Supporting Information

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SI Experimental Procedures

Generation and Characterization of Autoimmune STING-Deficient Mice. For mortality assessment, additional STING$^{+/-}$lpr/$^+$ mice were intercrossed to produce a second cohort of STING/lpr mice ($n = 15$, with 12 deaths during the observation period) and WT/lpr mice ($n = 9$, with five deaths), which were observed without intervention until the time of death. A second F2 cross also was established for fully backcrossed C57BL/6 STING$^{-/-}$ (kindly provided by D. Stetson, University of Washington, Seattle) and MRL.Fas^{lpr} mice. Identical results were obtained, and data are cumulative from both crosses. A similar cross was set up for IRF3$^{-/-}$ and MRL/lpr mice to generate cohorts of experimental F2 mice and analyzed as above for STING/lpr mice ($n \geq 10$ per group). In both cohorts severely moribund animals were killed for humane concerns and were included as deaths in the analysis. For the TMPD-induced peritonitis, 6-wk-old C57BL/6, Unc93b$^{3d/3d}$, and STING$^{-/-}$ mice were injected i.p. with TMPD as described previously (1) and were evaluated at day 14. The University of Massachusetts Medical School (UMMS) and Yale Institutional Animal Care and Use Committees approved all animal work.

Determination of Autoantibody Profiles. Images were captured on a Nikon E600 at 200× magnification and constant excitation and were processed in Adobe Photoshop. Autoantibody reactivity was examined further using an autoantigen proteomic array containing 88 autoantigens and 10 control proteins (2). Serum samples, diluted 1:100, were detected with Cy3-labeled anti-mouse IgG and Cy5-labeled anti-mouse IgM (Jackson ImmunoResearch), and Tiff images were generated. Genepix Pro-6.0 software was used to analyze the images and generate GenePix Results format (.GPR) files. Net fluorescence intensities were defined as the spot intensity minus background fluorescence intensity; data obtained from duplicate spots are averaged. The signal-to-noise ratio (SNR) was used as a quantitative measure of the ability to resolve true signal from background noise. A higher SNR indicates higher signal over background noise. SNRs equal to or higher than 3 are considered true signal-to-background noise ratios. The autoantibody levels were normalized to internal controls, all values were scaled relative to the minimum value $[x - \min(x)]$, and a pseudocount of 1 was added to each value. This operation sets the smallest value in the dataset to 1. All values were log10-transformed, and a heatmap was generated using open-source R-based software at UMMS. Hierarchical clustering of data as noted in the R program is reported.

Gene-Expression Analysis. RNA isolated from total spleen was used for quantitative RT-PCR using SYBR Green PCR Master Mix (Bio-Rad) with the following primer pairs: β-actin, sense, 5′-TGGCA-TAGAGGTCTTTACGGA-3′, antisense, 5′-TTGAACATGGCA-TTGTTACCAA-3′; TLR7, sense, 5′-ATGTGGACACGGAAGA-GACAA-3′, antisense 5′-GGTAAGGGTAAGATTGGTGGTG; TLR9, sense, 5′-ATGGTTCTCCGTCGAAGGACT-3′, antisense 5′-GAGGCTTCAGCTCACAGGG-3′; TLR3, sense, 5′-GTGAG-ATACAACGTAGCTGACTG-3′, antisense 5′-TCCTGCATCCA-AGATAGCAAGT-3′; GAPDH, sense, 5′-AGGTCGGTGTGAA-CGGATTTG-3′, antisense 5′-TGTAGACCATGTAGTTGAGG-TCA-3′; A20 (TNFAIP3), sense 5′-ACAGTGGACCTGGTAA-GAAAACA-3′, antisense 5′-CCTCCGTGACTGATGACAAG-AT-3′; SOCS1, sense 5′-CTGCGGCTTCTATTGGGGAC-3′, antisense 5′-AAAAGGCAGTCGAAGGTCTCG-3′; SOCS3, sense 5′-ATGGTCACCCACAGCAAGTTT-3′, antisense 5′-TCCAGT-AGAATCCGCTCTCCT-3′. For purified cell populations, BMMs were prepared as described (3). Activated cells were harvested

