Supporting Information

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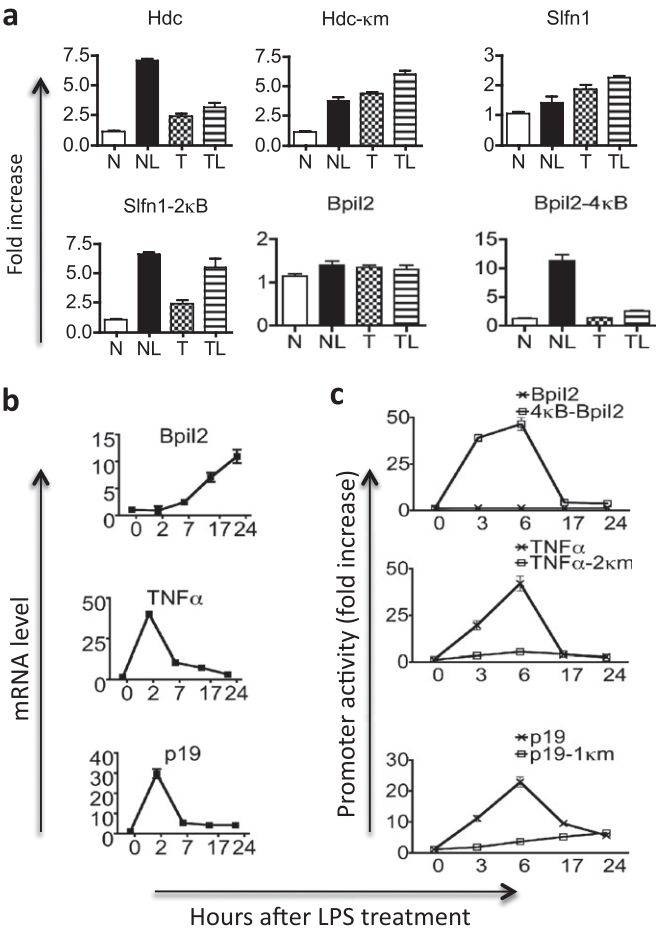

Fig. S1. NF-κB binding motifs dictate tolerance and primary gene responsiveness. (*A*) Wild-type and κB site-mutated *Hdc* (−496/+48), wild-type, and κB site-inserted *Slfn1* (−1025/+137) and *Bpil2* (−139/+67) promoter constructs were tested as in Fig. 1*B*. (*B*) Bone-marrow–derived macrophages were treated with 100 ng/mL of LPS for the indicated times, and mRNA levels of four LPS target genes were determined by real-time PCR. (*C*) Insertion of NF-κB binding motifs to the nontolerizable Bpil2 promoter rendered it the primary gene responsiveness, whereas mutation of the NF-κB binding motifs in tolerizable TNFα and p19 promoters had the opposite effect. The RAW246.7 macrophages were transiently transfected with specific reporter plasmids as indicated and treated with 100 ng/mL LPS for up to 24 h. At the selected time points, whole cell lysates were obtained and luciferase activities measured. Error bars depict SD of the mean. Experiments were repeated three times with similar results.

