

Supporting Information

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SI Materials and Methods

Patient Samples. Patients and controls were included in this study after giving informed consent under the National Institute of Arthritis and Musculoskeletal and Skin Diseases approved clinical research protocol 94-AR-0105. Patients who had TNF receptor-associated periodic syndrome (TRAPS) with structural mutations were heterozygous for structural TRAPS-associated mutation in *TNFRSF1A* (H22Y, C33Y, T50M, C33G, C52G, C52F, C30Y, or del c.193–14). Median age was 40.5 y (range 3–48 y). Patients with nonstructural TNFR1 mutations were heterozygous for TNFRSF1A R92Q or P46L mutations. All patients were asymptomatic and on prescribed medications at the time of blood collection and for at least 2 weeks preceding and following the collection. Peripheral blood mononuclear cells (PBMC) were collected by Ficoll separation.

TNFR1-Mutant Mice and Mouse Embryonic Fibroblasts. A targeting vector was prepared containing nucleotide mutations to produce the T50M and C33Y mutation in the mouse *tnfrsf1a* gene encoding TNF receptor 1 (TNFR1) (Fig. S1). ES cells harboring this mutation were selected and injected into blastocysts. Lines carrying this mutation were crossed to the E2A-Cre strain to remove the neomycin selection cassette and backcrossed to C57BL/6 for at least six generations before intercrossing to generate homozygous and heterozygous mutant mice. Presence of the mutant TNFR1 alleles was confirmed by resequencing cDNA from the final lines. Genotyping was performed with primers flanking the remaining LoxP site: T5GENF2, 5'-tgtgaaaccgtaccagat-3', and T3GENR3, 5'-cttggggacacaagctatcc-3'. TNFR1-knockout mice on a C57BL/6 background (tm1Imx) were obtained from Jackson Laboratory. Mouse embryonic fibroblasts (MEF) were generated from embryonic day 12–13 embryos of each genotype following established protocols. MEF homozygous for the C33Y and T50M TNFR1 mutations grew slowly with abnormally enlarged cell morphology and were found to be prematurely senescent as indicated by enhanced staining with β -galactosidase at neutral pH (1). Heterozygous TNFR1-mutant MEF did not display morphological features of senescence nor senescence-associated β -galactosidase staining (data not shown). Because of this possible confounding factor, we did not study homozygous TNFR1-mutant MEF. TNFR1-knockout MEF were a gift from Zheng-Gang Liu (National Cancer Institute, National Institutes of Health). All procedures in mice were carried out according to approved National Institute of Health animal protocols.

Cell Stimulation and Cytokine Assays. PBMC were isolated from patients and controls by Ficoll isolation from a venous blood sample as described above and were incubated in DMEM medium plus 10% heat-inactivated FCS with or without indicated concentrations of LPS (Ultrapure *Salmonella Minnesota* R595; List Biological Laboratories, Inc). After 24 h, supernatant was collected, centrifuged to remove cells and debris, and stored at -80°C for later analysis. Mouse peritoneal macrophages were collected by established methods from unmanipulated mice or 3 days after i.p. injection of 2 mL thioglycollate. Cytokine analysis was performed using BD Biosciences or Bio-Rad cytokine bead arrays according to the manufacturers' instructions. For LPS challenge and cytokine measurements, mice received an i.p. injection of the indicated amounts of LPS (*Escherichia coli* 0127: B8 LPS; Sigma L3129) at the indicated concentrations. For the LPS-D-galactosamine lethality study, animals were injected i.p. with a combination of *E. coli* LPS at the indicated concentrations and 20 mg/kg D-galactosamine (Sigma G0500).

Quantitative RT-PCR. Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen). First-strand cDNA was prepared using TaqMan Reverse Transcription Kit (Applied Biosystems). Equal amounts of cDNA were subjected to PCR to quantitative Bip, XBP-1, Chop, IL-6, TNFR1, and Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES) gene expression with the use of an ABI PRISM 7700 sequence-detection system. Predesigned primer/probe sets for each gene were purchased from Applied Biosystems. Probes specific for β 2-microglobulin or 18S rRNA were used as internal controls. Each measurement in WT cells without treatment was normalized to expression of β 2-microglobulin or 18S rRNA (delta Ct); then measurements of untreated WT and treated cells were compared (delta-delta Ct). The inverse log of the delta-delta Ct then was calculated to give the relative fold change.

