Supporting Information

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SI Materials and Methods

Generation of the Targeting Vector for *Brd4*-CKO Mice. DNA fragments for homologous arms of the *Brd4* targeting vector were amplified from 129s7/AB2.2 BAC clone bMQ74P02. Primers for the 5′ 3.5-kb arm of *Brd4* were 5′-CCG CTC GAG GCC AGT TAT CAG TGT CTC TTA CTT-3′ and 5′-AAC TCC CGG GGT GGT TGT GTG ATG GGA TAA G-3′; primers for the LoxP-flanked 1.35-kb fragment containing exon 1 of *Brd4* were 5′-CGG GAT CCC ATT CTC TAA AGG TCT GTA GG-3′ and 5′-CGG GAT CCT CCA CCT GCC TCT GCC TCC-3′; primers for the 3′ 3.0-kb arm of *Brd4* were 5′-CAG ATA TCT TTC TGA GTT CAA GGC CAG CC-3′ and 5′-AGG ATT TAA ATA TTA GTG ATG CCT GTA CGC GC-3′. The amplified fragments were sequentially cloned into ploxPFlpneo (a gift from James Shayman, University of Michigan, Ann Arbor, MI). The PGK-DTA cassette was derived from pBS-DTA.

Targeting Strategy for the *Brd4*-CKO Mice. The targeting vector was linearized with SwaI and electroporated into C57/6J ES cells. ES cell clones were screened for homologous recombination by long-range PCR. Correctly targeted ES cell clones were injected into C57 blastocysts to generate chimera mice followed by back-crossing to wild-type C57BL/6J for the generation of heterozygous *Brd4*-CKO mice. The latter then were intercrossed to obtain homozygous mutant. The neo cassette was removed by crossing to FLP mice.

The PCR primers used to screen for positive ES cell clones are as follows:

5′ homologous recombination of *Brd4* (a PCR fragment of 5,502 bp): F1′ 5′-CCC GAT TCC TTA CTA CTT TTT TAG AC-3′ (outside the 5′ homologous arm); R1 5′-CGG TGG GCT CTA TGG CTT CTG AG-3′ (*Neo* specific)

3′ homologous recombination of *Brd4* (a PCR fragment of 3,277 bp): F2 5′-CAG ACT GCC TTG GGA AAA GCG CCT-3′ (*Neo* specific); R2 5′-GAT GGA AAT TCA CAT GCT AAC AGG-3′ (outside the 3′ homologous arm)

Mice were genotyped by PCR using following primer sets: *Brd4* WT allele (430-bp PCR fragment): F1: 5′-CAT CCA ATC ACG AGA TCT GAC TCC-3′; R1: 5′-GCT GCA GTA ATC TCT GTA CAG G-3′

Brd4-null allele (582-bp PCR fragment): F1: 5′-CAT CCA ATC ACG AGA TCT GAC TCC-3′; R1: 5′-GCT GCA GTA ATC TCT GTA CAG G-3′

Or *Brd4* WT allele (361-bp PCR fragment): F3: 5′-GTG CAT AGG CCT TAT CTT AAT G-3′; R3: 5′-CTC AGT GGT AGA GTG CAT GC-3′

Brd4-null allele (485-bp PCR fragment): F3: 5′-GTG CAT AGG CCT TAT CTT AAT G-3′; R3: 5′-CTC AGT GGT AGA GTG CAT GC-3′

Mouse Infection Model. Group B *Streptococcus* strain O90R was grown in brain heart infusion broth (BHI) (CM1135; Oxoid) medium. Mice were i.p. administered with a bacterial suspension in PBS. At the indicated time after infection, the mice were killed, and their organs were collected. The organs were homogenized, and dilutions were plated onto agar plates to measure the cfu of the surviving bacteria.

Preparation of BMDMs. BMDMs were generated as described (43). Briefly, bone marrows were isolated from tibia and femur of mice. For differentiation of BMDMs, bone marrow cells were cultured in DMEM/F12 with 10% FBS, L-glutamine, penicillin/streptomycin, Hepes, and 20% conditioned medium of L929 cells. Macrophages were stimulated with 100 ng/mL LPS (*E. coli* O111:B4; L2630; Sigma). For in vitro infection with bacteria, cells were grown in medium without penicillin/streptomycin.