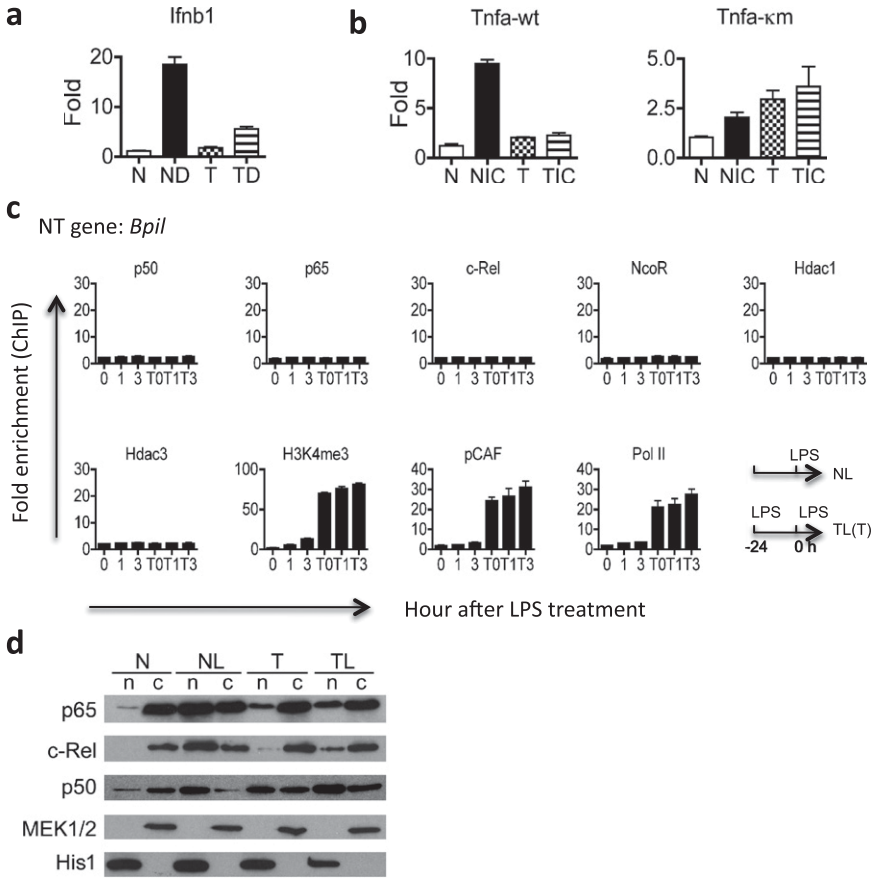

Fig. S2. LPS-induced immune receptor tolerance. (*A*) HEK293–TLR4 cells were transiently transfected with *Ifnb1* luciferase reporter plasmid. Cells were either (*i*) left untreated for 45 h (N), or (*ii*) rested for 25 h and transfected with 1 mg/mL of poly(dA-dT)poly(dT-dA) (synthetic double-stranded DNA) for an additional 20 h (ND), or (*iii*) treated with 100 ng/mL LPS for 24 h and then rested in fresh media for an additional 21 h (T), or (*iv*) treated with 100 ng/mL LPS for 24 h, rested in fresh media for 1 h, and then transfected with 1 mg/mL of poly(dA-dT)poly(dT-dA) for an additional 20 h (TD). Whole cell lysates were then prepared and luciferase activities measured. (*B*) RAW264.7 macrophages were transiently transfected with wild-type *Tnfa* (Tnfa-wt) or κB site-mutated *Tnfa* (Tnfa-κm) luciferase reporter plasmids. Cells were either (*i*) left untreated for 31 h (N), or (*ii*) rested for 24 h and treated with 5 μg/mL poly I:C (synthetic double-stranded RNA) for an additional 7 h (NIC), or (*iii*) treated with 100 ng/mL LPS for 24 h and then rested in fresh media for an additional 7 h (T), or (*iv*) treated with 100 ng/mL LPS for 24 h and restimulated with 5 μg/mL poly I:C for an additional 7 h (TIC). Whole cell lysates were then prepared and luciferase activities measured. (*C*) Naïve or tolerized (T) bone-marrow–derived macrophages were stimulated with 100 ng/mL LPS for 0, 1, or 3 h as indicated and tested by ChIP for the nontolerizable gene *Bpil2* as in Fig. 2. Error bars depict SD of the mean. (*D*) Increased nuclear p50 levels in tolerized cells. Bone-marrow–derived macrophages were either (*i*) left untreated for 25 h (naïve, N), or (*ii*) rested for 24 h and treated with 100 ng/mL LPS for an additional hour (NL), or (*iii*) treated with 100 ng/mL LPS for 24 h and then rested in fresh media for an additional hour (tolerized, T), or (*iv*) treated with 100 ng/mL LPS for 24 h and restimulated with 100 ng/mL LPS for another hour (TL). Nuclear (n) and cytoplamic (c) proteins were extracted and tested by Western blot. Experiments were repeated three times with similar results. *His1*, histone 1.