Western Blot Analyses. Cells were lysed in buffer containing 50 mM Tris at pH 7.4, 0.5% Triton X-100, 300 mM NaCl, 2 mM EDTA, 0.4 mM sodium vanadate, and protease inhibitor mixture (Roche). Forty micrograms of total protein were applied to each lane and subjected to SDS/PAGE and Western blotting via enhanced chemiluminescence (GE Healthcare). The following primary antibodies were used: anti-mouse TNFR1 (AF-425-PB; R&D Systems), JNK1 (554286; BD Pharmingen); phospho-JNK (Thr183/Tyr185, 9251), phospho-p38Thr180/Tyr182 (9215), phospho-ERK Thr202/Tyr204 (9101), p38 (9212), and ERK (9102) from Cell Signaling Technology; anti-human TNFR1 (H5, sc-8436), I κ B- α (sc-371), and Hsc 70 (B₆, sc-7298) from Santa Cruz Biotechnology Inc.; and actin (MAB1501R; Chemicon International). Multiple exposures were taken to select images with density within the dynamic range using Fuji Image Reader LAS-3000 software, and band intensity was quantified using Multi Gauge software (Fujifilm Global). Bands of interest were normalized to the corresponding nonphosphorylated bands or actin as indicated.

Analysis of Interferon Regulatory Factor 3 Dimerization. Native PAGE was performed essentially as previously described (2) using 7.5% Tris-HCl polyacrylamide gels. The gel was pre-run with 25 mM Tris and 192 mM glycine, pH 8.4, with 1% deoxycholate in the cathode chamber for 30 min at 40 mA. Cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and protease inhibitors then were ultracentrifuged at 435,000 \times g for 5 min to remove particulate debris. Fifteen micrograms of protein were loaded in native sample buffer (62.5 mM Tris-Cl, pH 6.8, 15% glycerol) and electrophoresed for 60 min at 25 mA. Interferon regulatory factor 3 (IRF3) monomers and dimers were visualized by Western blotting with anti-IRF3 (sc-9082; Santa Cruz Biotechnology Inc.).

NF- κ B and Cell Survival Assays. NF- κ B activation was analyzed and quantified by measuring ELISA-based DNA-binding activity of p65 using a TransAm transcription factor assay kit (Active Motif). Cell survival was assayed by measuring mitochondrial dehydrogenase activity via a method of transcriptional and translational (MTT) assay (ATCC).

Temperature Measurement. To monitor body temperature continuously, PDT4000 E-mitter temperature transmitters (Mini Mitter Company) were implanted surgically into the peritoneum of male mice. After at least 1 week of recovery, body temperature was recorded every 30–60 min, with ambient room temperature maintained at 30 $^{\circ}\text{C}$ with a 12-h light/dark cycle.

1. Itahana K, Campisi J, Dimri GP (2007) Methods to detect biomarkers of cellular senescence: The senescence-associated beta-galactosidase assay. *Methods Mol Biol* 371:21–31.

2. Iwamura T, et al. (2001) Induction of IRF-3/-7 kinase and NF-kappaB in response to double-stranded RNA and virus infection: Common and unique pathways. *Genes Cells* 6:375–388.

