΔM
Mapk14: +/+ +/+ +/+ +/+ ΔM/+ ΔM/+ ΔM/ΔM ΔM/ΔM
LPS: - + - + - + - +
p38α
TAK1
β-Actin
C Neutrophils
WT
Map3k7 ΔM/ΔM
Map3k7 ΔM/ΔM Mapk14 ΔM/ΔM
Map3k7 Flox
Map3k7 WT
Map3k7 Ex.
Mapk14 Flox
Mapk14 WT
Mapk14 Ex.
D
WT
Map3k7 ΔM/ΔM
Map3k7 ΔM/ΔM Mapk14 ΔM/ΔM
SP
CD69
CD4
2.12 5.78 36.1 56
3.82 17.5 33.9 44.8
1.95 7.98 49.9 40.1
E
Gated CD4+
WT
Map3k7 ΔM/ΔM
Map3k7 ΔM/ΔM Mapk14 ΔM/ΔM
SP
LN
IFN-γ
CD4
0.75 0.51 69.1 29.6
3.19 2.97 80.7 13.2
0.91 0.65 78.3 20.1
0.9 0.31 60.5 38.3
4.75 1.05 60.2 34
1.18 0.45 64.5 33.9
Gated CD8+
SP
LN
IFN-γ
CD8
0.45 0.34 80.5 18.7
2.57 1.76 88.6 7.11
0.56 0.47 83.5 15.7
0.26 0.66 78.3 20.8
1.12 3.59 68.6 26.7
0.39 0.81 77.6 21.2
F
Gated Gr-1+CD11b+
WT
Map3k7 ΔM/ΔM
Map3k7 ΔM/ΔM Mapk14 ΔM/ΔM
BM
SP
Counts
BrdU
28.6
36.1
25.7
14.4
29.7
14.1
G
Gated Gr-1+CD11b+
WT
Map3k7 ΔM/ΔM
Map3k7 ΔM/ΔM Mapk14 ΔM/ΔM
BM
SP
7-AAD
Annexin V
0.74 2.25 94.5 2.55
0.69 2.72 95.1 1.47
0.74 2.46 95.5 1.27
1.28 7.69 87.8 3.21
6.27 6.45 85 2.27
13.6 7.02 74.7 4.68
H
BMM
Day 3
WT
Map3k7 ΔM/ΔM
Map3k7 ΔM/ΔM Mapk14 ΔM/ΔM
3%
20%
7-AAD
Annexin V
4.35 9.71 64 21.9
4.13 9.56 21.3
3.95 18.4 19.5
1 8.25 16.7
2.38 11.1 38.3
0.99 7.94 12.9
I
Map3k7 ΔM/ΔM
Map3k7 ΔM/ΔM Mapk14 ΔM/ΔM
Day 3
Day 5
Counts
CFSE
J
Neutrophil
WT
Map3k7 ΔM/ΔM
Map3k7 ΔM/ΔM Mapk14 ΔM/ΔM
LPS (min) 0 10 30 0 10 30 0 10 30
P-IKKα/β
P-p38
P-JNK
Actin
K
Neutrophil
IL-6 (pg/ml)
LPS (hr) 0 1 3 6 12
TNF-α (pg/ml)
LPS (hr) 0 1 3 6 12
WT
Map3k7 ΔM/ΔM
Map3k7 ΔM/ΔM Mapk14 ΔM/ΔM
IL-1β (pg/ml)
LPS (hr) 0 3 3
ATP: - - +

Figure S3. Characterization of *Map3k7*$^{\Delta M/\Delta M}$ *Mapk14*$^{\Delta M/\Delta M}$ mice. (A) PCR analysis of macrophages using primers specific for *Map3k7* and *Mapk14* floxed, WT and excision alleles. (B) Immunoblot analysis of macrophages from WT, *Map3k7*$^{\Delta M/\Delta M}$ and *Map3k7*$^{\Delta M/\Delta M}$ *Mapk14*$^{\Delta M/\Delta M}$ mice using antibodies for p38α and TAK1. (C) PCR analysis of neutrophils using primers specific for *Mapk3k7* and *Mapk14* floxed, WT and excision alleles. (D and E) Flow cytometric analysis of CD4 and/or CD8 T cells in spleens and lymph nodes of WT and *Map3k7*$^{\Delta M/\Delta M}$ mice using CD69 (D) and IFN-γ (E) markers. (F) BrdU pulse labeling of mice and flow cytometric analysis of bone marrow and spleen cells using anti-BrdU. Gr-1$^+$CD11b$^+$ cells were gated to determine percentage of BrdU-positive cells. (G) Apoptosis was measured by Annexin V/7-AAD staining and flow cytometric analysis of cells isolated from the bone marrow and spleen. Gr-1$^+$CD11b$^+$ cells were gated to determine percentage of apoptotic cells. (H) Apoptosis was assayed using Annexin V/7-AAD staining and flow cytometric analysis of BMM cells cultured with M-CSF conditioned medium. F4/80$^+$ BMM cells were gated to determine percentage of apoptotic cells. (I) Cell proliferation was assessed in BMM cells by CFSE staining and flow cytometry. (J) Neutrophils were treated with LPS (100 ng/ml) for the indicated time points and immunoblot analysis was performed with the indicated antibodies. (K) IL-6, TNF-α and IL-1β secretion by neutrophils treated with or without LPS (100 ng/ml) for indicated time points was measured by ELISA. IL-1β production was stimulated with ATP (5 mM) for 1 h. Related to Figure 6 in the main text.

