Supporting Information

Chen and Ivashkiv 10.1073/pnas.1007816107

SI Materials and Methods

Reagents. LPS was purchased from Sigma, Pam_3Cys was purchased from EMC Microcollections, and IL-1β was from R&D Systems. The p38 inhibitor SB203580, JNK inhibitor SP600215, MAPK/ERK kinase (MEK) inhibitor U0126, and proteasome inhibitor MG-132 were from Calbiochem. Cycloheximide was from Sigma.

ELISA. ELISAs were performed with paired antibody sets as recommended by the manufacturer (BD Pharmingen).

Western Blotting. Cytoplasmic, nuclear, or whole-cell extracts were fractionated on 7.5–10% polyacrylamide gels by SDS-PAGE, transferred to polyvinylidene fluoride membranes (Millipore), and incubated with specific antibodies; enhanced chemiluminescence (Amersham) was used for detection. Antibodies to phospho-JNK (Thr183/185), phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-p38 (Thr180/Tyr182), IκBα, phospho–NF-κB p65 (Ser536), NF-κB p65, and IL-1 receptor-associated kinase M (IRAK-M) were from Cell Signaling. Antibodies to JNK1/3, p38α, TATA-binding protein (TBP), IRAK-1, SH2-containing inositol phosphatase 1 (SHIP-1), and MAPK phosphatase 1 (MKP-1) were from Santa Cruz Biotechnology.

Real-Time PCR. Total RNA was extracted with the RNeasy Mini Kit (Qiagen) and reverse-transcribed using the First Strand cDNA Synthesis kit (Fermentas). Quantitative real-time PCR was performed in triplicate using iQ SYBR Green Supermix and an iCycler iQ thermal cycler (Biorad). Relative expression was normalized for levels of GAPDH. Primary transcripts were measured with primers that amplified intronic sequences of the *IL6* and *TNF* genes. Primer sequences are available on request.

Densitometry. Restriction enzyme accessibility assay results were quantified using ImageJ software (National Institutes of Health). The percent of genomic DNA cut by restriction enzyme was calculated by dividing the mean intensity of the cut band by the sum of the mean intensities of the cut and uncut bands and then multiplying the result by 100.

Statistical Analysis. Statistical analysis was performed using paired Student's *t* test. *P* values <0.05 were considered statistically significant.

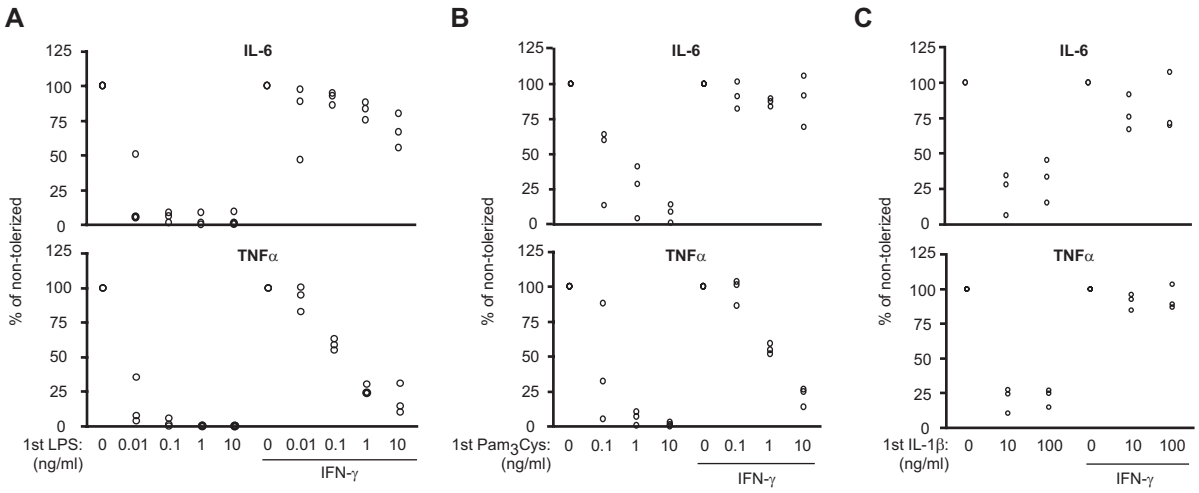

Fig. S1. IFN-γ blocks tolerization and cross-tolerization of IL-6 and TNFα. A summary of data for three independent donors is shown. Control and IFN-γ–activated monocytes were tolerized with increasing doses of (*A*) LPS (0.01–10 ng/mL), (*B*) Pam_3Cys (0.1–10 ng/mL), or (*C*) IL-1β (10–100 ng/mL) for 24 h as in Fig. 1 and then challenged with 10 ng/mL LPS. IL-6 and TNFα levels in culture supernatants were measured by ELISA. Percent tolerization of IL-6 and TNFα was calculated as described in Fig. 1.

