Supporting Information

Clark et al. 10.1073/pnas.1215450109

SI Materials and Methods

Materials. Protein kinase inhibitors were dissolved in DMSO and stored at −20 °C as 10 mM solutions. The TLR agonists Pam3CSK4, lipoteichoic acid (LTA), R837, and CpG (ODN1826) were from Invivogen and lipopolysaccharide (LPS) (Escherichia coli O55:B5) from Alexis Biochemicals. Mouse IL-1α was obtained from Sigma. Mouse and human recombinant M-CSF was purchased from R&D Systems. Murine IL-4 and IFNγ were from PeproTech.

Chemical Synthesis. MRT67307 was synthesized as described (1). KIN112 is compound 28 in the article by Martin et al. (2) and was synthesized according to the authors’ instructions. To generate MRT699665, 7-[(1S)-4-hydroxy-2,3-dihydro-1H-inden-1-yl]-5,5-dimethyl-2-(methylsulfonyl)-5,7-dihydro-6H-pyrrolo[2,3-d]pyrimidin-6-one was synthesized as reported (3). This intermediate (75 mg, 0.2 mmol) and 3-(pyrrolidin-1-ylmethyl)aniline (106 mg, 0.6 mmol) were added to NMP (0.5 mL), and the resulting mixture was irradiated for 1 h at 180 °C in a Biotage I-60 microwave reactor. The mixture was cooled, filtered, and the filtrate was purified by preparative LCMS [HPLC column: 4.6 x 50 mm (5 μm) C-18 Xbridge; flow rate: 3 mL/min; run time: 3.2 min; solvent A: 0.1% ammonium hydroxide in water; solvent B: methanol; gradient: 10–100% B; gradient time: 2.35 min. HPLC retention time (2.15 min)] to give a brown solid (20 mg, 21%).

Synthesis of HG-9-91-01. The urea formation was performed by using a Biotage Initiator+ Microwave Synthesizer. All reactions were monitored by TLC with 0.25 mm E. Merck precoated silica gel plates (60 F254) and Waters LCMS system (Waters 2489 UV/Visible Detector, Waters 3100 Mass, Waters 515 HPLC pump, Waters 2545 Binary Gradient Module, Waters Reagent Manager, and Waters 2767 Sample Manager) by using SunFire C18 column (4.6 x 50 mm, 5 μm particle size); solvent gradient = 100% A at 0 min, 1% A at 5 min; solvent A = 0.05% TFA in Water; solvent B = 0.035% TFA in MeOH; flow rate: 2.5 mL/min. Purification of reaction products was carried out by flash chromatography using CombiFlashRFi with Teledyne Isco RediSepRFi High Performance Gold or Silicycle SiliaSep High Performance columns (4, 12, 24, 40, or 80 g) and Waters LCMS system using SunFire Prep C18 column (19 x 50 mm, 5 μm particle size); solvent gradient = 80% A at 0 min, 10% A at 8 min; solvent A = 0.035% TFA in water; solvent B = 0.035% TFA in MeOH; flow rate: 25 mL/min. The purity of all compounds was more than 95% and was analyzed with Waters LCMS system. 1H NMR and 13C NMR spectra were obtained by using a Varian Inova-600 (600 MHz for 1H, and 125 MHz for 13C) spectrometer. Chemical shifts are reported relative to chloroform (δ = 7.24) for 1H NMR or dimethyl sulfoxide (δ = 2.95) for 1H NMR and dimethyl sulfoxide (δ = 39.51) for 13C NMR. Data are reported as br = broad, s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet.

Scheme of HG-9-91-01.

Synthesis of HG-9-91-01.