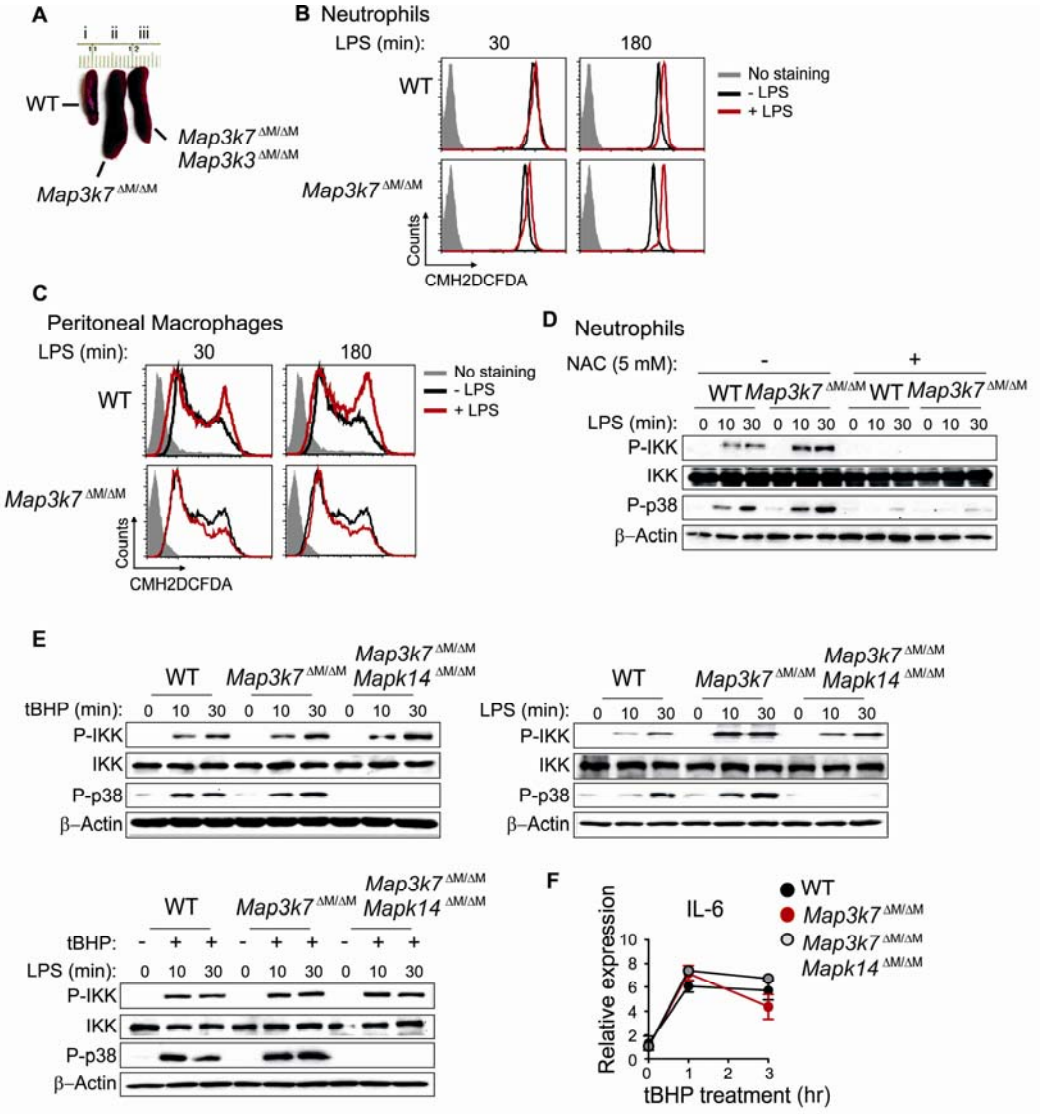

Figure S4. Spleen phenotype of *Map3k7*$^{\Delta M/\Delta M}$ *Map3k3*$^{\Delta M/\Delta M}$ mice, ROS production and Effects of ROS inducer on IKK phosphorylation in WT, *Map3k7*$^{\Delta M/\Delta M}$ and *Map3k7*$^{\Delta M/\Delta M}$ *Mapk14*$^{\Delta M/\Delta M}$ neutrophils. (A) Genetic deletion of *Map3k3* partially rescues splenomegaly in *Map3k7*$^{\Delta M/\Delta M}$ mice. (B and C) ROS release was measured in neutrophils (B) and macrophages (C) treated with or without LPS (100 ng/ml) for 30 min and 180 min. (D) Neutrophils were pretreated with or without NAC (5 mM) for 30 min followed by LPS stimulation for different time points. Cell lysates were collected and used for immunoblot analysis with the indicated antibodies. (E) Neutrophils from WT, *Map3k7*$^{\Delta M/\Delta M}$ and *Map3k7*$^{\Delta M/\Delta M}$ *Mapk14*$^{\Delta M/\Delta M}$ mice were treated with tBHP (200 μM), LPS (100 ng/ml), or both treatment for 0, 10 min and 30 min and cell lysates were collected and used for immunoblot analysis with the indicated antibodies. (F) Neutrophils from WT, *Map3k7*$^{\Delta