into RLT buffer containing 2-mercaptoethanol for subsequent processing with the RNeasy Mini kit (Qiagen). Each RNA sample was adjusted to contain the same quantity by using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific). RNA then was hybridized and quantified with the NanoString nCounter analysis system (NanoString Technologies) per the manufacturer's protocol. The gene-expression data first were normalized to an internal positive control set, then to an internal negative control set, and then to seven housekeeping genes, i.e., GAPDH, β-glucuronidase, β-actin, hypoxanthine phosphoribosyltransferase 1, tubulin β, phosphoglycerate kinase 1, and clathrin H chain 1. All values were log10-transformed, and a heatmap was generated using the open-source R-based software at UMMS as stated above.

Flow Cytometry. The following conjugated anti-mouse Abs were used: peridinin chlorophyll (PerCP)-Cy5.5 anti-CD19 (1D3), phycoerythrin (PE)-Cy7 anti–Ly-6G (1A8), allophycocyanin (APC)-e780 anti-CD11b (M1/70), PerCP-Cy5.5 anti-TCRβ (H57.597), and PE-Cy7 anti-CD95 (Jo-2) (BD Biosciences); PerCP-Cy5.5 anti-F4/80 (BM8), V500 anti-CD4 (GK1.5), eFluor605NC anti-CD8α (53-6.7), and efluor450 anti-CD45R (RA3-6B2) (eBioscience); efluor450 anti-CD11c (3.9) and APC anti–Ly-6C (ER-MP20) (Serotec); FITC PNA (Vector Labs); APC anti-CD93 AA4.1, PE anti-CD138 (281-2), APC anti-NK1.1 (PK136), FITC anti-CD86 (GL1), efluor450 anti–IFN-γ (XMG1.2), APC-Cy7 anti-CD44 (IM7), PE-Cy7 anti-CD69 (H1.2F3), APC anti–Siglec-H (eBio440c), and PE anti-CD317 (Bst-2, ebio927) (eBioscience); and FITC anti–IFN-α (RMMA-1; PBL IFN Science). Treg cells were stained using APC-efluor780 anti-CD4 (RM 4–5; eBioscience), APC anti-CD25 (PC61; eBioscience), and FITC anti-FOXP3 (FJK-16s; eBioscience). Live-Dead Blue and Aqua dead cell stains (Invitrogen) were used to distinguish between live and dead cells.

Cells were incubated in either CD16/32 (Fc Block; BD Biosciences) or 2.4G2 supernatant (2.4G2 hybridoma, ATCC) before staining. Cellular DNA was determined by flow cytometry using propidium iodide or 7-aminoactinomycin D (BD Biosciences). Intracellular staining of IFN-γ and IFN-α was carried out on splenic single-cell suspensions preincubated with Monensin (BD Biosciences) for 2 h and then with Fc block (2.4G2) for 15 min. Cells were permeabilized and fixed with BD Cytofix-Cytoperm buffer and subsequently incubated with FITC-conjugated rat monoclonal antibody to IFN-α (Clone RMMA-1; PBL) or e450-conjugated monoclonal antibody to IFN-γ (eBioscience). Cells were acquired on a LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Cell Cultures. BMMs were derived as described previously (3). Briefly bone marrow extracts were expanded in vitro in the presence of L929 supernatants for 7 d. The rested BMMs then were stimulated with TLR ligands, CpG B (1826 ODN; 5 μM; IDT), CLO97 (300 ng; InvivoGen), pI:C (100 μg/mL; InvivoGen), CpG A (2336 ODN; 1 μM IDT), Sendai virus (Cantrell strain; Charles River Laboratories; 200 high activity units), and poly(dA-dT) (1 μg/mL; Sigma), ISD as previously described (4) (3μM IDT) for 20 h or for the times specified. Transfection of intracellular ligands was done with Lipofectamine 2000 from Invitrogen. Full-length murine STING or a scrambled sequence was cloned into the pRetro-Zeocin (pRZ) vector and tagged with HA and citrine. The production of viral particles and transduction of target cells were conducted according to the protocols at www.broad.mit.edu/genome_bio/trc/publicProtocols.html. Zeocin selection (encoded

in the pRZ vector) was used until stable expression was obtained. Overexpression of STING was verified by Western blot analysis.