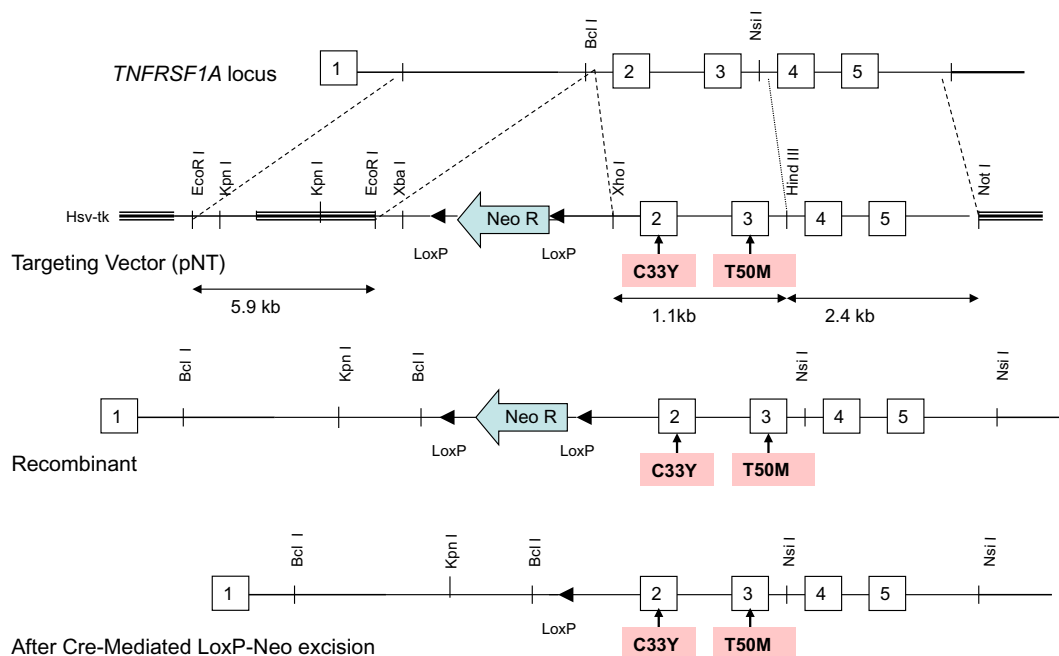


Fig. S1. Construction of targeted *TNFRSF1A* alleles. A 1.1-kb *Xho*I/*Hind*III fragment containing the C33Y point mutation in exon 2 or the T50M mutation in exon 3 was generated by site-directed mutagenesis. These fragments were ligated to a 2.3-kb fragment extending from intron 3 to intron 5. This cassette was ligated into the pTNT targeting vector to form the 3.3-kb 3' homology arm. A 5.1-kb 5' homology arm containing exon 1 and intron 1 was inserted upstream of the Neo/*loxP* selection cassette. The linearized targeting vector was introduced into ES cells by electroporation, and 170 colonies resistant to G418 were selected. PCR using two sets of primers outside the 5' and 3' end of the construct and in the Neo cassette identified two independent ES cell clones with the mutation in *tnfr1* targeted by homologous recombination (Recombinant). The mutant *TNFR1* allele after removal of the Neo cassette by crossing to Cre-expressing mice is shown at the bottom. Mutant mice were identified by PCR using the nucleotides encoded by the *loxP* sequence as an indicator of the mutant allele. Both mutations are shown here on the same vector for illustrative purposes, but the two strains were made independently, with two separate founder lines generated for each allele.

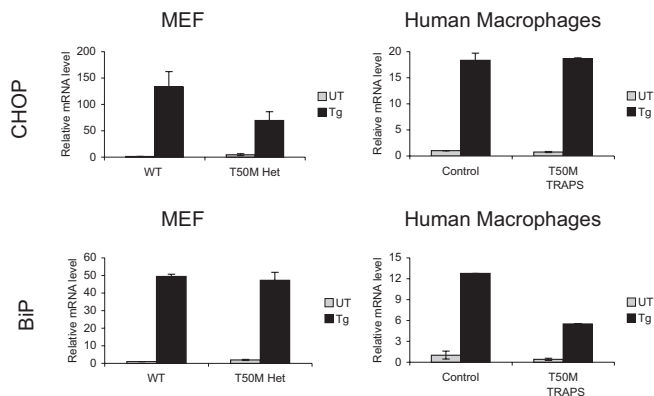


Fig. S2. Normal endoplasmic reticulum (ER) stress responses in cells harboring TRAPS-associated *TNFR1* mutations. Induction of the ER stress-responsive genes *CHOP* and *BiP* were compared between WT and T50M heterozygous (T50M Het) MEF (Left) or monocyte-derived macrophages from TRAPS patients heterozygous for the T50M mutation (Right) treated (Tg) or not treated (UT) with 10 μ M thapsigargin for 6 h. Gene expression measured by quantitative RT-PCR with species-specific probes for *BiP* or *CHOP* and normalized to 18S rRNA. Fold changes were calculated as described in *SI Materials and Methods*. The data are averages of values from two independent experiments.