6-Chloro-N-(2,4-Dimethoxyphenyl)Pyrimidin-4-Amine. To a solution of 4,6-dichloropyrimidine (1.2 g; 8.11 mmol) in 2-propanol (34 mL) was added 2,4-dimethoxyaniline (1.03 g, 6.75 mmol) and N,N-diisopropylethylamine (2.82 mL, 16.22 mmol). The reaction mixture was stirred at 50 °C for 24 h and partitioned between ethyl acetate and saturated aqueous sodium bicarbonate solution. The organic layer was washed with brine, dried over MgSO4, filtered through a pad of celite, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (1:3:7, ethyl acetate/hexane) to afford 6-chloro-(2,4-dimethoxyphenyl)pyrimidin-4-amine (1.4 g; 78% yield) as an off-white solid. Rt = 3.80 min; 1H-NMR 600 MHz (CDCl3) δ 8.39 (s, 1H), 7.44 (bs, 1H), 7.37 (bs, 1H), 6.49–6.52 (m, 3H), 3.81 (m, 6H); 1H NMR 600 MHz (DMSO-d6) δ 9.02 (s, 1H), 8.23 (s, 1H), 7.36 (bs, 1H), 6.60 (d, J = 2.4 Hz, 1H), 6.47 (m, 1H), 3.68 (m, 6H); MS m/z: 266.13 [M+1].

N4-(2,4-Dimethoxyphenyl)-N6-(4-(4-Methylpiperazin-1-yl)Phenyl)Pyrimidine-4,6-Diamine. To a solution 6-chloro-N-(2,4-dimethoxyphenyl)pyrimidin-4-amine (1.0 g, 3.77 mmol) in 2-butanol (9 mL) and...
trifluoroacetic acid (1 mL) was added 4-(4-methylpiperazin-1-yl) aniline (685 mg, 3.58 mmol, AK Scientific). The reaction mixture was stirred at 100°C for 24 h, and the solvent concentrated under reduced pressure. The reaction mixture was diluted with dichloromethane and washed with saturated aqueous potassium carbonate solution and brine. The organic layer was dried over MgSO₄, filtered through a pad of celite, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (1.90–9.79, ammonia solution 7.0 M in methanol/dichloromethane; Sigma Aldrich) to afford N4-(2,4-dimethoxyphenyl)-N6-(4-(4-

methylpiperazin-1-yl)phenyl)pyrimidine-4,6-diamine (1.2 g, 80% yield) as an off-white solid. Rt = 2.18 min; 1H NMR 600 MHz (DMSO-d₆) δ 6.87 (s, 1H), 8.05 (s, 1H), 8.01 (s, 1H), 7.56 (dd, J = 8.4 Hz, 1H), 7.30 (d, J = 8.4 Hz, 2H), 6.87 (d, J = 9.6 Hz, 2H), 6.63 (d, J = 3.0 Hz, 1H), 6.51 (dd, J = 9.0 Hz, J = 3.0 Hz, 1H), 5.70 (s, 1H), 3.77 (s, 3H), 3.75 (s, 3H), 3.08 (m, 4H), 2.56 (m, 4H), 2.30 (s, 3H); 13C NMR 125 MHz (DMSO-d₆) δ 161.87, 160.80, 157.45, 157.31, 157.76, 146.20, 132.56, 124.40, 121.45, 120.93, 113.07, 110.07, 103.48, 99.17, 83.76, 73.71, 55.56, 55.32, 54.26, 48.36, 45.13; MS m/z: 421.45 [M+H]+.

1-(2,4-Dimethoxyphenyl)-3-(2,6-Dimethylphenyl)urea, were generated in a ratio of 4:1 by HPLC analysis.

Bone marrow-derived macrophages were differentiated from mouse embryonic fibroblasts (MEFs) from TBK1/IKKe−/−, TBK1/IKKe−/−, LKB1+/−, and LKB1−/− mice were cultured in DMEM containing 10% (vol/vol) FCS, 20 μg/mL LPS, and 1 μg/mL CpG. For knockdown of SIK1, SIK2, and SIK3, shRNAs targeting SIK1 (TRCN0000079130), and the negative control (SHC001) were used in the Mission Lentiviral Packaging Mix were purchased from Sigma.