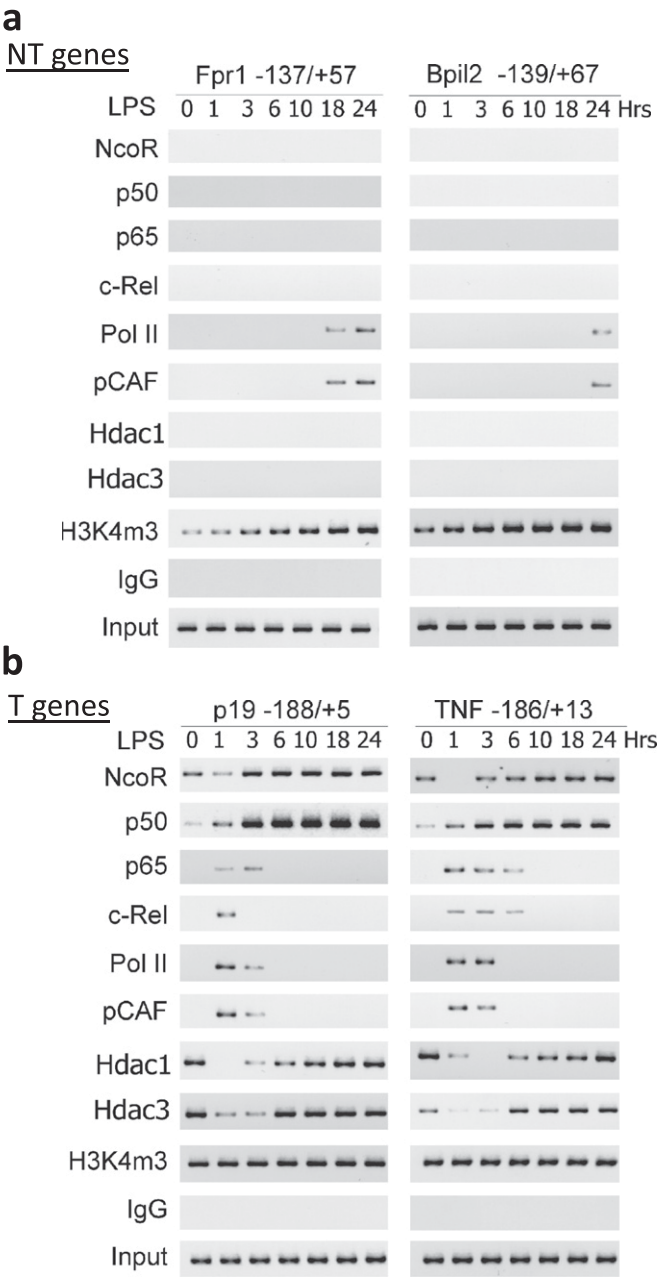

Fig. S3. LPS-induced formation of the repressosome at tolerizable, but not nontolerizable, gene promoters. Bone-marrow–derived macrophages were left untreated (0) or treated with 100 ng/mL LPS for up to 24 h. Chromatin IP was performed for tolerizable gene promoters p19 (−188/+5) and TNFα (−186/+13) and nontolerizable gene promoters Fpr1 (−137/+57) and Bpil2 (−139/+67). Experiment was repeated at least three times with similar results.

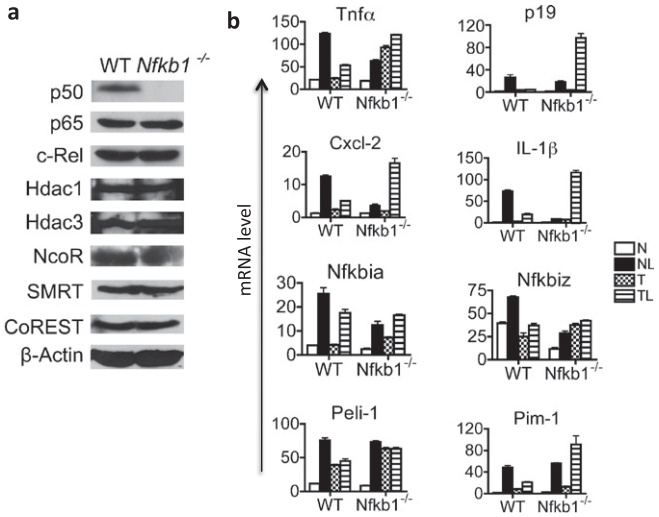

Fig. S4. Lack of LPS tolerance in *Nfkb1*$^{-/-}$ macrophages. (*A*) Bone-marrow–derived macrophages from WT and *Nfkb1*$^{-/-}$ mice (n = 4) were generated, and their whole cell lysates examined by Western blot. Except p50, the expression of p65, c-Rel, Hdac1, Hdac3, NcoR, SMRT, and CoREST was not affected by the *Nfkb1* gene mutation. (*B*) *Nfkb1*$^{-/-}$ macrophages were resistant to tolerance. Bone-marrow–derived macrophages from WT and *Nfkb1*$^{-/-}$ mice (n = 4) were treated with LPS as described in Fig. 4*D*. mRNA levels of eight LPS-induced genes were determined by real-time PCR. Error bars depict SD of the mean. Experiments were repeated three times with similar results.