Determination of Autoantibody Profiles. ANAs were detected by immunofluorescence on HEp-2 slides (Antibodies, Inc.) as previously described (1).

Cytokine Assays. A Luminex screen was performed on the serum using a Bio-Plex 23-plex kit (Bio-Rad) and a Bio-Plex 200 reader (Bio-Rad) per the manufacturer's instructions.

ELISA. Serum levels of IL-6, TNF-α, and IFN-γ protein levels were determined by ELISA (eBioscience). The murine IFN-β kit was as described (3). An anti-mouse albumin ELISA kit was used to measure urine protein (Bethyl Laboratories).

Determination of Clinical Disease. H&E-stained kidney sections were scored in a blinded fashion by three independent observers to determine a glomerular and interstitial inflammation score as described (5). Briefly, a mean glomerular score was calculated for each mouse by grading injury in 20 glomeruli. Glomeruli were scored as follows: 0, normal; 1, mesangial expansion; 2, endocapillary proliferation; 3, capillaritis or necrotic changes; 4, crescents.

The interstitial score was determined by examining 20 high-power fields and scoring the interstitial inflammation on a scale from 0 to 4 as absent or involving <25, 25–50, or >50% of the interstitium.

IDO-1 Staining. Spleen tissue was snap-frozen in optimum cutting temperature (OCT) medium. For IDO-1 staining cryostat sections were fixed in cold methanol and processed as previously described (6). Anti-mouse IDO-1 (Rockland Immunochemicals) and anti-mouse DyLight-488 (Sigma) were used to visualize IDO-1 levels in the spleen. Nuclei were counterstained with DAPI (Invitrogen). Images were captured on a Leica SP8 confocal microscope.

ELISPOT Assay. To detect antibody-forming cells (AFCs) that make κ light-chain IgG antibodies, 96-well multiscreen filter plates were coated overnight at 4 °C with 5 μg/mL polyclonal goat-anti mouse κ (1050–01; Southern Biotech). Nonspecific binding was blocked with 1% BSA in PBS, and serially diluted cell suspensions were plated and incubated at 37 °C for 8 h. Biotinylated secondary antibodies to IgG (1030-08; Southern Biotech) were detected with alkaline phosphatase-conjugated streptavidin and bromo-4-chloro-3-indolyl phosphate substrate (Southern Biotech).

