Supplementary Data

Materials and Methods

Gene targeting

The 5′ and 3′ homology arms of the targeting vector were amplified by long-range polymerase chain reaction (PCR) using the BAC clone RP23-401A2 (Invitrogen) as a template with the following primers: 5′ arm (4.3 kb), 5′-TTGGCG CGCCTGTGCTCTGCTGACCGTTTC-3′ and 5′-ACGCGTC GACTGAAGAGGCGCTTGCCGGCT-3′; 3′ arm (7.4 kb), 5′-CTAGCTAGCAGACAGGTATTACAGGCTGGC-3′ and 5′-CC GCTCGAGATCAGGAGTCATAGATGCAAG-3′ (restriction sites used for cloning are underlined). The amplified 5′ and 3′ arms were ligated into the *Asc*I-*Sal*I sites and *Nhe*I-*Xho*I sites of the PGK-Neo-pA/DT-A vector (http:// cdb.riken.jp/arg/cassette.html), respectively. The linearized targeting construct (digested with *Asc*I) was transfected into TT2 embryonic stem cells (5), and G418-resistant clones were screened by PCR using SC1 (5′-CTGGCTTGCTTCATATCGCTTGCTTGCTCA-3′, external to the targeting construct) and SC2 (5′-GGGCCAGCT CATTCCTCCCACTCATGATCT-3′, in the PGK-Neo cassette). Homologous recombination was verified by Southern blot analysis with the 5′ and 3′ flanking probes. Probe labeling, hybridization, and signal detection were carried out using the AlkPhos Direct labeling and detection system (GE Healthcare). Animals were genotyped by PCR using a mixture of three primers: GT1 (5′-AAACTACCTCGGAGAGCCAGGGGGTGA C-3′), GT3 (5′-CGACGAGGCTCAGTGCACGCTTAGATAG-3′), and SC2. The primer set GT1/GT3 was used for the detection of a 935-bp fragment specific for the wild-type allele, while the GT1/SC2 set amplified a 530-bp fragment of the mutant allele. Germline chimeras were obtained from two independent recombinant embryonic stem clones.

Northern blot and reverse transcription–polymerase chain reaction analysis

Northern blot analysis was carried out as described previously (1). The RNA blot was hybridized with mouse transmembrane thioredoxin-related protein (TMX) cDNA probes labeled with AlkPhos Direct (GE Healthcare). For reverse transcription–polymerase chain reaction analysis, 1 μg of RNA was reverse transcribed into cDNA using the SuperScript first-strand synthesis system (Invitrogen). The following primers were used for PCR amplification of mouse TMX, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal control: TMX (597 bp), 5′-TCCCTGGTGTC CTGCTTGTCA-3′ and 5′-CTATCTTCTGCCTCCTCTTCT-3′; GAPDH (807 bp), 5′-GGTCATCATCTCCGCCCCTTCTG-3′ and 5′-TTCAAGAGAGTAGGGAGGGCTCC-3′.

Reagents and antibodies

Recombinant mouse tumor necrosis factor-α (TNF-α) (T7539) was from Sigma. Rabbit anti-TMX polyclonal antibody was used as described previously (3). Rabbit polyclonal antibodies specific for ERp57 (SPA-585), calnexin (SPA-860), protein disulfide isomerase (SPA-890), and TNF receptor 1 (CSA-815) were obtained from Assay Designs. Mouse monoclonal antibinding protein (BD Biosciences) and mouse monoclonal anti-α-tubulin (Sigma) were used. Rabbit monoclonal anti-cleaved caspase-3 (Asp175, #9664), rabbit polyclonal anti-cleaved caspase-8 (Asp387, #9429), anti-poly(ADP–ribose) polymerase (#9542), anti-Bcl2-associated X protein (#2772), anti-FLICE inhibitory protein (#3210), anti-phospho-IκB-α (Ser32, #9241), anti-IκB-α (#9242), anti-phospho-SAPK/c-Jun N-terminal kinase (JNK) (Thr183/Tyr185, #9251), and anti-SAPK/JNK (#9252) were obtained from Cell Signaling. For flow cytometry, cells were stained with the following fluorescence-labeled antibodies: phycoerythrin (PE)-conjugated anti-mouse T cell receptor b (H57-597), anti-mouse CD11b (M1/70), anti-mouse CD19 (6D5), anti-mouse Toll-like receptor 4 (MTS510), fluorescein isothiocyanate (FITC)-conjugated anti-mouse major histocompatibility complex class I (28-14-8), and allophycocyanin-conjugated anti-mouse CD11b (M1/70) (eBioscience). PE-conjugated anti-mouse CD4 (H129.19) and FITC-conjugated anti-mouse CD8a (53-6.7) were obtained from BD Pharmingen. Anti-mouse TNF-α polyclonal antibody (P350; Thermo Scientific) was used for the neutralization studies in mice.