Antibodies and Inhibitor. Primary antibodies used were anti-Brd4 (A301-985A; Bethyl Laboratories, Inc.); anti-IκBα (C-21) (sc-371; Santa Cruz Biotechnologies); anti–NF-κB p65 (A) (sc-109; Santa Cruz Biotechnologies); anti–β-tubulin (T0198; Sigma); anti–Phospho-Mnk (Thr197/202) (2111S; Cell Signaling Technology); anti-Mnk2 (sc-6964; Santa Cruz Biotechnologies); anti–phospho-eIF4E (Ser209) (9741; Cell Signaling Technology); anti-eIF4E (9742; Cell Signaling Technology); anti-HDAC1 (sc-7872; Santa Cruz Biotechnologies); Phospho-MAPK Family Antibody Sampler Kit (9910; Cell Signaling Technology); MAPK Family Antibody Sampler Kit (9926; Cell Signaling Technology); 4E-BP Antibody Sampler Kit (9955; Cell Signaling Technology); anti–phospho-p70 S6 kinase (Thr389) (9205; Cell Signaling Technology); and anti-p70 S6 kinase (9202; Cell Signaling Technology). The Mnk2 inhibitor used was cercosporamide (45-005-00U; Fisher Scientific).

Flow Cytometry. Mice were i.p. injected with 5 mg/kg LPS (*E. coli* O111:B4; Sigma). Twenty-four hours later, mice were anesthetized with ketamine and xylazine. To prepare single-cell suspensions, total lung tissue was harvested, digested in 1640 medium containing 0.13 mg/mL Liberase Blendzyme (05401119001; Roche) and 20 U/mL DNase (10104159001; Roche), and filtered through a 40-μm cell strainer. Single-cell suspensions of the lungs were incubated with anti-mouse CD16/CD32 Fc Block (2.4G2, 553141; BD) to prevent nonspecific antibody binding. For flow cytometry, cells were stained with the following antibodies: Brilliant Violet 605 anti-mouse CD11c (N418, 117333; BioLegend), anti-mouse F4/80 antigen PE (BM8, 12-4801; eBioscience), anti-mouse CD11b APC-eFluor 780 (M1/70, 47-0112; eBioscience), and anti-mouse Ly-6G (Gr-1) FITC (RB6-8C5, 11-5931; eBioscience). For exclusion of dead cells from the analysis, samples were labeled with propidium iodide staining solution (00-6990; eBioscience). For calculation of absolute cell numbers, AccuCount Fluorescent Particles were used (ACFP-70; Spherotech).

Quantitative Real-Time PCR. Total RNA was isolated with the Aurum Total RNA Mini Kit (7326820; Bio-Rad) and was reverse transcribed with the iScript cDNA Synthesis Kit (170-8891; Bio-Rad). Subsequently, gene expression was analyzed with iTaq Universal SYBR Green Supermix (172-5124; Bio-Rad). Primer sequences are available upon request.

ELISA. Cytokine levels in serum and cell supernatants were measured using mouse ELISA Ready-SET-Go! for IL-23 (88-7230; eBioscience), IL-12 p70 (88-7121; eBioscience), IL-6 (88-7064; eBioscience), IL-1 α (88-5019; eBioscience), and mouse IFN-γ with the Quantikine ELISA Kit (MIF00; R&D Systems). ELISAs were performed according to the manufacturer's instructions.

Lung Histology. Mice were killed 24 h after the injection of 30 mg/kg LPS or PBS, and lungs were fixed with 10% formalin via tracheal injection, harvested, and resuspended in 10% formalin. Formalin-fixed tissues were embedded in paraffin. Four-micrometer-thick sections were stained with H&E and examined for apoptosis. Inflammation in each lung was scored by a single pathologist (D.H.)

blinded to the group. LPS-induced apoptosis was determined using the TACS 2 TdT Fluorescein Kit (4812-30-K; Trevigen).

