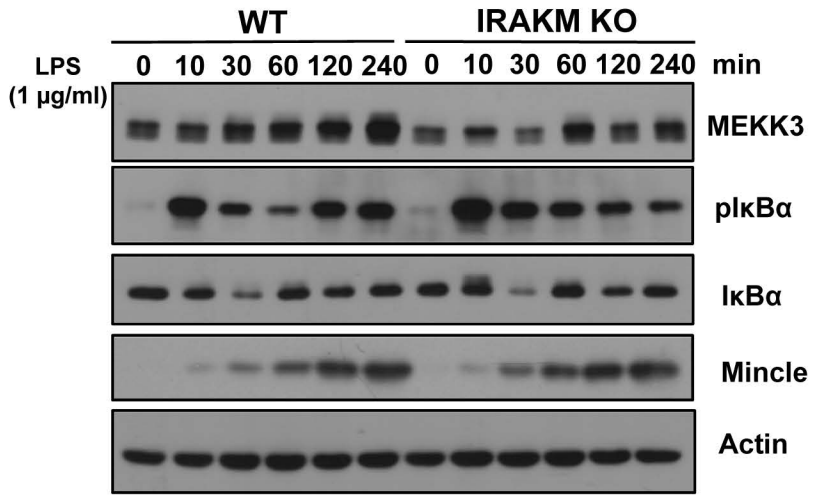
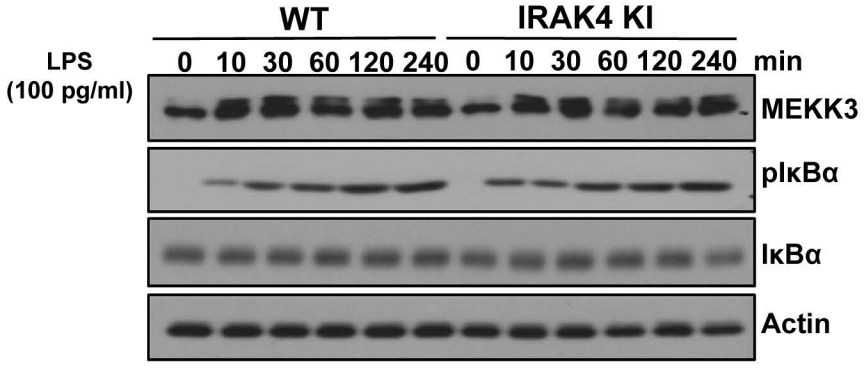
Suppl. Fig. 1