Cell Culture. Bone marrow-derived macrophages were differentiated for 7 d in DMEM supplemented with 5 ng/mL recombinant M-CSF (R&D Systems), 2 mM glutamine, 10% (vol/vol) FCS, penicillin and streptomycin. RAW264.7 cells stably expressing SIK2 and SIK3 were generated by retroviral transduction using a Murine Moloney Leukemia virus-based system prepared with VSV-G envelope protein. Retrovirus particles were prepared according to the manufacturer's instructions (Conlutech). Viruses encoding the gene of interest and the Tet-On protein were harvested 48 h after transfection, diluted fourfold with fresh media, and incubated with RAW264.7 cells in the presence of 2 μg/mL protamine sulfate (Sigma) for 24 h. Fresh media containing 1 mg/mL G418 (Tet-On) and 3 μg/mL R837, or 2 μg/mL G418 (Tet-On) and 3 μg/mL puromycin (gene of interest) was added to select the transduced cells. To induce gene expression, cells were cultured in the presence of 20–1,000 ng/mL doxycyclin for 8–24 h.

Retrovirual Transduction of RAW264.7 Cells. RAW264.7 cells stably expressing CRTC3 and SIK2 were generated by retroviral transduction using a Murine Moloney Leukemia virus-based system prepared with VSV-G envelope protein. Retrovirus particles were prepared according to the manufacturer’s instructions (Conlutech). Viruses encoding the gene of interest and the Tet-On protein were harvested 48 h after transfection, diluted fourfold with fresh media, and incubated with RAW264.7 cells in the presence of 2 μg/mL protamine sulfate (Sigma) for 24 h. Fresh media containing 1 mg/mL G418 (Tet-On) and 3 μg/mL puromycin (gene of interest) was added to select the transduced cells. To induce gene expression, cells were cultured in the presence of 20–1,000 ng/mL doxycyclin for 8–24 h.

RAW264.7 cells or BMDMs were transfected with 100 pmol of SMARTpool siRNA oligos against CREB, CRTC1, CRTC2, CRTC3, or a nontargeting control (Dharmacon) by using AMAXA nucleofection. Cells were cultured for 24 h before incubation with inhibitors for 1 h followed by stimulation for 1 h with 100 ng/mL LPS. Gene expression was measured by real-time quantitative PCR (qPCR). For shRNA knockdown of SIKs, lentivirus was generated according to the manufacturer’s instructions.
(Sigma), and RAW264.7 cells were infected by using virus harvested 48 and 72 h after transfection. Infected cells were selected using 3 μg/mL puromycin.

**qPCR.** mRNA was extracted from cells by using the RNeasy Micro Kit following the manufacturers’ instructions (Qiagen). cDNA was generated from 1 μg of total RNA by using the iScript cDNA synthesis kit and quantified by qPCR using the SsoFast EvaGreen Supermix on a CFX96 real-time system (Bio-Rad Laboratories). The relative expression of each gene was calculated from Ct values by using the Pfaffl method (5) and was normalized against the mRNA levels of 18S RNA or GAPDH. Results are reported relative to untreated control cells, which was set to 1. The primers used were the following:

- **IL-10-F, CCCCATACTGGTCTTCCCTC**
- **IL-10-R, GATCTCCCTGTTTTCCTCTCC**
- **c-Fos-R, CTCACCTTCTGAGCACTAG**
- **c-Fos-R, CTCACCTTCTGAGCACTAG**
- **Nurr1-R, CCAATCAATCCAAACG**
- **Nurr1-R, CCAATCAATCCAAACG**
- **Nurr77-F, GGAGGACGAGGAGGAGT**
- **Nurr77-R, GGAGGACGAGGAGGAGT**
- **CRTC3-F, ACTCAAAGAAGGCGGGTTCC**
- **CRTC3-R, ATTCCCATCAAACTGTCTCC**
- **CRTC2-F, TGCGACTGGCTTATACAAGG**
- **CRTC2-R, GAGTGCTCCGAGATGAATCC**
- **SIK2-F, AAATAAATGGCAGCAAAG**
- **SIK2-R, AAATAAATGGCAGCAAAG**
- **YM1-R, GTCTTGCTCATGTGTGTAAGTGA**
- **YM1-F, AGAAGGGAGTTTCAAACCTGGT**
- **Mgl2-R, GGCCTCCAATTCTTGAAACCT**
- **Mgl2-R, GGCCTCCAATTCTTGAAACCT**
- **Arg1-F, CTCCAAGCCAAAGTCCTTAGAG**
- **Arg1-R, AGGAGCTGTCATTAGGGACATC**
- **LIGHT-R, GATACGTCAAGCCCCTCAAG**
- **LIGHT-F, CTGCATCAACGTCTTGGAGA**
- **SPHK1-F, ACACAGTGTGCAGTTGATGA**
- **SPHK1-R, ACACAGTGTGCAGTTGATGA**
- **18S-R, CCATCCAATCGGTAGTAGCG**
- **18S-F, GTAACCCGTTGAACCCCATT**
- **GAPDH-F, GGACACGAGGAGGAGT**
- **GAPDH-R, GGACACGAGGAGGAGT**
- **Nurr77-F, CCTGTTGCTAGAGTCTGCCTTC**
- **Nurr77-R, GGAGGACGAGGAGGAGT**
- **c-Fos-R, AACATTGACGCTGAAGGACTAC**
- **c-Fos-R, AACATTGACGCTGAAGGACTAC**
- **IL-10-F, ACCTTATTGTGCTTCCCTC**
- **IL-10-R, GATCTCCCTGTTTTCCTCTCC**
- **pSer370 (S253D bleed 2) of CRTC3** were raised against the peptide CWKEEKHPGFR (S277D bleed 2) and coupled to Protein G-Sepharose. To detect proteins in cell lysates, 20 μg of protein extract was separated by SDS/PAGE. After transfer to PVDF membranes, proteins were detected by immunoblotting and visualized by treating the blots with ECL (Amersham) followed by autoradiography. The following antibodies were used for immunoblotting: pSer133 CREB, pSer171 CRTC2, total CRTC2, GAPDH, total STAT3, and pTyr705 STAT3 were purchased from Cell Signaling Technology; FLAG (M2 clone) was obtained from Sigma; CRTC3 was from Abcam; HA (3F10) was from Roche, 14-3-3 was obtained from Abcam; FLAG (M2 clone) was obtained from Sigma; CRTC3 was from Abcam; HA (3F10) was from Roche, 14-3-3 was obtained from Abcam; 14-3-3 was obtained from Abcam; GAPDH, total STAT3, and pTyr705 STAT3 were purchased from Cell Signaling Technology.

**Luciferase Assays.** HEK293 cells were cotransfected with DNA encoding FLAG-CRTC3 or phosphomutants along with a CREB firefly luciferase reporter construct (pCRE-luc from Stratagene) and pTK-Rl, which encodes renilla luciferase under the control of a constitutive promoter. At 24 h after transfection, cells were treated with or without 10 μM KIN112 for a further 24 h. Firefly luciferase activity was measured with a dual luciferase assay system (Promega) and was normalized to Renilla luciferase activity.

**Immunoblotting.** Cells were rinsed in ice-cold PBS and extracted in lysis buffer (50 mM Tris HCl at pH 7.4, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium β-glycerol 1-phosphate, 1 mM DTT, 1 mM sodium orthovanadate, 0.27 M sucrose, 1% (vol/vol) Triton X-100, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 mM phenylmethylsulphonyl fluoride). Cell extracts were clarified by centrifugation at 14,000 × g for 10 min at 4 °C, and protein concentrations were determined by using the Bradford assay. FLAG-CRTC3 was purified on anti-FLAG M2 agarose, whereas endogenous CRTC3 was immunoprecipitated from cell extracts by using anti-CRTC3 raised against the peptide CWKEEKHPGFR (S277D bleed 2) and coupled to Protein G-Sepharose. To detect proteins in cell lysates, 20 μg of protein extract was separated by SDS/PAGE. After transfer to PVDF membranes, proteins were detected by immunoblotting and visualized by treating the blots with ECL (Amersham) followed by autoradiography.