Microarray. BMDMs were stimulated with 100 ng/mL LPS or PBS for 4 h. Total RNA was extracted with the Aurum Total RNA Mini Kit (7326820; Bio-Rad) and was checked for quality using the Agilent Bioanalyzer. Total RNA (200 ng) was labeled using the Agilent two-color Low Input Quick Amp Labeling kit (Agilent Technologies) according to the manufacturer's protocols. Labeled samples were hybridized to a mouse 4x44K array and scanned on an Axon 4000B microarray scanner (Molecular Devices) at 5-μm resolution. Spot finding was carried out using GenePix 6.1 image analysis software (Molecular Devices).

Microarray data preprocessing and statistical analyses were done in R (v 3.2.1) (44) using the limma package (v 3.24.14) (45). Median foreground values from the three arrays were read into R, and any spots that had been manually flagged (−100 values) were given a weight of zero (46). The background values were ignored because investigations showed that trying to use them to adjust for background fluorescence added more noise to the data. The individual Cy5 and Cy3 values from all the samples were normalized together using the quantile method and then were $\log_2$-transformed (46). The Mouse Whole Genome 4x44K v2 Microarray from Agilent interrogates 24,079 genes using 39,030 probes spotted one time (1×) and 399 probes spotted 10 times (10×) each. Correlations between the replicate spots per probe were high, so they were simply averaged for each sample. The positive and negative control probes were used to assess what minimum expression level could be considered "detectable above background noise" (six on the log2 scale) and then were discarded. A mixed effect statistical model (47) was fit on the 39,429 unique probes and also adjusted for the random effect of array pairing (48). Pairwise comparisons between all logical pairings of the six total groups were calculated along with the interaction term, which tested whether the mutant and WT strains differed in their expression pattern over time. After making the comparisons, 15,467 probes were discarded because they did not have expression values greater than six in at least two samples. For the remaining 23,962 probes, raw *P* values were adjusted separately for each comparison using the false discovery rate method (49). Annotation information for the probes was taken from Bioconductor's MmAgilentDesign026655.db_3.1.2 annotation package (50).

Polysome Fractionation and RNA Purification. Polysome fractionation was performed as described (51). Briefly, BMDMs were stimulated with 100 ng/mL LPS (*E. coli* O111:B4; Sigma L2630) for 0 or 1 h. Ribosomes were stalled by the addition of 100 μg/mL cycloheximide (CHX) for 5 min and were washed twice with PBS containing 100 μg/mL CHX. Cells then were lysed in polysome lysis buffer [15 mM Tris·HCl (pH 7.5), 15 mM MgCl2, 300 mM NaCl, 1% Triton X-100, 2 mM DTT, 200 U/mL RNasin ribonuclease inhibitor (Promega), and protease inhibitor (88666; Thermo)]. Nuclei were removed by centrifugation (16,000 × *g*, 4 °C, 7 min), and the lysate was loaded onto a sucrose density gradient [10–50% in 15 mM Tris·HCl (pH 7.5), 15 mM MgCl2, 300 mM NaCl, and 2 mM DTT]. After ultracentrifugation at 174,900 × *g* in a SW32 Ti rotor for 3.75 h, gradients were collected into 16 fractions. RNA from the polysome parts then was extracted by TRIzol LS and was reverse transcribed with random primers.

Cytoplasmic and Nuclear Extracts. Cytoplasmic extracts were separated from nuclear extracts using the CelLytic NuCLEAR Extraction Kit (NXTRACT; Sigma) according to the manufacturer's instructions.

ChIP. The ChIP assay was performed as described previously with minor modifications (18, 42). The sequences of ChIP primers and a detailed protocol will be provided upon request.

EMSA. Whole-cell extracts were prepared as previously described (52). EMSA was done using a LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology, Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. Briefly, whole-cell extracts were incubated with biotin-labeled consensus κB enhancer oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (Integrated DNA Technologies) for 30 min at room temperature and were resolved in a native polyacrylamide gel. The protein–DNA–biotin complexes were blotted onto a positively charged nylon membrane followed by crosslinking on a transilluminator. The complexes were revealed with streptavidin-HRP conjugate and LightShift chemiluminescent substrate. Comparability of the various cell extracts was assessed by EMSA with a biotin-labeled Oct1 probe (5′-TGTCGAATGCAAATCACTAGAA-3′).