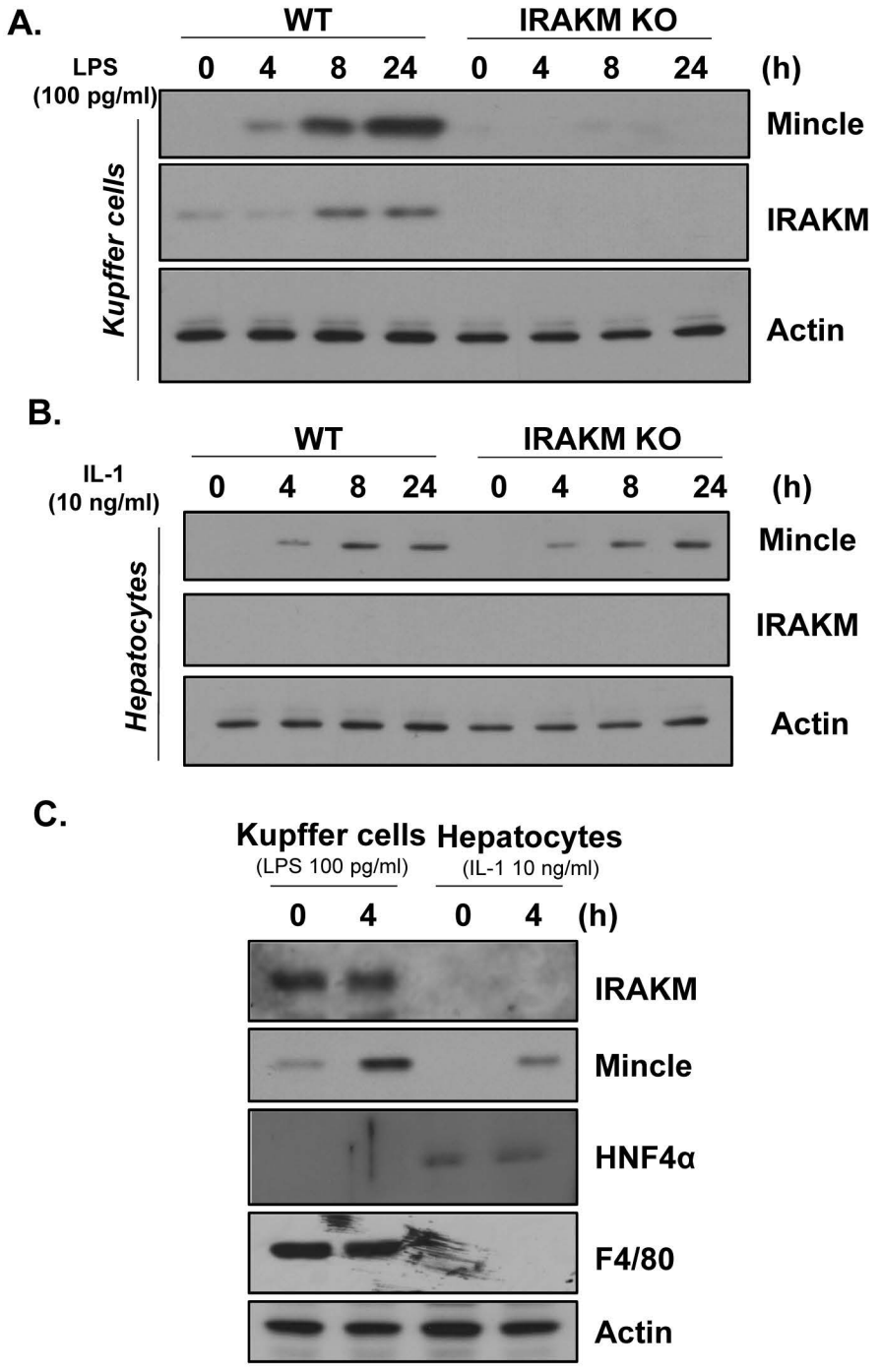
Suppl. Fig.1 IRAKM is required for late phase high dose LPS-mediated NFκB activation. Cell lysates from wild-type (WT) and IRAKM KO bone marrow-derived macrophages (BMDMs) untreated or treated with high doses LPS (1 μg/ml) for the indicated times were analyzed by Western blot analysis. The experiments were repeated for three times with similar results

Suppl. Fig. 2



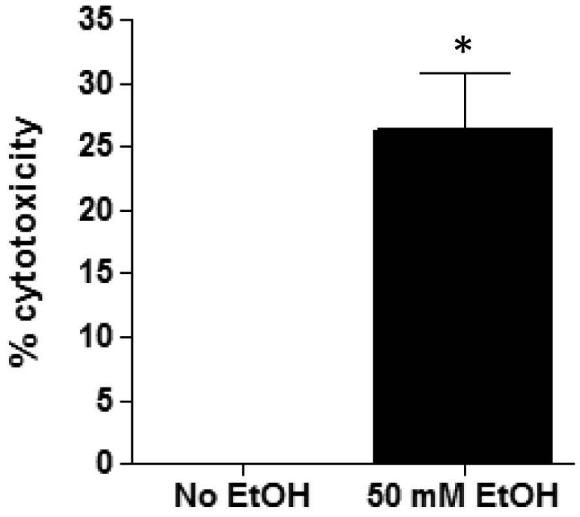
Suppl. Fig.2 IRAK4 kinase activity is not required for low dose LPS-mediated NFκB activation. Cell lysates from wild-type (WT) and IRAK4 kinase activity inactive knockin (IRAK4 KI) bone marrow-derived macrophages (BMDMs) untreated or treated with low dose LPS (100 pg/ml) for the indicated times were analyzed by Western blot analysis. The experiments were repeated for three times with similar results.