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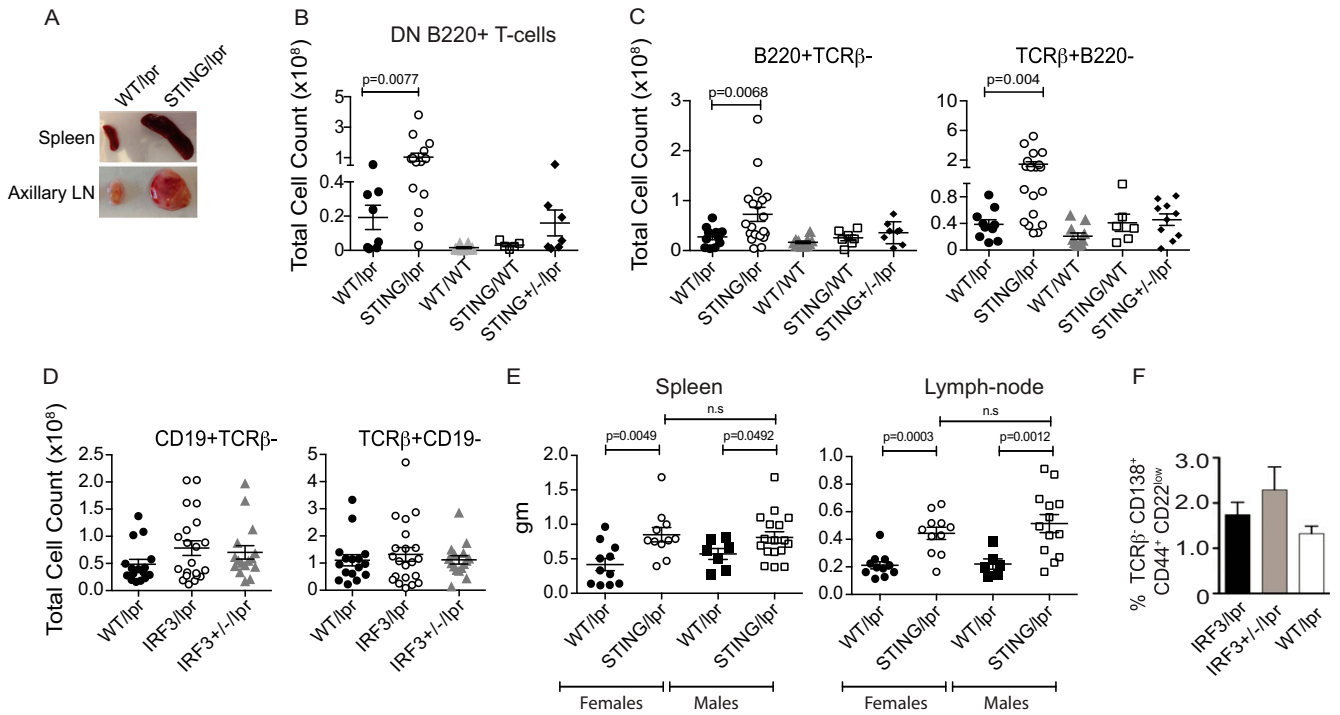
Fig. S1. STING deficiency but not IRF3 deficiency promotes disease severity in MRL/lpr mice (related to Figs. 1 and 2). (*A*) Representative images of spleen and axillary lymph node from 16-wk-old WT/lpr and STING/lpr mice. (*B*) Total numbers of splenic B220$^+$ TCRβ^+ CD4$^-$ CD8$^-$ DN cells in 16- to 25-wk-old WT/lpr, STING/lpr, WT/WT, STING/WT, and STING$^{+/-}$/lpr mice. (*C*) Total numbers of B220$^+$ TCRβ^- B cells and B220$^-$ TCRβ^+ T cells in the spleens of 16- or 25-wk-old STING/lpr mice and littermates as determined by flow cytometry. (*D*) As in C; total cell numbers of splenic CD19$^+$ TCRβ^- B cells and CD19$^-$ TCRβ^+ T cells in WT/lpr, IRF3/lpr, and IRF3$^{+/-}$/lpr mice (*E*) Weights of spleens and largest axillary lymph nodes) from F2 littermates segregated by sex. (*F*) Percentage of live TCRβ^- CD44$^+$ CD138$^+$ CD22^{low} AFCs in the spleens of WT/lpr, IRF3/lpr, and IRF3$^{+/-}$/lpr mice. $n = 16$ or more mice per