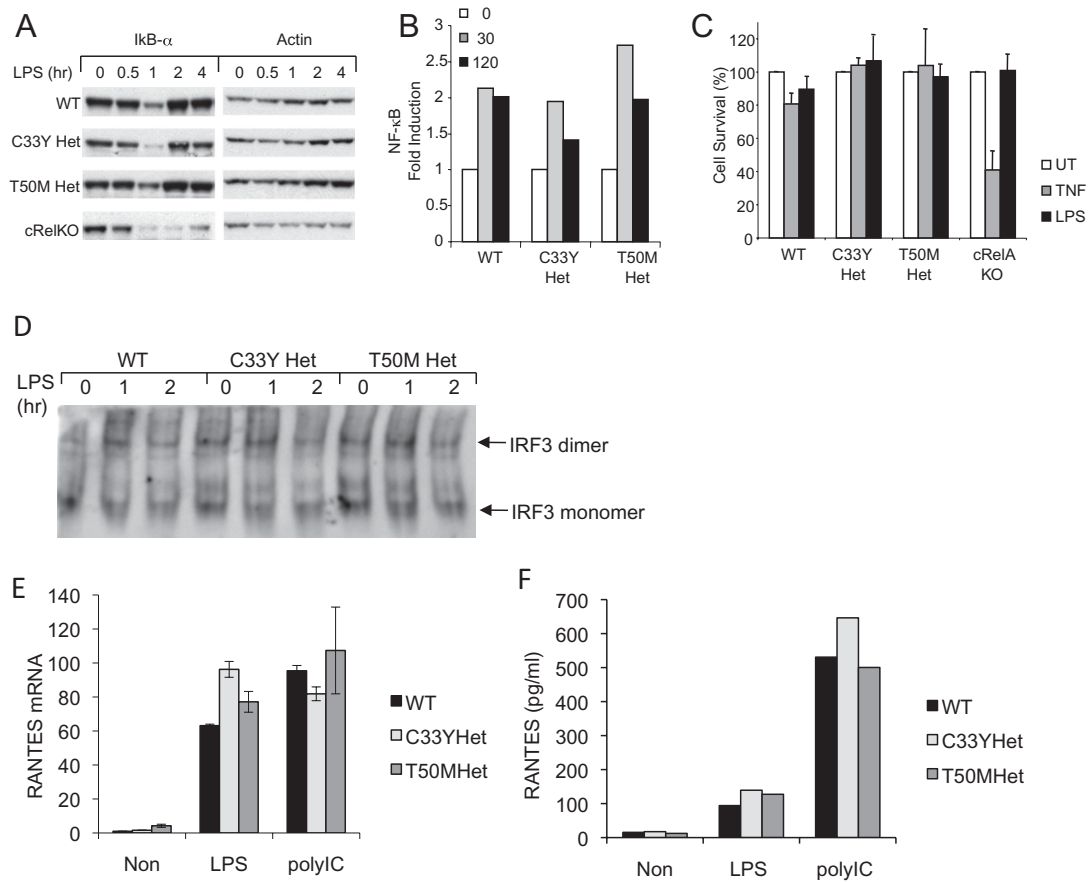


Fig. S3. Normal IRF3 activation and RANTES induction in heterozygous TNFR1-mutant cells. (A) Kinetics of NF- κ B activation in TNFR1-mutant MEF. Levels of I- κ B α were analyzed at the indicated times after LPS treatment of MEF of the indicated genotypes. (B) Fold induction of NF- κ B nuclear activity as measured by an ELISA for NF- κ B p65 binding to specific oligonucleotides in nuclear extracts from MEF of the indicated genotypes. (C) Cell death was measured in MEF of the indicated genotypes treated with TNF (30 μ g/mL) or LPS (100 ng/mL) for 24 h, and cell viability was quantitated by MTT assay after normalization to 100% for untreated control cells. (D) Resident peritoneal macrophages pooled from six mice of the indicated genotype were stimulated with 100 ng/mL LPS for 0, 1, or 2 h. Protein lysates were applied to native gels followed by Western blot analysis using anti-IRF3 antibody to visualize IRF3 monomers and dimers. (E) RANTES mRNA was measured by quantitative RT-PCR in resident peritoneal macrophages pooled from six mice of the indicated genotype followed by treatment with 100 ng/mL LPS or 50 μ g/mL polyIC for 4 h. The fold change in RANTES mRNA relative to WT unstimulated cells is shown. (F) RANTES was measured in the supernatant of cell cultures of resident peritoneal macrophages pooled from six mice of the indicated genotype after treatment with 100 ng/mL LPS or 50 μ g/mL polyIC for 24 h.