M/\Delta M}$ and *Map3k7*$^{\Delta M/\Delta M}$ *Mapk14*$^{\Delta M/\Delta M}$ mice were treated with tBHP (200 μM) for the indicated time points. Total RNA from the treated cells were isolated and used for measuring IL-6 expression determined by quantitative RT-PCR. Related to Figure 7 in the main text.

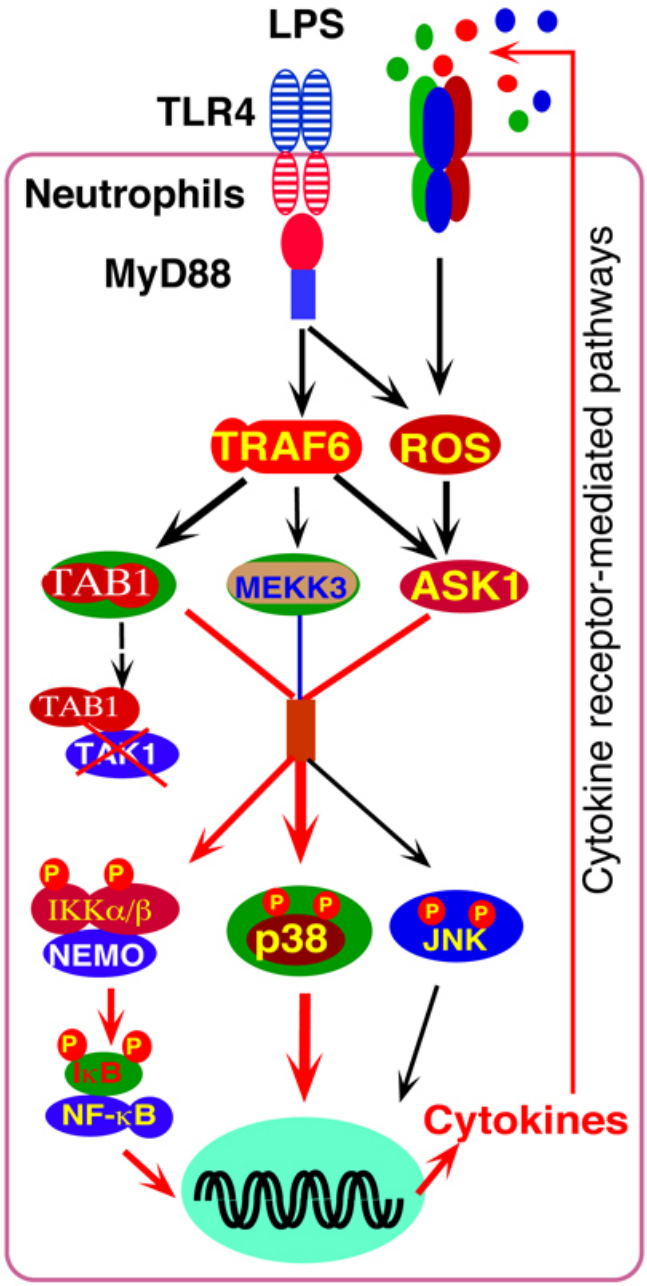

Figure S5. A proposed working model that illustrates how TAK1 deletion leads to increased phosphorylation IKK, p38 and JNK activation in neutrophils. Among them, p38 phosphorylation is a key component of innate signaling regulation in TAK-deficient neutrophils. TAB1, ROS-ASK1 and MEKK3 are involved in the increased p38 and IKK phosphorylation, but TAB1- and ROS-mediated p38 phosphorylation may be more important than MEKK3 in LPS-induced innate immune responses. p38-mediated signaling of p38 and downstream target cytokines are important for LPS-induced ROS production, which stimulates both IKK and p38 phosphorylation, forming a positive feedback regulatory loop. Related to discussion in main text.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Antibodies, reagents and kits