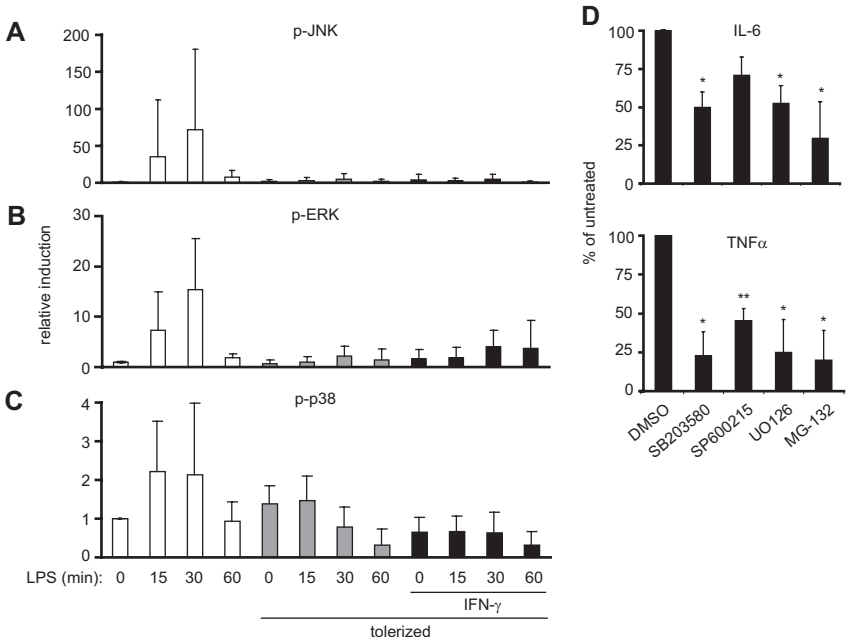

Fig. S2. IFN-γ does not alter upstream Toll-like receptor (TLR) signaling defects in tolerized monocytes. Cumulative data for multiple donors are shown. Control and IFN-γ–activated monocytes were tolerized with 0.1 ng/mL LPS and then stimulated with 10 ng/mL LPS for the indicated times. Densitometry was used to quantify relative induction (mean + SD) of (*A*) phospho-JNK (n = 10 at the 0-, 15-, and 30-min time points; n = 4 at the 60-min time point), (*B*) phospho-ERK (n = 9 at the 0-, 15-, and 30-min time points; n = 3 at the 60-min time point), and (*C*) phospho-p38 (n = 12 at the 0-, 15-, and 30-min time points; n = 4 at the 60-min time point). (*D*) IFN-γ–treated tolerized monocytes are still dependent on MAPK signals for induction of IL-6 and TNFα. IFN-γ–activated tolerized monocytes were incubated with 0.1% DMSO, 10 μM SB203580, 20 μM SP600215, 20 μM UO126, or 20 μM MG-132 for 30 min before stimulation with 10 ng/mL LPS for 3 h. IL-6 and TNFα levels in culture supernatants were measured by ELISA. Data are shown as percent of cytokine levels in DMSO-treated cells and are expressed as mean + SD of three independent donors. P values were calculated by paired Student's t test. $^{*}P < 0.05$; $^{**}P < 0.01$.

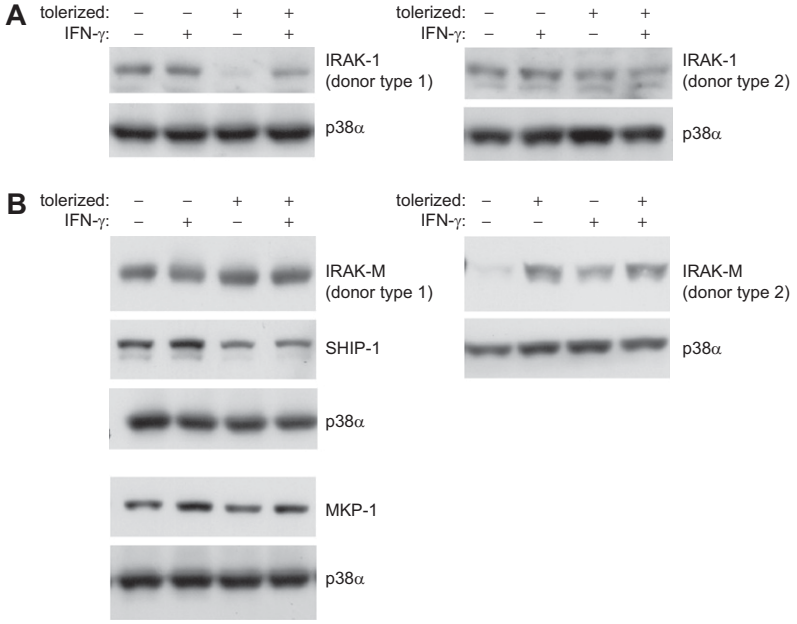

Fig. S3. IFN-γ does not inhibit the expression of reported mediators of tolerance. Control and IFN-γ–activated monocytes were tolerized with 0.1 ng/mL LPS, and cell extracts were subjected to Western blotting for (*A*) IRAK-1 and (*B*) IRAK-M, SHIP-1, and MKP-1. p38α was used as the loading control. Data are representative of four to seven independent donors.