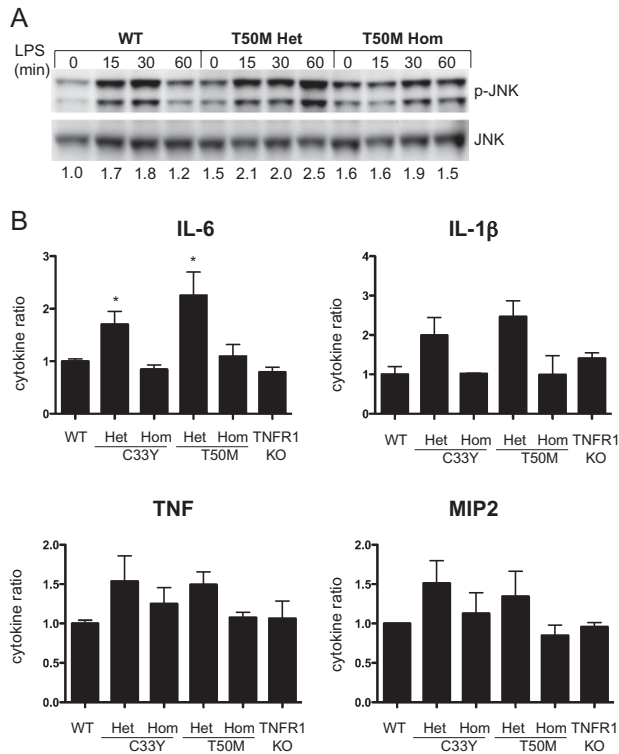


Fig. 54. Signaling and cytokine secretion abnormalities in immune cells from TNFR1-mutant mice. (A) Kinetics of JNK phosphorylation after LPS treatment of splenocytes from mice of the indicated genotypes. Densitometric analysis of JNK phosphorylation is shown for each time point. (B) Cytokine and chemokine secretion by resident peritoneal macrophages from mice of the indicated genotype stimulated with 100 ng/mL LPS. For measurement of IL-1 β , 5 mM ATP was added for the final 15 min of stimulation. TNF was measured at 2 h and other cytokines at 6–24 h. Data are compiled from three independent experiments with the average amount of cytokine secreted by WT cells in each experiment normalized to 1.0. Ranges of cytokines produced by WT macrophages were IL-6 1,612–14,000 pg/mL for IL-6; 460–4,417 pg/mL for IL1 β ; 104–993 pg/mL for TNF; and 4,619–9,677 pg/mL for MIP-2. *, comparisons to WT cells with $P < 0.05$ by unpaired Student's *t* test. Hom, homozygous.