Anti-TAK1 and anti-TAB1 antibodies were purchased from Santa Cruz Biotechnology; anti-β-actin antibody was purchased from Sigma. Anti-MEKK3 antibody was purchased from BD Biosciences. Antibodies against phospho-IKK (Ser 176/180), IκBα, phospho-p38, p38, p38α, TAB1, phospho-JNK1/2, and phospho-ERK1/2 were purchased from Cell Signaling Technology. Specific anti-mouse antibodies utilized for flow cytometry include Gr1-FITC, CD11b-PECy7 (Mac-1), F4/80-PE, CD4-PerCP-Cy5.5, CD8-APC, CD69-PE, CD44-Pacific blue, CD62L-FITC, IFN-γ-PE, CD3-PE, and B220-FITC (eBioscience). LPS (0111:B4, TLR4 ligand), N-acetyl-$_L$-cysteine (NAC) and *tert*-Butyl hydroperoxide (tBHP) were purchased from Sigma-Aldrich. Adenosine triphosphate (ATP) was purchased from Invivogen. CM-H_2DCFDA was purchased from Invitrogen. Immunohistochemical staining was performed using anti-CD11b (AbCam) and Ki-67 (Vector Laboratories) antibodies. TUNEL staining was performed using a TdT In Situ Apoptosis Detection Kit (R&D Systems) following manufacturer's instructions. Clodronate liposomes were purchased from Encapsula NanoSciences. Granulocyte-colony stimulating factor (G-CSF) was purchased from Shenandoah Biotechnology Inc.

Proliferation and apoptosis assays. Proliferation was assessed using a BrdU labeling kit (BD Biosciences) according to manufacturer's instructions. Briefly, mice were injected with BrdU (100 mg/kg, i.p.). Tissues were harvested, stained with GR-1-PE, CD11B-PE-CY7, and APC-BrdU antibodies and analyzed by flow cytometry. Annexin V and 7AAD staining kit (BD Biosciences) was used to detect apoptotic cells following manufacturer's instructions.

T cell proliferation was assessed using [^{3}H] thymidine assay using irradiated neutrophils as antigen presenting cells (APCs). Briefly, $CD4^+$ naïve T cells (1×10^5) were isolated from spleens and co-cultured with irradiated $Gr1^+CD11b^+$ cells (2×10^5) with anti-CD3 antibody for 56 h in 96 well plates. T cell proliferation was determined by labeling cultured cells with [^{3}H] thymidine for 16 h and radioactivity was measured using a scintillation counter.

Carboxyfluorescein diacetate, succinimidyl ester (CFSE) staining was performed by labeling bone marrow macrophage (BMM) cells with CFSE (5 μM) for 10 min. Cells were washed with PBS, plated in RPMI media containing 10% FBS and 1% (volume/volume) penicillin-streptomycin supplemented with 10% M-CSF conditioned media and incubated at 37 °C until they were analyzed by flow cytometry.

Real-time PCR analysis. Total RNA was isolated from cells and first-strand cDNA synthesis was performed using total RNA, oligo-dT primers and reverse transcriptase II according to manufacturer's instructions (Invitrogen). Real-time PCR was done using SYBR GreenER qPCR Super Mix Universal (Invitrogen) and specific primers using the ABI Prism 7000 analyzer (Applied Biosystems).

The sequences of the primers are as following:

Primers for mouse *Gapdh*: Forward 5'-TTGTCTCCTGCGACTTCAACAG-3'

Reverse: 5’-GGTCTGGGATGGAAATTGTGAG-3’

Primers for mouse *Tnf-α*: Forward: 5’-ACAGAAAGCATGATCCGCG-3’

Reverse: 5’-GCCCCCCATCTTTTGGG-3’

Primers for mouse *Il-6:* Forward: 5’-CCAGAAACCGCTATGAAGTTCC-3’

Reverse: 5’- TTGTCACCAGCATCAGTCCC-3’

Primers for mouse *Il-1β*: Forward: 5’-GTGGCTGTGGAGAAGCTGTG-3’

Reverse: 5’- GAAGGTCCACGGGAAAGACAC-3’