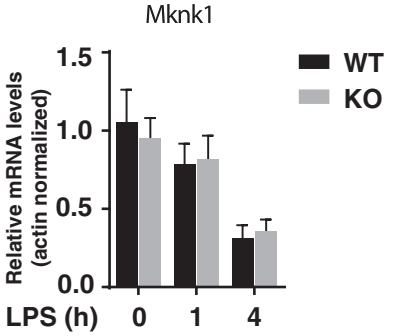

Fig. S1. WT or *Brd4*-deficient BMDMs were stimulated with LPS (100 ng/mL) for the indicated time periods, and the expression of *Mknk1* was analyzed by real-time PCR.

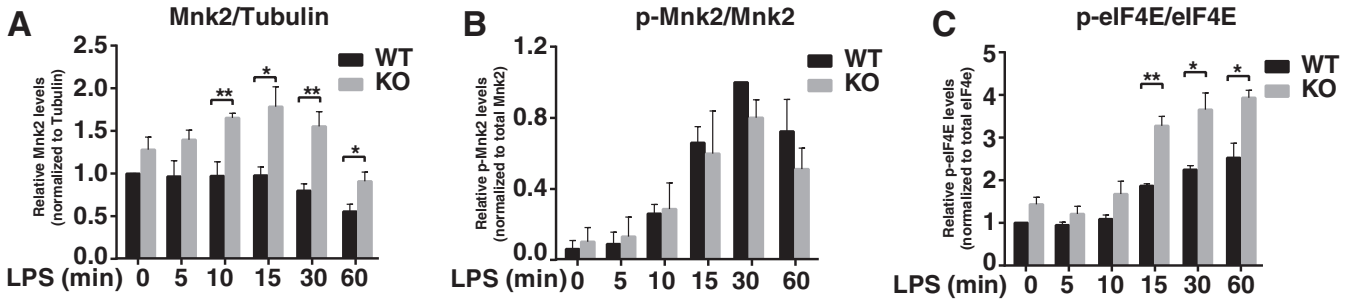

Fig. S2. (*A*) Densitometry analysis showing band intensity of Mnk2 relative to tubulin. Data are shown as mean ± SEM, $n = 4$ (*$P < 0.05$, **$P < 0.01$). (*B*) Densitometry analysis showing band intensity of p-Mnk2 relative to total Mnk2. Data are shown as mean ± SEM, $n = 3$. (*C*) Densitometry analysis showing band intensity of p-eif4E relative to total eif4E. Data are shown as mean ± SEM, $n = 3$ (*$P < 0.05$, **$P < 0.01$).

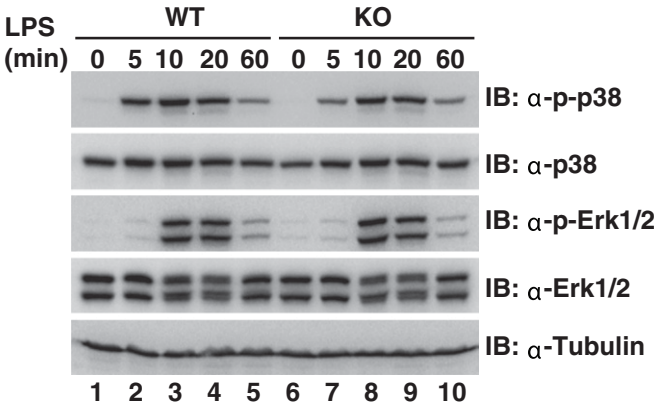

Fig. S3. WT or *Brd4*-deficient BMDMs were stimulated with LPS (100 ng/mL) for the indicated time periods, followed by immunoblotting with the indicated antibodies. IB, immunoblot.