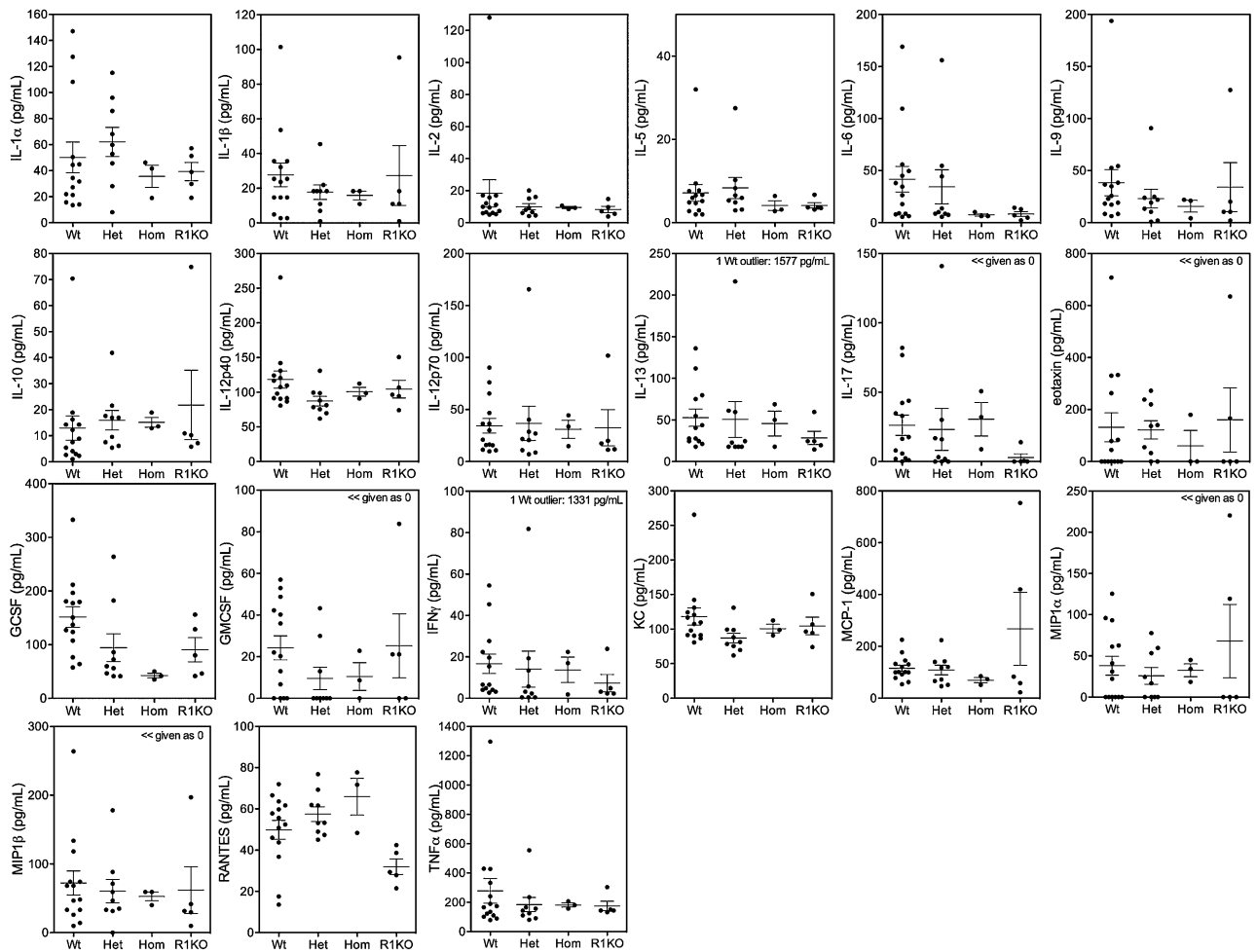


Fig. 55. Multiplex analysis of serum cytokines in TRAPS-knockin mice. Blood samples were taken randomly from unstimulated mice of the indicated genotypes. Twenty-three cytokines were measured; IL-3 and IL-4 were below detection in all mice and therefore have been left out from the figure. No significant differences from values in WT mice were seen in any of these cytokine comparisons. R1KO, TNFR1-knockout mice.

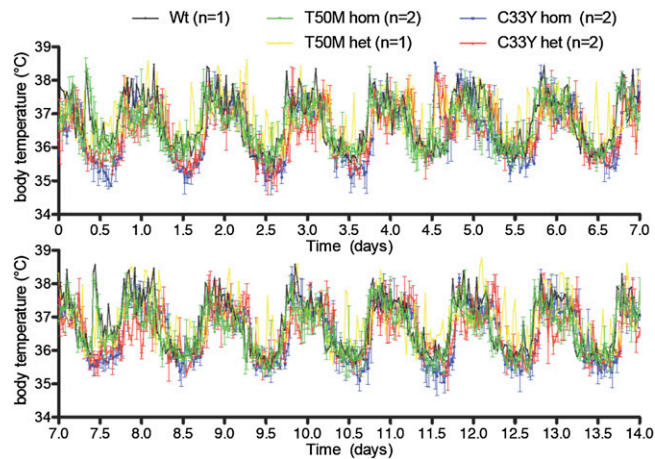


Fig. 56. Baseline body temperature measurements in T50M and C33Y TNFR1-mutant mice. Body temperature in cohorts of mice of the indicated genotypes ($n = 2$) were measured every 30 min with i.p. implanted chips over the course of 21 days. Average body temperature of mice of each genotype is shown for each measurement, with days measured midnight to midnight. Data are representative of three independent groups of mice monitored continuously for up to 28 days.