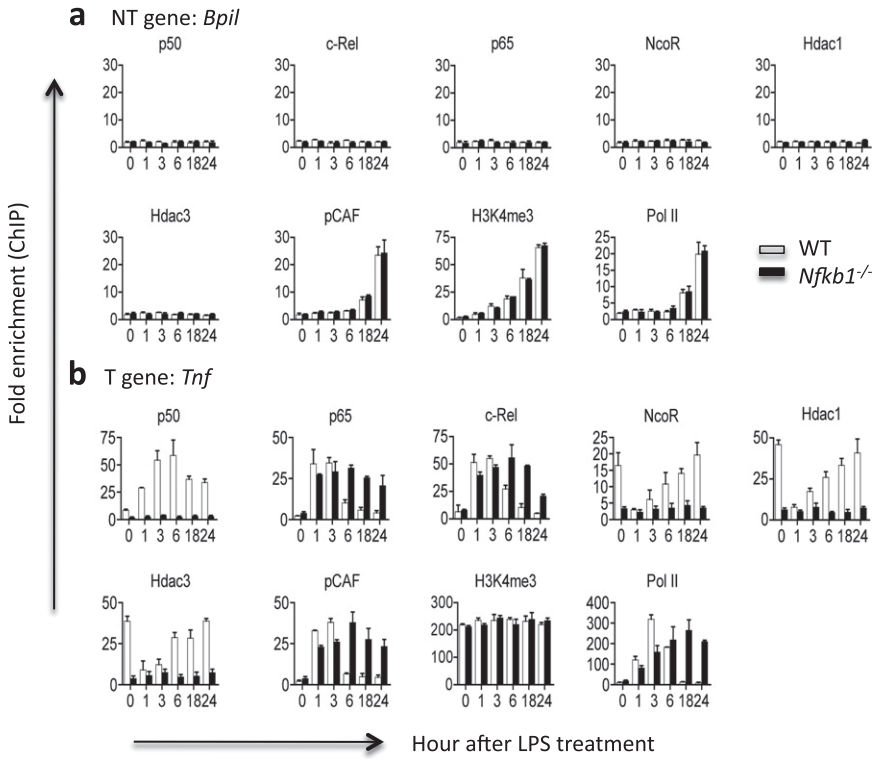

Fig. S5. *Nfkb1* null mutation selectively affects NcoR and histone deacetylase binding to tolerizable genes. Bone-marrow–derived macrophages from WT and *Nfkb1*$^{-/-}$ mice (n = 4) were either left untreated (0) or treated with 100 ng/mL LPS for up to 24 h as indicated. Cells were then fixed and chromatin IP was performed for the indicated genes and factors as described in *Materials and Methods*. Error bars depict SD of the mean. Experiments were repeated three times with similar results.