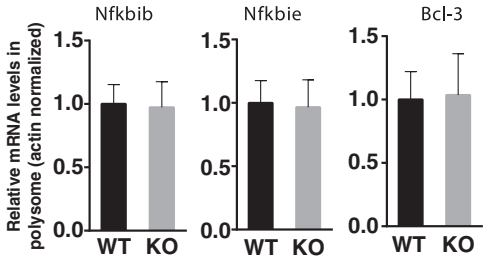

Fig. S4. WT or *Brd4*-deficient BMDMs were stimulated with LPS (100 ng/mL) for 1 h. Levels of *Nfkbib*, *Nfkbibe*, and *Bcl-3* mRNA from polysomal fractions were analyzed by real-time PCR. Data are representative of three independent experiments.

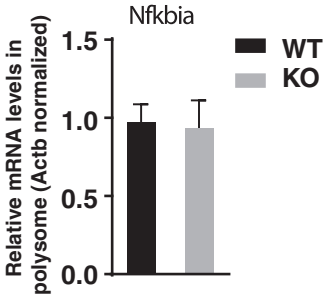

Fig. S5. Levels of *Nfkbia* mRNA isolated from polysomal fractions of unstimulated WT or *Brd4*-deficient BMDMs were analyzed by real-time PCR.

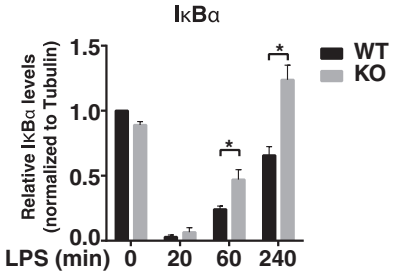

Fig. S6. Densitometry analysis showing band intensity of IκBα relative to tubulin. Data are shown as mean ± SEM, $n = 3$ (*$P < 0.05$).

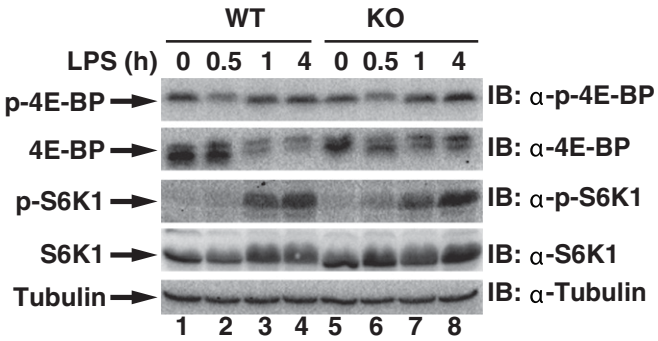

Fig. S7. WT or *Brd4*-deficient BMDMs were stimulated with LPS (100 ng/mL) for the indicated time periods, followed by immunoblotting with the indicated antibodies.

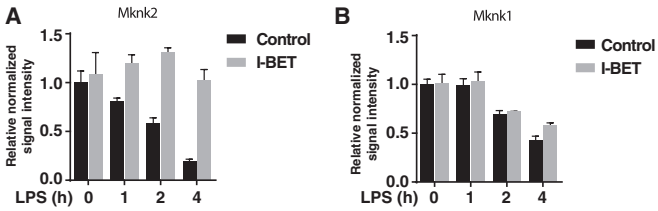

Fig. S8. The relative signal intensity of *Mknk2* (*A*) and *Mknk1* (*B*) in the microarray data described by Nicodeme et al. (20).