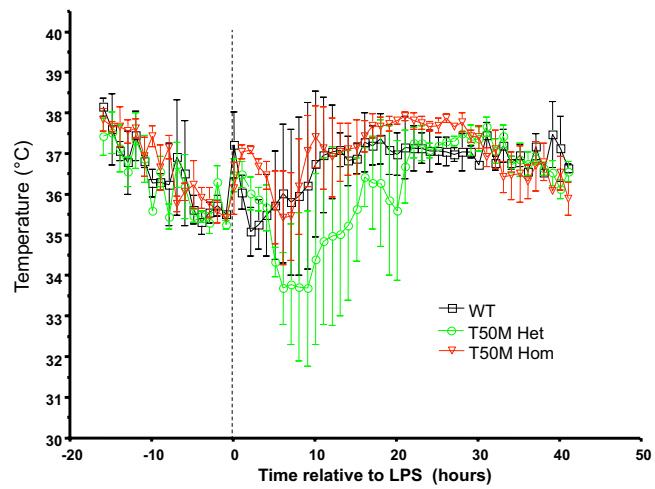


Fig. S7. Exaggerated temperature response to LPS in heterozygous TNFR1-knockin mice. TNFR1 T50M-knockin mice were injected with 2.5 μ g LPS/g body weight, and body temperatures were monitored with i.p. implanted microchips. Data show average \pm SEM for groups of two or three mice per genotype. Dashed line indicates time of injection (0 hour = 3 PM actual time). The temperature curve of heterozygous mice was significantly different from that of the other genotypes ($P = 0.0463$) by two-way ANOVA.