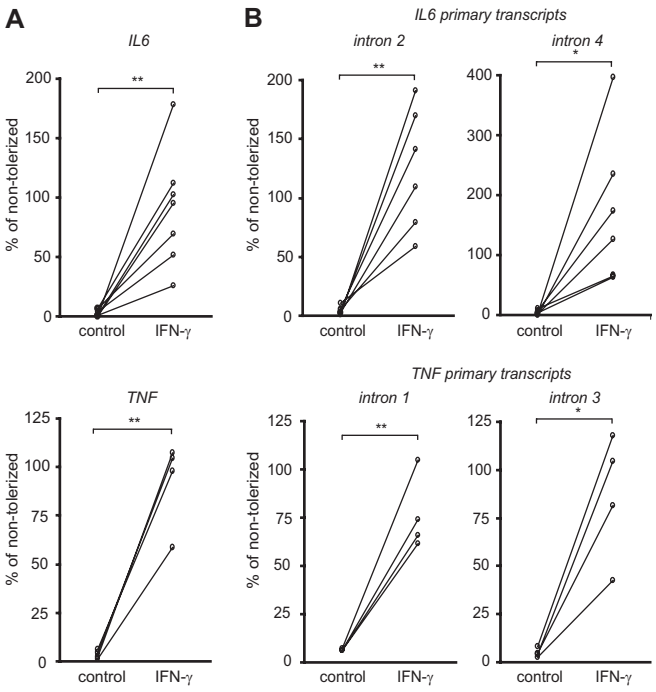

Fig. S4. IFN-γ blocks tolerization of IL-6 and TNFα at the level of transcription. Cumulative data for multiple donors are shown. Control and IFN-γ–activated monocytes were tolerized with 0.1 ng/mL LPS and then stimulated with 10 ng/mL LPS for 1 or 3 h for analysis of *TNF* mRNA and *IL6* mRNA, respectively. Real-time PCR was used to measure (*A*) steady-state mRNA levels and (*B*) primary transcript levels. Data show percent tolerization of *IL6* and *TNF* mRNA levels as described in Fig. 1 and are a summary of four to seven independent donors. *P* values were calculated by paired Student's *t* test. $*P < 0.05$; $**P < 0.01$.

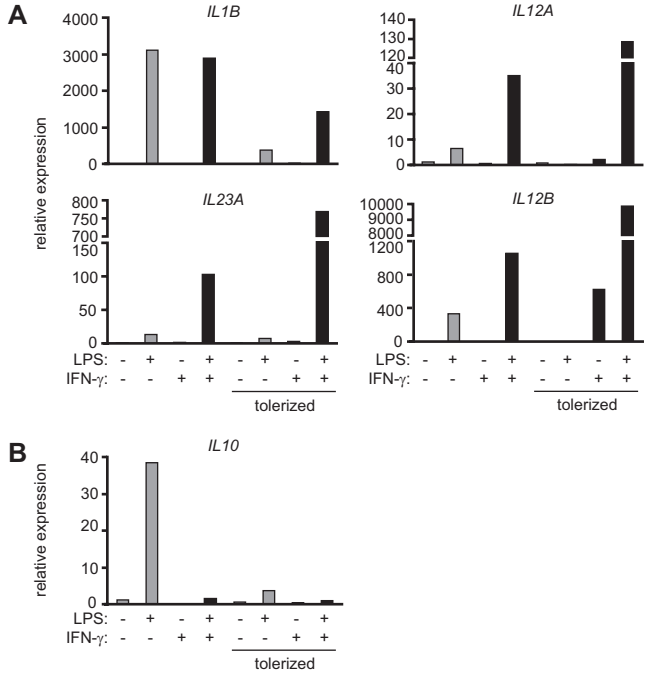

Fig. S5. IFN-γ broadly restores inflammatory gene expression in tolerized human monocytes. Control and IFN-γ–activated monocytes were tolerized with 0.1 ng/mL LPS and then stimulated with 10 ng/mL LPS for (*A*) 1 h (for analysis of *IL1B* and *IL23A* mRNA), 3 h (for analysis of *IL12A* and *IL12B* mRNA), and (*B*) 6 h (for analysis of *IL10* mRNA). mRNA levels were measured by real-time PCR. Data are representative of at least three independent donors.