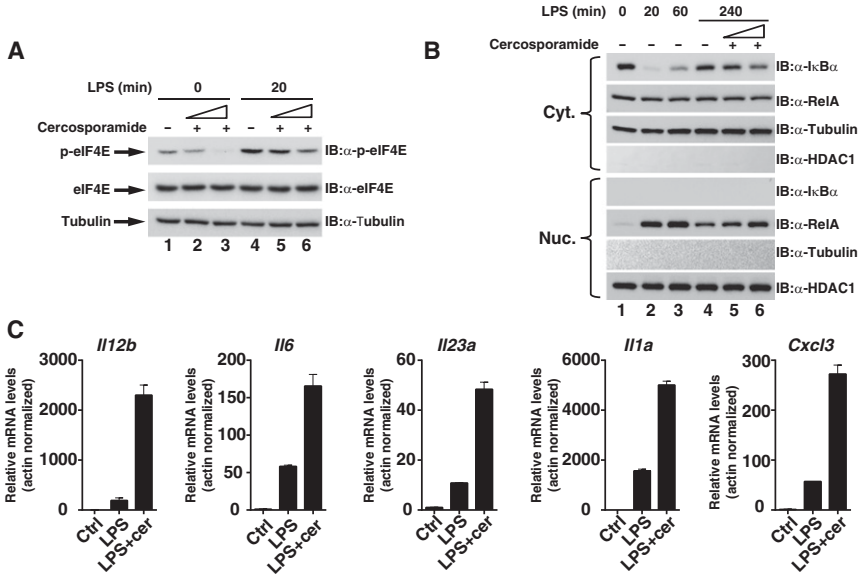

Fig. S9. (*A*) *Brd4*-deficient BMDMs were or were not pretreated with cercosporamide (1 μM and 5 μM) followed by the stimulation with LPS (100 ng/mL) for the indicated time periods and immunoblotting with the indicated antibodies. (*B*) *Brd4*-deficient BMDMs were or were not pretreated with cercosporamide (1 μM and 5 μM) followed by treatment with LPS (100 ng/mL) at the indicated time points. The cytoplasmic (Cyt.) and nuclear (Nuc.) extracts were immunoblotted for the levels of IκBα and RelA. HDAC1 and tubulin were used as nuclear and cytoplasmic protein controls, respectively. (*C*) *Brd4*-deficient BMDMs were pretreated or not with cercosporamide (5 μM for *Il23a*, *Il1a*, and *Cxcl3*; 20 μM for *Il12b* and *Il6*) followed by stimulation with LPS (100 ng/mL) for 1 h (*Il12b* and *Il6*) or 4 h (*Il23a*, *Il1a*, and *Cxcl3*). The expression of indicated genes was analyzed by real-time PCR. For all experiments, the total treatment time of cercosporamide was 5 h.

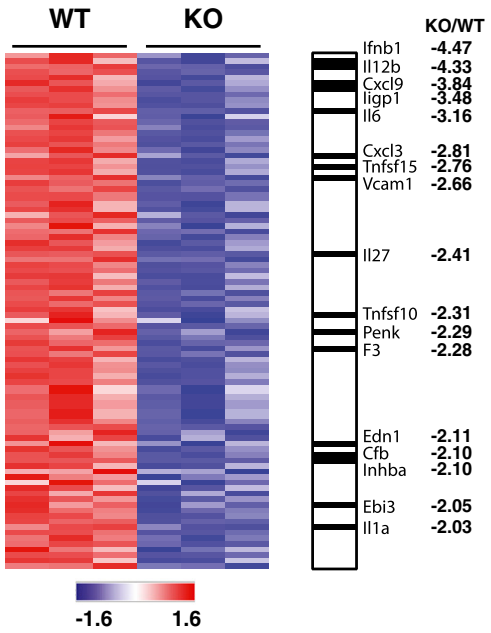

Fig. S10. Microarray analysis of genes that were LPS inducible in the WT macrophages and were down-regulated in the KO macrophages compared with WT after LPS stimulation for 4 h, presented as relative expression level (scaled Z-score), based on log2-normalized expression levels (*Left*), including NF-κB targets (dark boxes, *Center*). The NF-κB target genes (listed from top to bottom) were *Ifnb1*, *Il12b*, *Cxcl9*, *Iigp1*, *Il6*, *Cxcl3*, *Tnfsf15*, *Vcam1*, *Il27*, *Tnfsf10*, *Penk*, *F3*, *Edn1*, *Cfb*, *Inhba*, *Ebi3*, and *Il1α*.

Dataset S1. Total results of the microarray study with or without LPS stimulation

Dataset S1

Dataset S2. Genes regulated by Brd4 after LPS stimulation (0 h and 4 h)

Dataset S2