Phosphorylation of CRTC3 by the salt-inducible kinases controls the interconversion of classically activated and regulatory macrophages


Macrophages acquire strikingly different properties that enable them to play key roles during the initiation, propagation, and resolution of inflammation. Classically activated (M1) macrophages produce proinflammatory mediators to combat invading pathogens and respond to tissue damage in the host, whereas regulatory macrophages (M2b) produce high levels of anti-inflammatory molecules, such as IL-10, and low levels of proinflammatory cytokines, like IL-12, and are important for the resolution of inflammatory responses. A central problem in this area is to understand how the formation of regulatory macrophages can be promoted at sites of inflammation to prevent and/or alleviate chronic inflammatory and autoimmune diseases. Here, we demonstrate that the salt-inducible kinases (SIKs) restrict the formation of regulatory macrophages and that their inhibition induces striking increases in many of the characteristic markers of regulatory macrophages, greatly stimulating the production of IL-10 and other anti-inflammatory molecules. We show that SIK inhibitors elevate IL-10 production by inducing the dephosphorylation of cAMP response element-binding protein (CREB)-regulated transcriptional coactivator (CRTC) 3, its dissociation from 14-3-3 proteins and its translocation to the nucleus where it enhances a gene transcription program controlled by CREB. Importantly, the effects of SIK inhibitors on IL-10 production are lost in macrophages that express a drug-resistant mutant of SIK2. These findings identify SIKs as a key molecular switch whose inhibition reprograms macrophages to an anti-inflammatory phenotype. The remarkable effects of SIK inhibitors on macrophage function suggest that drugs that target these protein kinases may have therapeutic potential for the treatment of inflammatory and autoimmune diseases.

Toll-like receptor | AMPK-related kinases | MRT67307 | MRT199665 | HG-9-31-01

The ability of macrophages to adapt their physiology in response to extracellular cues allows them to play key roles throughout the inflammatory process from its onset to its resolution (1, 2). Tissue-resident macrophages are among the first leukocytes to respond to foreign pathogens. These macrophages express a variety of receptors, including Toll-like receptors (TLR), which recognize core components of bacteria, viruses, fungi, and protozoa (3). The ligation of TLRs stimulates the formation of classically activated macrophages (M1) that produce proinflammatory mediators leading to the recruitment of other leukocytes, such as neutrophils, which help to combat the invading pathogens (1, 2, 4). However, after the host has cleared the infection, the inflammatory response must be resolved effectively to repair the damaged tissue and avoid the development of chronic inflammation. To this end, macrophages acquire distinct anti-inflammatory phenotypes to promote tissue repair and the resolution of inflammation. For example, IL-4 and IL-13 induce wound-healing macrophages (M2a), which deposit extracellular matrix to repair the damaged tissue, whereas stimulation of macrophages with TLR agonists and costimuli such as apoptotic cells and immune complexes induce regulatory macrophages (M2b), which promote the resolution of inflammation (1, 2, 4, 5).

Regulatory macrophages are characterized by the production of high levels of the anti-inflammatory cytokine IL-10 and low levels of the proinflammatory cytokine IL-12 (1, 5). The ratio of IL-10 to IL-12 production provides the best marker for the detection of regulatory macrophages, but these cells also express other molecules, such as the tumor necrosis factor (TNF) family member LIGHT and sphingosine kinase 1 (SPHK1) (6). Importantly, injection of regulatory macrophages into mice ameliorates the cardinal features of septic shock, colitis, and experimental autoimmune encephalomyelitis (5, 7). Thus, understanding the signaling pathways that control the conversion of classically activated macrophages into regulatory macrophages will provide further insight into the molecular mechanisms regulating the resolution of inflammation and may also identify novel drug targets for the treatment of chronic inflammatory diseases. Here, we report the unexpected observation that inhibition of the salt-inducible kinases (SIKs) induces a macrophage population with all of the hallmarks of regulatory macrophages and dissect the molecular mechanism by which the SIKs restrict the conversion of classically activated to regulatory macrophages.

Results

MRT67307 Increases TLR-Stimulated Production of Anti-Inflammatory Cytokines While Suppressing Proinflammatory Cytokine Secretion.

We developed the protein kinase inhibitor MRT67307 (8, 9) (Fig. S1A) and, while characterizing it, discovered that macrophages exposed to this compound secreted far higher levels of the anti-inflammatory cytokines IL-10 and IL-1ra and much lower levels of proinflammatory cytokines in response to bacterial lipopolysaccharide (LPS), a ligand for TLR4 (Fig. S1B). Similar results were obtained when macrophages were stimulated with ligands that activate other TLRs (Fig. 1B). These striking findings led us to investigate the molecular mechanism by which MRT67307 elevates IL-10 because this anti-inflammatory cytokine is an essential marker of regulatory macrophages.

MRT67307 Increases IL-10 Production via a cAMP Response Element-Binding Protein (CREB)-regulated Transcriptional Coactivator (CRTC) 3 Dependent Mechanism.