**Cytokine Secretion.** After stimulation with ligands, the cell culture medium was removed, clarified by centrifugation for 10 min at 14,000 × g, and the concentration of TNFα, IL-6, IL-10, IL-12p40, and RANTES were measured by using the Bio-Plex Pro Assay system from Bio-Rad. IL-1ra and IFNβ were measured by using ELISA kits from R&D Systems.
kinase assays were performed as described by Hastie et al. (6) but using a peptide derived from sequences surrounding Ser171 of CRTC2 (ALNRTSSDALHRRR) as a substrate. Kinase profiling was performed as described (7) and performed by the International Centre for Kinase Profiling (www.kinase-screen.mrc.ac.uk).

14-3-3 Binding Assay. CRTC3 was immunoprecipitated from cell extracts, resolved by SDS/PAGE, and transferred onto PVDF membrane. The membrane was incubated with purified yeast 14-3-3 that had been coupled to digoxigenin (DIG) (1 μg/mL) overnight. An interaction with 14-3-3 was detected by subsequently incubating the membrane with anti-DIG–horseradish peroxidase conjugate followed by ECL and autoradiography.

Phosphoproteomics. RAW264.7 cells were labeled by using the stable isotope labeling of amino acids in cell culture (SILAC) method. Cells were treated for 1 h with 2 μM MRT67307 or vehicle control and, subsequently, left unstimulated or stimulated for 30 min with 1 μg/mL Pam3CSk4. The cells were lysed in detergent-free lysis buffer (50 mM Tris-HCl at pH 8.2, 10 mM glycerol 2-phosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM DTT, 1 mM PMSF, 1 μg/mL aprotinin, and 1 μg/mL leupeptin) containing 8 M urea, the extract was clarified by centrifugation, and protein concentrations were determined by using the Bradford method. Two milligrams of cell extract protein from each experimental condition were mixed in a 1:1 ratio and then alkylated for 30 min with 50 mM iodoacetamide. The sample was diluted with 0.1 M ammonium bicarbonate to a final urea concentration of 1.5 M, and the proteins were digested overnight with 100 μg of trypsin at 37 °C. The digests were acidified, desalted by using C18 Sep-Pak cartridges (Waters), and the peptides were dried by using a SpeedVac. The digests were acidified, desalted by using C18 Sep-Pak cartridges (Waters), and the peptides were dried by using a SpeedVac. The peptides were dissolved in 750 μL of 80% (vol/vol) ACN/0.1% (vol/vol) trifluoroacetic acid and fractionated by hydrophilic (HILIC) chromatography (8). The samples were loaded on a TSKgel Amide-80 column (TOSOH, dimension: 4.6 mm × 25 cm), and the gradient was developed as described (9). Under these conditions, the phosphopeptides eluted from the HILIC column between 20–70 min. Phosphopeptides from these samples were enriched by using a standard Fe²⁺–IMAC enrichment protocol (8, 9) and measured by LC-MS/MS. Samples were separated on a Proxeon Easy-nLC system (Thermo Fisher Scientific) by using a 20 cm long, 75 μm internal diameter PicoFrit column (New Objective) home packed with Magic C18 reverse phase material (Monday Bioresources) and the following gradient: (i) 0–170 min from 35 to 80% buffer B; (ii) 170–187 min from 35 to 80% buffer B; (iii) 187–188 min from 80 to 90% buffer B; 188–198 min isocratic at 90% buffer B; 198–199 min from 90 to 2% buffer B; 199–204 min isocratic at 2% buffer B. The composition of buffer A was as follows: 0.1% (vol/vol) formic acid, 2% (vol/vol) acetonitrile. The nano-LC system was online with a Thermo Fisher Scientific LTQ Orbitrap Velos instrument set to perform top-15 data-dependent CID analysis in the 350–1,600 m/z range by using a resolution of 60,000 for the precursor scan and a minimal intensity for sequencing of 10,000 counts. Monoisotopic precursor selection was used and +1 as well as unassigned charge states were excluded from sequencing. Dynamic exclusion was set to a repeat count of 2 within 30 s, with exclusion duration of 90 s and an exclusion mass width of 10 ppm. The data were analyzed by using MaxQUANT (10).