Suppl. Fig. 3



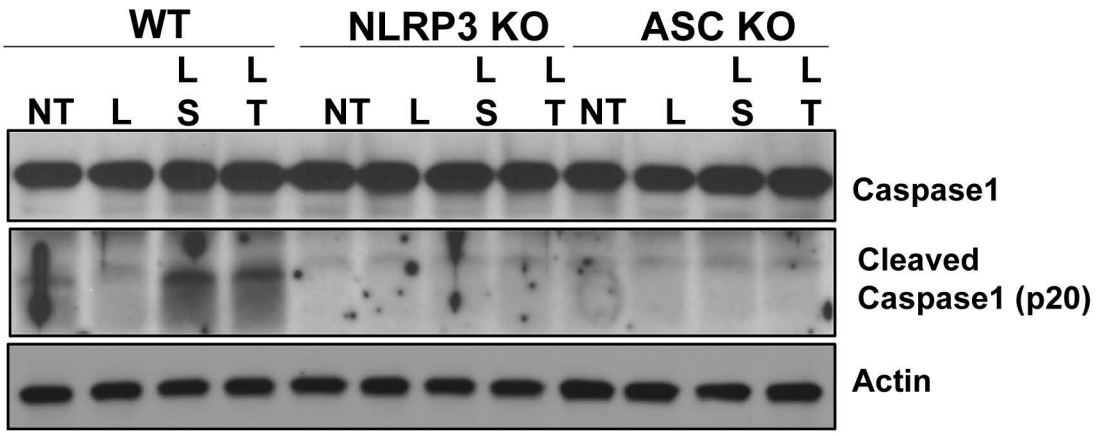
Suppl. Fig. 3 Mincel and IRAKM expression in primary Kupffer cells and hepatocytes. Primary Kupffer cells and hepatocytes from WT and IRAKM KO mice were treated with LPS (100 pg/ml) or IL-1 (10 ng/ml) for the indicated times. The cell lysates were collected and analyzed by the Western blot analysis. The experiments were repeated for three times with similar results.

Suppl. Fig. 4



Suppl. Fig. 4. **Ethanol-induced cell death in primary hepatocytes.** Primary hepatocytes from WT mice were treated with EtOH (50 mM) for 24 hours.. Cytotoxicity was measured utilizing a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay kit. The experiments were repeated for three times with similar results. Data represent mean ± SEM; *, $P<0.05$.

Suppl. Fig. 5



Suppl. Fig. 5 SAP130/TDM induced inflammasome activation is dependent on NLRP3 and ASC. BMDMs from WT, NLRP3 KO and ASC KO mice were treated with PBS (NT), LPS (100 pg/ml) for 24 hours (L), LPS (100 pg/ml, 24 hours) + SAP130 (5 µg/ml, 6 hours) (LS) or LPS (100 pg/ml, 24 hours) + TDB (100 µg/ml, 6 hours). Cell lysates and supernatants were collected together and were immunoblotted with the antibodies against caspase1, cleaved caspase1 (p20) and actin. The experiments were repeated for three times with similar results.

Supporting Information

Animal procedures. All mice were on a C57BL/6 background. IRAKM KO and Mincle deficient mice were bred in house and their respective littermate controls were used as wild-type (WT) mice. Female mice (10-12 weeks old) were randomized into ethanol-fed and pair-fed groups and then adapted to a control liquid diet for 2 days. Mice were housed in shoe-box cages (2 animals/cage) with microisolator lids. Standard microisolator handling procedures were used throughout the study.