group. Wider horizontal bars represent the means of each group, and error bars represent SEM. WT/lpr, IRF3/lpr, and IRF3$^{+/-}$/lpr mice were analyzed at age 22 wk; WT/lpr and STING/lpr mice were analyzed at age 16 wk. *P* values for WT/lpr vs. STING/lpr in *B* and *C* were calculated using two-way ANOVA.
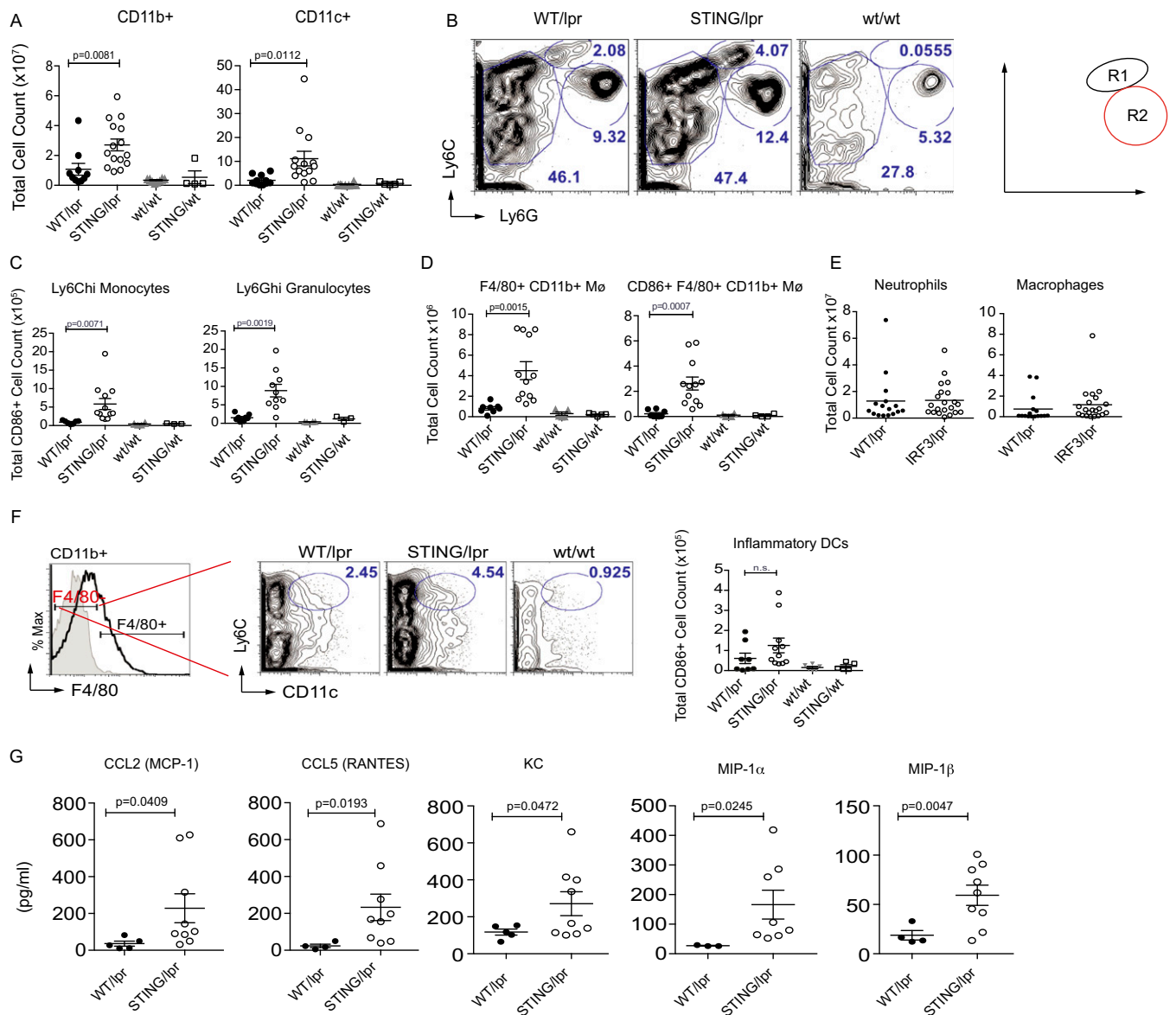
Fig. S2. STING deficiency but not IRF3 deficiency promotes the expansion and activation of myeloid-derived cell populations (related to Fig. 4). (*A*) Total number of cells positive for CD11b and CD11c. (*B*) Identification of Ly6C^{hi} CD11b$^+$ inflammatory monocytes (R1) and Ly6C^{int} Ly6G^{hi} CD11b$^+$ N1 neutrophils (R2). (*C*) Total CD86$^+$ cell counts of CD11b$^+$ Ly6C^{hi} monocytes and CD11b$^+$ Ly6G^{hi} granulocytes. (*D*) Counts of total F4/80$^+$ and CD86$^+$ F4/80$^+$ macrophages in STING/lpr mice and littermates. Mice were 16 wk old unless otherwise noted. (*E*) Total counts of splenic neutrophils and macrophages in 22-wk-old IRF3/lpr mice and littermates. (*F*) Identification