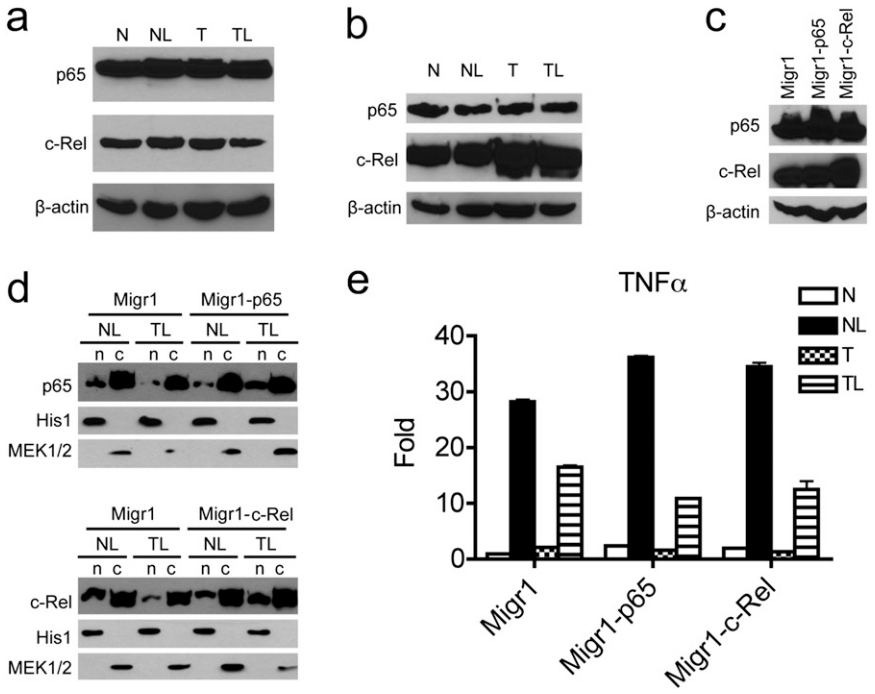

Fig. S6. Retrovirus-mediated p65 and c-Rel gene transfer does not abolish LPS tolerance. (*A*) Bone-marrow–derived macrophages were either (*i*) left untreated for 25 h (naïve, N), (*ii*) rested for 24 h and treated with 100 ng/mL LPS for an additional hour (NL), (*iii*) treated with 100 ng/mL LPS for 24 h and then rested in fresh media for an additional hour (tolerized, T), or (*iv*) treated with 100 ng/mL LPS for 24 h and restimulated with 100 ng/mL LPS for another hour (TL). Whole cell lysates were obtained and analyzed by Western blotting. (*B*) RAW264.7 macrophages were treated and analyzed as described in *A*. (*C*) RAW246.7 macrophages were infected with Migr1-based retroviruses that carry enhanced green fluorescent protein (EGFP) cDNA (Migr1) or EGFP cDNA plus murine p65 (Migr1–p65) or c-Rel (Migr1–c-Rel) full-length cDNA. Whole cell lysates were prepared 2 d later and analyzed by Western blotting. (*D*) RAW246.7 macrophages were first treated with retroviruses as in *C* and then subjected to LPS tolerance as described in *A*. Nuclear (n) and cytosolic (c) proteins were fractionated using the Active Motif Nuclear Extraction kit, and the same amounts of proteins were analyzed in each lane by Western blotting. (*E*) RAW246.7 macrophages were first treated with retroviruses as in *C*, and then either (*i*) left untreated for 27 h (naïve, N), (*ii*) rested for 24 h and treated with 100 ng/mL LPS for an additional 3 h (NL), (*iii*) treated with 100 ng/mL LPS for 24 h and then rested in fresh media for an additional 3 h (tolerized, T), or (*iv*) treated with 100 ng/mL LPS for 24 h and restimulated with 100 ng/mL LPS for an additional 3 h (TL). Total RNA was obtained and the relative TNFα mRNA levels were determined by real-time PCR.

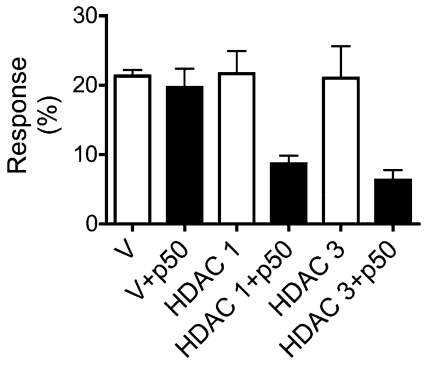

Fig. S7. p50 is required for Hdac-induced Tnf gene repression. RAW246.7 macrophages were transiently transfected with Tnfa promoter luciferase plasmid (V) with or without p50, Hdac1, or Hdac3 expression plasmids as indicated. Cells were then treated with or without 100 ng/mL LPS for 24 h and restimulated with 100 ng/mL LPS for an additional 7 h before they were tested for luciferase activities. Data presented are percentage of responses of tolerized macrophages (i.e., TL) over macrophages that did not receive the first LPS treatment but were stimulated with LPS for 7 h (i.e., NL). Only with the p50 cotransfection, Hdac1 and Hdac3 expression significantly reduced the Tnfa promoter activity ($P < 0.001$). Error bars depict SD of the mean. Experiments were repeated three times with similar results.