Statistical Analysis. Quantitative data are presented as the mean ± SD. Statistical significance of differences between experimental groups was assessed by using the Student t test or two-way ANOVA with the Bonferroni post test. Differences in means were considered significant if P < 0.05. Unless otherwise indicated, the symbols represent the following *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. S1. Chemical structures of pharmacological inhibitors of SIKs. (A) MRT67307. (B) MRT199665. (C) KIN112. (D) HG-9-91-01.

Fig. S2. The effects of MRT67307 on CREB-dependent gene transcription are not caused by inhibition of the IKK-related kinases. (A) A much higher concentration of MRT67307 is needed to enhance LPS-stimulated IL-10 mRNA production than to suppress IFNβ mRNA formation. (n = 4, mean ± SD). Red arrow indicates EC50 values. (B) MRT67307 elevates Nurr77 mRNA levels similarly in IL-1–stimulated TBK1/IKKe+/− and TBK1/IKKe−/− MEFs (mean ± SD, n = 3, **P < 0.01).
Fig. S3. Selectivity of MRT67307, MRT199665, KIN112, and HG-9-91-01 for different protein kinases. The activities of 108 protein kinases were measured in the presence of 1 μM MRT67307 (A), 1 μM MRT199665 (B), 1 μM KIN112 (C), and 0.1 μM HG-9-91-01 (D). The data are reported as the percent of activity remaining in the presence of the inhibitor relative to the activity measured in the absence of inhibitor. The red bars indicate the protein kinases inhibited most potently and members of the AMPK subfamily are highlighted in blue (n = 2, mean ± SD).
**Fig. S4.** Effect of SIK inhibitors on TLR-stimulated cytokine production. (A) MRT67307, MRT199665, KIN112, and HG-9-91-01 stimulate IL-10 secretion in a dose-dependent manner. BMDMs were treated for 1 h with the indicated concentrations of MRT67307, MRT199665, KIN112, and HG-9-91-01, then stimulated for 6 h with LPS. IL-10 levels in culture supernatants were measured by using the Bio-Plex kit (n = 4, mean ± SD). (B) Effect of KIN112 on LPS-stimulated mRNA and cytokine production. BMDMs were treated for 1 h with 10 μM KIN112 and then stimulated for 1 h with 100 ng/mL LPS (mRNA expression) or for 6 h (protein secretion) (mean ± SD, n = 4, ***P < 0.001). (C) Effect of HG-9-91-01 on cytokine production in macrophages costimulated with LPS and IFNγ. BMDMs were incubated for 1 h with 500 nM HG-9-91-01 then stimulated with 10 ng/mL IFNγ and/or 100 ng/mL LPS. IL-10 mRNA levels were measured by qPCR after stimulation for 1 h and cytokine secretion measured after 6 h of stimulation (mean ± SD, n = 4). **P < 0.01 and ***P < 0.001 compared with samples treated with vehicle control.

**Fig. S5.** siRNA knockdown of CRTC3 abolishes the effects of HG-9-91-01 on IL-10 production in primary macrophages. BMDMs were transfected with siRNA oligonucleotides against CRTC1, CRTC2, CRTC3, or nontargeting oligonucleotides as a negative control. Cells were incubated for 1 h without or with or 500 nM HG-9-91-01, then stimulated for a further 1 h with 100 ng/mL LPS. Gene expression was quantified by qPCR and reported relative to mRNA levels measured in untreated cells (1.0). Knockdown efficiency was monitored by qPCR (mean ± SD, n = 4, *P < 0.05, **P < 0.01, ***P < 0.001).
Fig. S6. Conservation of the effect of SIK inhibitors on IL-10 production in myeloid cells and across species. (A and B) Effect of MRT67307 and KIN112 on LPS-stimulated CREB-dependent gene transcription and IL-10 production in dendritic cells. Bone marrow was differentiated into dendritic cells (BMDCs) by incubation for 7 d in the presence of GM-CSF. BMDCs were incubated for 1 h with 2 μM MRT67307 or 10 μM KIN112 and then stimulated for 1 h with 100 ng/mL LPS (mRNA expression) (A) or the culture supernatant was harvested at the times indicated and protein secretion measured (B) (mean ± SD, n = 4). (C and D) Effect of SIK inhibitors on mRNA production in human cells. (C) THP-1 monocytes were treated with 1 μM MRT199665 or 10 μM KIN112 for 1 h and then stimulated with 1 μg/mL Pam3CSK4 for 1 h. (D) Primary human macrophages were incubated for 1 h with 1 μM MRT199665 or 500 nM HG-9-91-01 and then stimulated for 1 h with 100 ng/mL LPS. mRNA levels were measured by qPCR (mean ± SD, n = 4). For all graphs, statistical significance is reported as follows: **P < 0.01, ***P < 0.001.