of CD11b$^+$ F4/80$^-$ Ly6C^{hi} CD11c$^+$ iDCs and total CD86$^+$ cell counts of iDCs. (*G*) Serum levels of chemokines determined by Luminex-multiplex array. *P* values were determined by Student's *t* test.
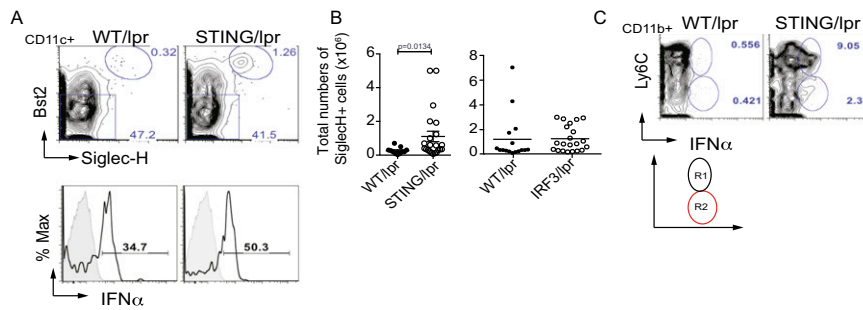
Fig. S3. STING deficiency but not IRF3 deficiency promotes the enrichment of IFN-α⁺ lymphoid-derived pDCs and Ly6C⁺ cells (related to Fig. 4). (*A*) Gating strategy for cells positive for intracellular staining of IFN-α within the CD11c⁺ Bst-2⁺ SiglecH⁺ pDC subset. (*B*) Total numbers of SiglecH⁺ pDCs in the spleens of 16-wk-old WT/lpr and STING/lpr mice and in 22-wk-old IRF3/lpr mice and WT/lpr littermates. (*C*) Identification of cells positive for intracellular staining of IFN-α within the Ly6C^{hi} CD11b⁺ inflammatory monocyte and Ly6C^{lo} CD11b⁺ monocyte populations. R1 and R2 gates were used to designate Ly6C^{hi} and Ly6C$^{+/lo}$ cells. *P* values are calculated using two-way ANOVA and are reported for STING/lpr vs. WT/lpr.
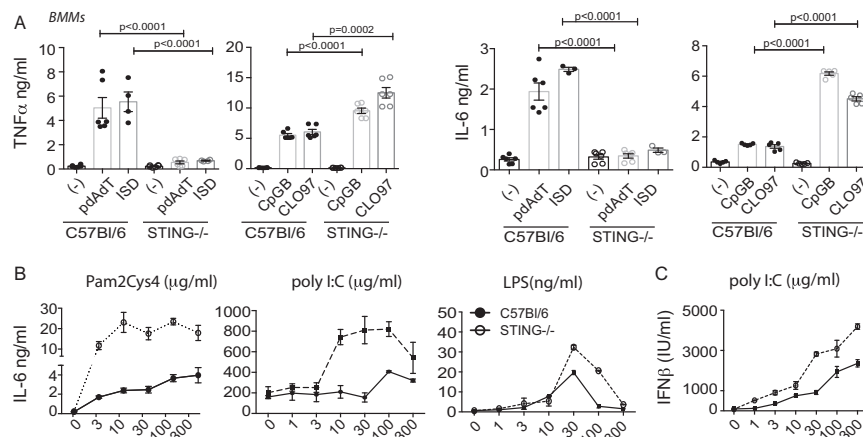