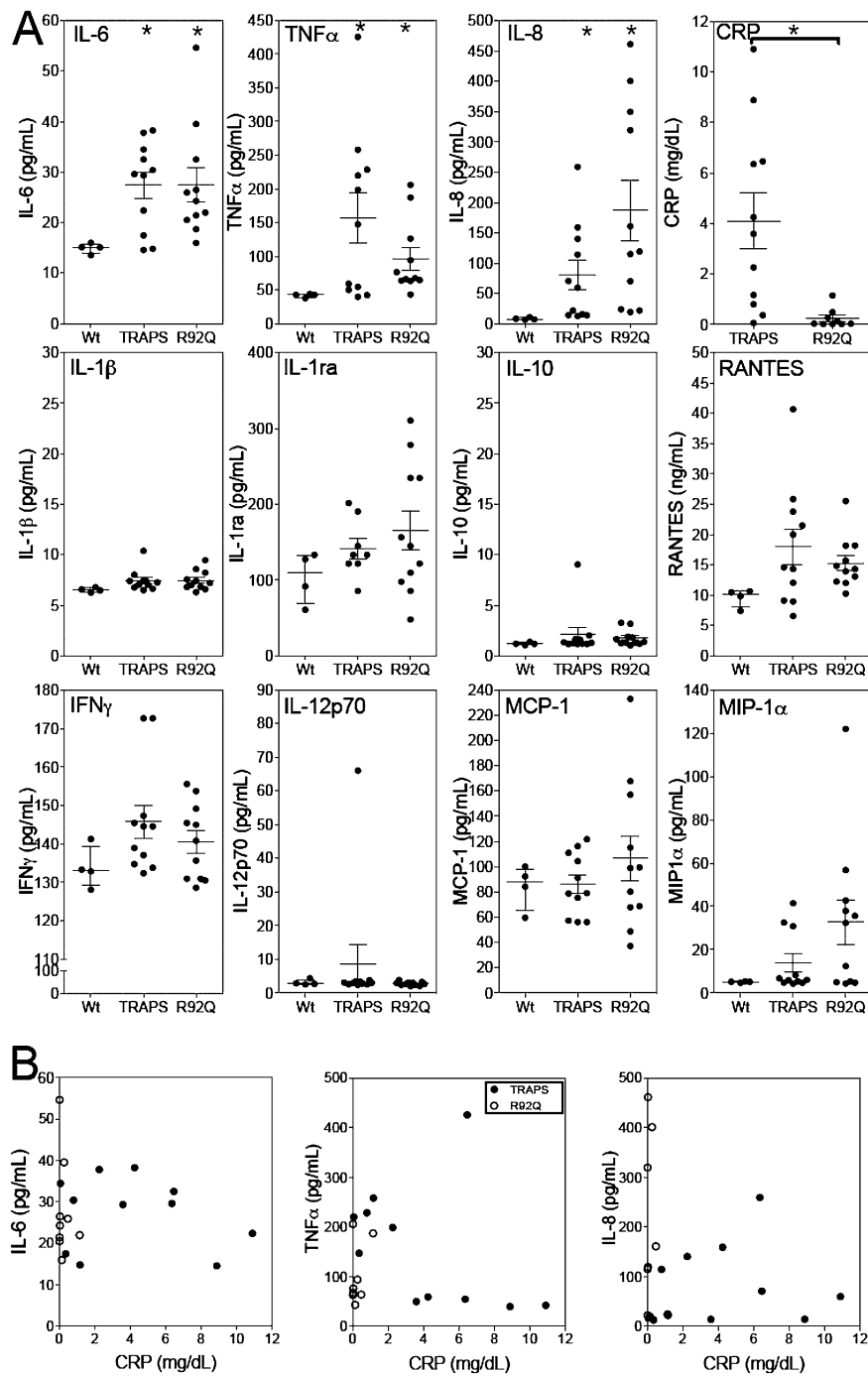


Fig. S8. Serum cytokine measurements in TRAPS patients. (*A*) Serum samples were collected from 11 TRAPS patients (TRAPS), 11 patients with R92Q or P46L TNFR1 polymorphisms (R92Q), and 4 healthy volunteers (Wt) and were analyzed by multiplex cytokine analysis. *, $P < 0.05$ by Mann-Whitney comparison with normal donors. (*B*) Lack of correlation of IL-6, IL-8, and TNF with C-reactive protein (CRP) concentration in these patients.

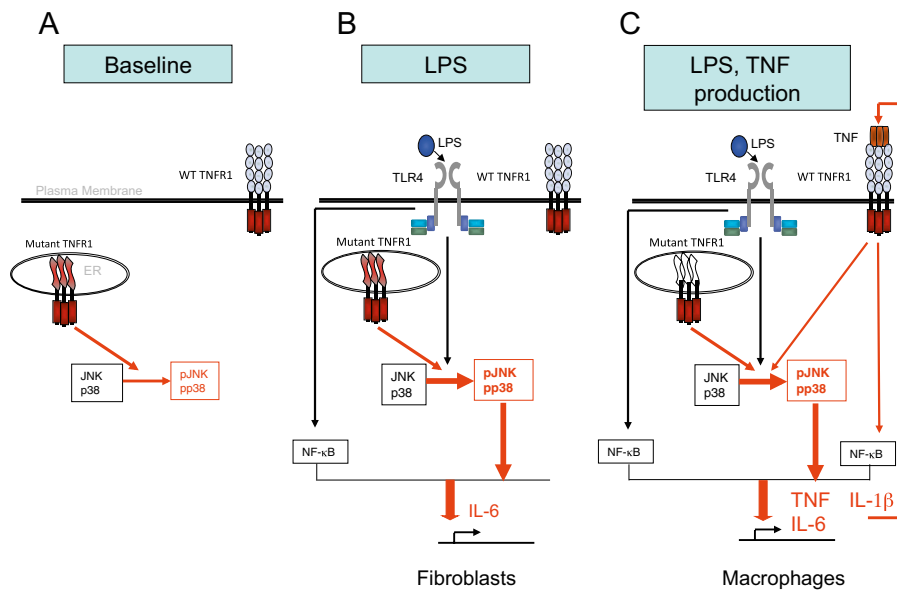


Fig. S9. Functional cooperativity between WT and mutant TNFR1 in TRAPS. (A) MAPK is activated by ER-retained mutant TNFR1. However, this activation is not sufficient to trigger spontaneous cytokine production. (B) After LPS stimulation, signals generated through TLR4 synergize with the elevated MAPKs and generate excess cytokine production, principally IL-6 in fibroblasts. (C) In immune cells, autocrine or paracrine TNF signaling through the WT TNFR1 is necessary for maximal cytokine production in heterozygous TNFR1-mutant cells.