Fig. S7. SIK inhibitors induce the dephosphorylation of CRTC3 at Ser62, Ser162, Ser329, and Ser370 and its translocation to the nucleus. (A) HEK293 cells were transfected with FLAG-CRTC2, FLAG-CRTC3, or FLAG-CRTC3[S162A]. Aliquots of the cell extracts were subjected to SDS/PAGE and immunoblotting by using an antibody that recognizes CRTC2 phosphorylated at Ser171. The figure shows that this antibody also recognizes CRTC3 phosphorylated at Ser162. (B) HEK293 cells were transfected with FLAG-CRTC3, FLAG-CRTC3[S62A/S162A/S329A/S370A] (termed CRTC3-4A), or with empty vector (FLAG-ev). After transfection, cells were incubated for 1 h without (control) or with 2 μM MRT67307, 1 μM MRT199665, 10 μM KIN112, or 500 nM HG-9-91-01. (C) Inhibition of SIKs promotes the translocation of endogenous CRTC3 to the nucleus. RAW264.7 cells were treated with MRT67307, MRT199665, KIN112, or HG-9-91-01 for 1 h and stained for CRTC3 (red), tubulin (green), and DNA (DAPI-blue).
Fig. S8. shRNA knockdown of SIKs sensitizes macrophages to HG-9-91-01. (A) mRNA abundance of SIK1, SIK2, and SIK3 in RAW264.7 macrophages. Gene expression was measured by qPCR. The number of copies of each SIK mRNA was normalized to GAPDH (mean ± SD, n = 3). (B) The efficiency of SIK knockdown. RAW264.7 cells were transduced with lentiviral vectors encoding a control shRNA or shRNAs targeting SIK1, SIK2, and SIK3. The expression of SIK1, SIK2, and SIK3 was measured by qPCR and normalized to that of control cells using GAPDH (mean ± SD, n = 3). (C) The concentration of HG-9-91-01 required to stimulate IL-10 mRNA is reduced in cells with decreased SIK expression. Cells were incubated for 1 h with increasing concentrations of HG-9-91-01, then stimulated for 1 h with 1 μg/mL Pam3CSK4. The levels of IL-10 mRNA were measured by qPCR and normalized by using GAPDH. Data are presented with the highest level of expression set to 1 (mean ± SD, n = 3). IC50 for control cells (filled circles) was 260 ± 10 nM and SIK1/2/3 shRNA cells (open circles) was 150 ± 20 nM (P < 0.01). (D) Increased IL-10 expression in cells with decreased SIK expression. Same as C, except that HG-9-91-01 concentration was held at 100 nM (mean ± SD, n = 3). For all graphs, statistical significance is reported as follows: *P < 0.05, **P < 0.01.

Fig. S9. Gene expression profile of wound-healing macrophages (M2a). BMDMs were stimulated with 10 ng/mL IL-4 for the times indicated and gene expression measured by qPCR (mean ± SD, n = 4, **P < 0.01, ***P < 0.001).
Fig. S10. IL-10 signals in an autocrine manner in macrophages treated with SIK inhibitors. (A) MRT67307 increases LPS-induced phosphorylation of STAT3. BMDMs were treated for 1 h without or with 2 μM MRT67307, then stimulated with LPS for the times indicated. (B) Phosphorylation of STAT3 does not occur in BMDMs from IL-10−/− mice. As in A, except that cells were stimulated for 2 h with 100 ng/ml LPS.