Biological Reagents and Cell Culture. LPS (Escherichia coli 055:B5) and ATP (A2383) were purchased from Sigma-Aldrich. TDB was purchased from Invivogen. Antibodies against phosphorylated IκBa(Ser32/S36), JNK, IKKa/b(Ser176/180), SYK(Tyr323), and total IκBa were purchased from Cell signaling. Antibodies to IRAK-4 and SYK were purchased from Enzo Life Science. Antibody to FLAG (anti-FLAG) and hemagglutinin (anti-HA) were purchased from Sigma-Aldrich. Antibody to MEKK3 was purchased from BD Bio-sciences Pharmingen. Antibody to IRAKM was purchased from Novus Biologicals. Antibody to SAP130 was purchased from Abnova. Antibodies against IRAK1, TAK1 and Actin were from Santa Cruz Biotechnologies. Anti-IL-1β antibody (3ZD) was obtained from the Biological Resources Branch of the NIH. Anti-caspase-1 (p20) antibody was generated as previously described by L. Franchi and G. Nunez (1). Anti-Mincle antibodies were generated as previously describes (2, 3). V5-tagged recombinant SAP130 was overexpressed in HEK-293 cells and purified as previously described (2). Bone-marrow derived macrophages were obtained from the bone marrow of tibia and femur by flushing with DMEM. The cells were cultured in DMEM supplemented with 20% fetal bovine serum (FBS), penicillin G (100 µg/ml), streptomycin (100

μg/ml) with GM-CSF (20 ng/ml) (Fig. 1A, 2A, 4A, 4B) or 30% L929 condition medium (Fig. 1C, 1E, 2B, 2C, 2D, 6B, 6C).

Plasmids and retroviruses. Mouse IRAKM and IRAK1 cDNA were purchased from Open Biosystems. IRAK1/IRAKM chimeric constructs were generated by overlapping PCR. The wild-type IRAK1, IRAKM and IRAK1/IRAKM chimeric mutant were cloned into pMX retroviral expression vector and transfected into phoenix cell for viral packaging. Immortalized IRAK1/2/3 triple deficient BMDMs were infected by the packaged retrovirus for 3 days and selected by puromycin (2μg/ml) for 2days for stable viral integration. For all PCR reactions high fidelity Pfu Turbo polymerase was used (Stratagene).

Inflammasome activation. After stimulation of BMDM, as described in Figure legends, cell-free supernatant was then either collected for ELISA analysis, or cell lysates and supernatants were collected together for immunoblotting by the addition of 1% Nonidet-P40 supplemented with complete protease inhibitor 'cocktail' (Roche) and 2 mM dithiothreitol directly to the well. Cells were scraped and lysed on ice for 30 minutes, then spun at 13,000 rpm. Protein concentration was measured. 4X Laemlli buffer was then added, samples were boiled and approximately 20 μg of sample was run on a 15% SDS-PAGE gel.

Quantitative real-time PCR. Total RNA was isolated using TRIzol reagent (Invitrogen). 3μg of total RNA was then used for reverse transcription reaction using SuperScript-reverse transcriptase (Invitrogen). Q-PCR was performed in AB 7300 RealTime PCR System, and the gene expression of mouse CXCL1 (KC), TNFα, IL-6, MCP-1 Mincle, 18s RNA and β-actin was examined by SYBR® GREEN PCR Master Mix (Applied Biosystems). PCR amplification was

performed in triplicate, and water was used to replace cDNA in each run as a negative control. The reaction protocol included pre-incubation at 95°C to activate FastStart DNA polymerase for 10 min, amplification of 40 cycles that was set for 15 s at 95°C, and annealing for 60 s at 60°C. The results were normalized with the housekeeping gene β-actin or 18s RNA.

ELISA assay. Supernatants from cell cultures were collected and measured for the level of mouse cytokines IL-1β, CXCL1, IL-6 and TNFα using Duoset ELISA kits (R&D system) according to manufacturer's instructions.

Sample collection. At the end of the feeding protocols, mice were anesthetized, blood samples taken into non-heparinized syringes from the posterior vena cava and livers excised. Portions of each liver were then either fixed in formalin or frozen in optimal cutting temperature (OCT) compound (Sakura Finetek) for histology, frozen in RNAlater (Qiagen) or flash frozen in liquid nitrogen and stored at -80°C until further analysis. Blood was transferred to EDTA-containing tubes for the isolation of plasma. Plasma was then stored at -80°C. Histopathology and immunohistochemistry of mouse liver: Formalin-fixed tissues were paraffin-embedded, sectioned, coded and stained with hematoxylin and eosin. Frozen liver sections were used for staining F4/80 and Mincle. All images presented in the results are representative of at least 3 images per liver and 4 mice per experimental condition.