Fig. S4. STING deficiency modulates TLR signaling strength (related to Fig. 6). (*A*) Levels of TNF-α and IL-6 secreted by BMMs from WT and STING$^{-/-}$ mice in response to TLR9 (CpGB, 5 μM), TLR7 (CLO97, 300 ng/mL), poly (dA:dT) (1 μg/mL), and ISD (3 μM) ligands. Supernatants were collected 24 h after stimulation. (*B*) Levels of IL-6 secreted by C57BL/6 and STING$^{-/-}$ BMMs treated with noted concentrations of various TLR ligands: Pam$_2$Cys$_4$ (TLR2), poly(I:C) (TLR3), and LPS (TLR4) for 24 h. (*C*) Levels of IFN-β secreted by C57BL/6 and STING$^{-/-}$ BMMs treated with a concentration range of poly(I:C) for 24 h.
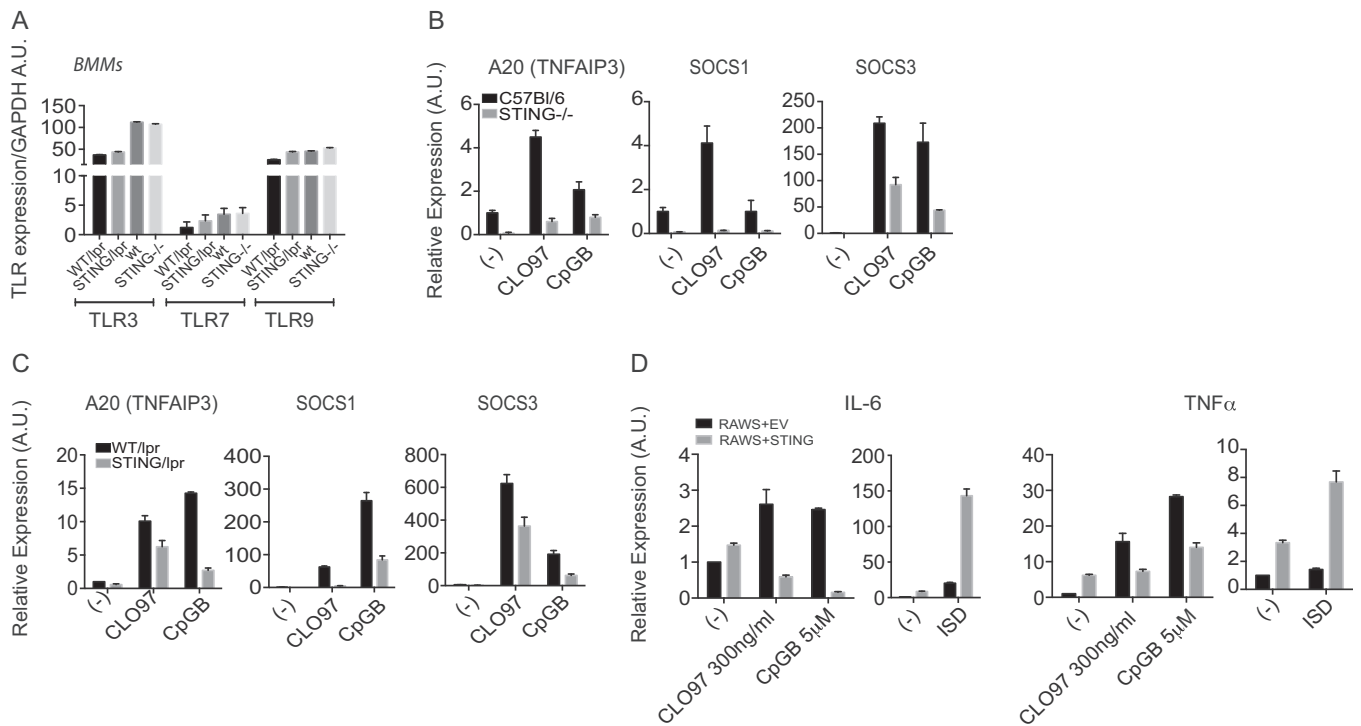
Fig. S5. STING expression regulates basal levels of key negative regulators and TLR-signaling strength but not TLR levels (related to Fig. 7). (*A*) Real-time PCR analysis of resting levels of TLR3, -7, and -9 in BMMs derived from WT/lpr, STING/lpr, WT/WT, and STING/WT littermate mice relative to the housekeeping gene *GAPDH*. (*B* and *C*) Protein levels of A20 and SOCS1 in C57BL/6 and STING$^{-/-}$ BMMs (*B*) or WT/lpr- and STING/lpr-derived BMMs (*C*) either left untreated or treated with endosomal TLR ligands CpGB (5 μM) and CLO97 (300 ng/mL) for 3 h. (*D*) Secreted levels of IL-6 and TNF-α from the RAW 264.7 macrophage cell line stably overexpressing STING in a retrovector (pRetroZeocin) or a control empty retrovector (EV), treated with CpG-B, CLO97, and ISD (3 μM), 3 h after stimulation.