Neutralization of TNF-α in vivo

For neutralization of TNF-α, mice were intraperitoneally injected with 100 μg of anti-TNF-α antibody in a volume of 110 μl per animal. At 6 h after administration of the antibody, mice were challenged with lipopolysaccharide (LPS) (3 ng/body) and D-(+)-galactosamine (10 mg/body).

Preparation of resident peritoneal macrophages

Cells were harvested from mouse peritoneal cavities and cultured in an RPMI-1640 medium (Sigma) with 10% fetal calf serum (Invitrogen) for 2 h on temperature-responsive RepCell plates (CellSeed). After washing with a prewarmed culture medium to remove nonadherent cells, the RepCell plates were cooled to room temperature; the detached cells collected comprised the macrophage-enriched fraction. Macrophages were plated at 1×10^6 cells per well in 24-well culture plates and incubated for 24 h before treatment with LPS.

Preparation of fibroblasts from mouse tail-tips

Tail-tip fibroblasts (TTFs) were prepared as described previously (4), and maintained in the Dulbecco's modified Eagle's medium with 10% fetal calf serum. TTFs were treated with 10 ng/ml TNF-α for different times as indicated in the figure, and lysed in the lysis buffer supplemented with the PhosSTOP phosphatase inhibitor cocktail (Roche).

Microarray analysis

Total RNA was isolated from liver samples using miRNeasy kit (Qiagen). Fluorescent-labeled probes were prepared and hybridized to 3D-Gene Mouse Oligo chip 24k (Toray). Microarray data have been deposited in the NCBI

Gene Expression Omnibus (GEO) database (accession number: GSE28438).

Preparation of TMX-conjugated magnetic beads

Catalytic thioredoxin-like domain of TMX was produced as a secreted protein in FreeStyle 293-F cells (Invitrogen) cultured in a serum-free medium. The plasmid encoding N-terminal portion of TMX [pcDNA3.1-TMX140-mycHis (2)] was transfected into 293-F cells using FreeStyle MAX Reagent, and cells were incubated at 37°C in a humidified atmosphere of 8% CO_2. Forty-eight hours after transfection, protein was purified from culture supernatants using Ni-NTA agarose (Qiagen). Purified protein ($\sim$15 kDa) was dialyzed against the binding buffer (10 m*M* Hepes, 50 m*M* KCl, 1 m*M* ethylenediaminetetraacetic acid, and 10% glycerol), and protein purity was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Coomassie staining. Tosyl-activated magnetic beads (Tamagawa Seiki) loaded with TMX protein were incubated for 17 h at 4°C, and the beads were further incubated overnight in 1 *M* Tris–HCl, pH 8.0, to deactivate remaining free tosyl groups. The resulting TMX-conjugated beads were resuspended in a binding buffer and stored at 4°C until used.

Purification of TMX-binding proteins

Subcellular extraction of liver samples was performed using a ProteoExtract subcellular proteome extraction kit (S-PEK; EMD Biosciences) following the manufacturer's instructions with slight modifications. Briefly, fragmented tissue from a wild-type mouse was dounce-homogenized in S-PEK extraction buffer I and centrifuged at 16,000 *g* for 10 min. The precipitates were resuspended in extraction buffer II and incubated for 20 min at 4°C, followed by centrifugation at 20,000 *g* for 30 min to pellet insoluble material. The resulting supernatant was collected as a membrane-enriched fraction used for affinity purification of TMX-binding proteins. Control or TMX-conjugated beads were added to the liver extracts and incubated for 4 h at 4°C. After washing with S-PEK extraction buffer II, proteins were eluted with 1 × lithium dodecyl sulfate sample buffer with 50 m*M* dithiothreitol. Eluted proteins were resolved in 4–12% NuPAGE Bis-Tris gel (Invitrogen), and visualized by SYPRO Ruby staining. Selected protein bands were subjected to tryptic digestion and matrix assisted laser desorption ionization time of flight mass spectrometry analysis.

Supplementary References

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