ALT/AST and triglyceride measurement. Plasma samples were assayed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using commercially available

enzymatic assay kits (Diagnostic Chemicals) following the manufacturer's instructions. Total hepatic triglycerides were assayed using the Triglyceride Reagent Kit from Pointe Scientific Inc. (Lincoln Park, MI).

Immunohistochemistry. Freshly isolated liver tissues were buried with O.C.T. compound (Sakura Finetek) and froze in liquid nitrogen. Frozen liver sections were blocked with 2% bovine serum albumin (diluted in PBS) containing 0.1% sodium azide for 1 hour followed by overnight incubation with the primary antibody (1:1000 dilution for anti-Mincle and 1 : 50 for anti-F4/80) at 4 Celsius degree. After washing in PBS, slides were incubated with the fluorochrome-conjugated secondary antibody (Alexa fluor-488 labeled donkey-anti-rat IgG and Alexa fluor-594 labeled mouse-anti-rabbit IgG, or Alexa fluor-488 labeled mouse-anti-rabbit IgG and Alexa fluor-594 labeled donkey-anti-rat IgG 1:200 diluted in blocking buffer) for 2 hr in the dark at room temperature, washed again in PBS and mounted with VECTASHIELD containing anti-fade reagent (Vector Laboratories). Fluorescent images were acquired using a LEICA confocal microscope. No specific immunostaining was seen in sections incubated with PBS rather than the primary antibody.

Cell sorting. Liver single-cell suspensions were generated by using gentleMACS Dissociator (Miltenyi Biotec), according to manufacturer's protocol. Non-parenchymal mouse liver cells were isolated by gradient centrifugation using Histodenz (Sigma-Aldrich) under sterile conditions. Then the cells were stained in FACS buffer using APC-conjugated anti-CD11b and PE-conjugated anti-F4/80 from eBiosciences. After labeling, cell subsets were sorted on a

FACSAria (BD Biosciences). Sorted cells were washed with PBS immediately frozen at −80°C for RNA isolation.

Kupffer cell and hepatocyte isolation. The wildtype and IRAKM KO mice were anesthetized with pentobarbital sodium (30 mg/kg ip), and the portal vein was cannulated under aseptic conditions. The liver was subsequently perfused with EGTA solution (5.4 mM KCl, 0.44 mM KH_2PO_4, 140 mM NaCl, 0.34 mM Na_2HPO_4, 0.5 mM EGTA, and 25 mM Tricine, pH 7.2), and digested with DMEM containing 0.075% collagenase solution. Kupffer cells were isolated by differential centrifugation in Histodenz (Sigma). The cells were cultured overnight in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin G (100 µg/ml), and streptomycin (100 µg/ml), and then replaced with serum-free medium for 4 h, followed by stimulation with LPS for various time periods. Hepatocytes were isolated by differential centrifugation in Percoll (GE). The cells were cultured overnight in Williams' Medium E, containing 2 mM L-glutamine , 5% FBS, 100 nM insulin, 100 nM dexamethasone, penicillin G (100 µg/ml), and streptomycin (100 µg/ml)

.

Cytotoxicity assay. Cytoxicity was measured utilizing a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) based cytotoxicity assay kit (Sigma), following the manufacturer’s instructions.

Reference

1. Franchi L, Nunez G. The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1beta secretion but dispensable for adjuvant activity. Eur J Immunol 2008;38:2085-2089.

2. Yamasaki S, Ishikawa E, Sakuma M, Hara H, Ogata K, Saito T. Mincle is an ITAM-coupled activating receptor that senses damaged cells. Nat Immunol 2008;9:1179-1188.

3. Wells CA, Salvage-Jones JA, Li X, Hitchens K, Butcher S, Murray RZ, Beckhouse AG, et al. The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to Candida albicans. J Immunol 2008;180:7404-7413.