Initial experiments revealed that MRT67307 greatly increased the formation of IL-10 mRNA in TLR-stimulated macrophages but not in unstimulated macrophages (Fig. 2A). The


The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

1To whom correspondence should be addressed. E-mail: p.cohen@dundee.ac.uk.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1215450109/-/DCSupplemental.
effects were rapid and transient, IL-10 mRNA levels reaching a maximum after 1 h and returning to near basal levels after 6 h (Fig. 2B). Because one pathway by which TLR ligands stimulate transcription of the IL-10 gene involves the activation of the transcription factor CREB (10), we initially studied whether MRT67307 could enhance the formation of the mRNA encoding other CREB-dependent genes, such as those encoding the transcription factor c-fos and the nuclear orphan receptors Nurr1 and Nurr77. MRT67307 indeed increased mRNA encoding these proteins with similar kinetics to IL-10 in TLR-stimulated, but not unstimulated, macrophages (Fig. 2B). Moreover, siRNA knockdown of CREB prevented MRT67307 from potentiating the induction of IL-10 mRNA by LPS (Fig. 2C). Our results therefore indicated that MRT67307 enhanced IL-10 production by stimulating CREB-dependent gene transcription in TLR-stimulated macrophages.

The activation of CREB by TLR ligands is known to require its phosphorylation at Ser133, which is catalyzed by the mitogen and stress-activated kinases 1 and 2 (11), and generates a docking site

Fig. 1. MRT67307 increases IL-10 production and suppresses proinflammatory cytokine production in macrophages. (A) MRT67307 increases IL-10 and IL-1ra secretion while suppressing the release of proinflammatory cytokines in LPS-stimulated bone marrow-derived macrophages (BMDMs). In the absence of LPS, cytokine concentrations in the culture supernatant were below 10 pg/mL, even in the presence of MRT67307 (6 h, n = 4, mean ± SD, ***$P$ < 0.001). (B) Effect of MRT67307 on cytokine production in BMDMs stimulated with different TLR agonists (n = 6, mean ± SD). TLR1/2-Pam3CSK4, TLR2/6-lipoteichoic acid (LTA), TLR4-LPS, TLR7-R837, TLR9-CpG DNA (ODN1826). All data $P$ < 0.001 relative to 0 except for IL-6 regulation by R837 and CpG. ns, not significant.

Fig. 2. MRT67307 increases CREB-dependent gene transcription by promoting the dephosphorylation of CRTC3. (A) Effect of MRT67307 on IL-10 mRNA levels in TLR-stimulated BMDMs (n = 4, mean ± SD). (B) Effect of MRT67307 on transcription of CREB-dependent genes in LPS-stimulated BMDMs (n = 4, mean ± SD). (C) siRNA knockdown of CREB prevents the increase in IL-10 expression by MRT67307 in LPS-stimulated RAW264.7 macrophages (n = 3, mean ± SD). (D) Effect of MRT67307 on CREB and ATF1 phosphorylation in response to LPS in RAW264.7 macrophages. (E) Phosphoproteomics pipeline. (F) Mass spectrum showing phosphopeptide precursor ions corresponding to CRTC3[329–339], which is dephosphorylated at Ser329 in RAW264.7 macrophages treated with MRT67307. Ions are marked with white, grey, and black circles to denote the origin of the ion as described in E. (G) Summary of phosphoproteomic results showing the phosphorylation of Ser62, Ser329, and Ser370 of CRTC3 induced by MRT67307 (L, light; M, medium; H, heavy). (H) CRTC3 phosphorylation at Ser370 is unaffected by stimulation of BMDM for 15 min with LPS but suppressed by MRT67307. (I) siRNA knockdown of CRTC3 blocks the induction of IL-10 mRNA in RAW264.7 macrophages stimulated with LPS in the presence of MRT67307. Knockdown efficiency was measured by qPCR and immunoblotting (n = 3, mean ± SD). For all graphs, statistical significance is reported as follows: *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001.
for the cofactors CREB-binding protein (CBP) and the closely related p300 (12). CREB-dependent gene transcription can be further enhanced by interactions with the CRTC family. Studies in other mammalian cells and in our experiments with CRTC2, have shown that the dephosphorylation of CRTC2 releases them from 14-3-3 proteins by facilitating their entry into the nucleus where they associate with CREB to promote CREB-dependent gene transcription (12). We found that MRT67307 had little effect on TLR-stimulated phosphorylation of CREB at Ser133 or the closely related ATF1 at Ser63 (Fig. 2D). However, in a phospho-proteomic study to identify proteins whose phosphorylation was suppressed by MRT67307 (Fig. 2E), we found that the phosphorylation of CRTC3 was unaffected by stimulation with the TLR1/2 agonist Pam3CSK4, but nevertheless was robustly enhanced transcription of the CREB-dependent Nurr77 gene in macrophages were incubated with MRT67307 (Fig. 2F and G). Similarly, phosphorylation of CRTC3 at Ser370 was unaffected by LPS stimulation but blocked by MRT67307 (Fig. 2F). siRNA knockdown of CRTC3, but not CRTC1 or CRTC2, suppressed the effects of MRT67307 on TLR-stimulated IL-10 production in macrophages (Fig. 2F). Collectively, these results suggested that the effects of MRT67307 on CREB-dependent gene transcription, including IL-10 transcription, were mediated by the inactivation of a protein kinase leading to the dephosphorylation and activation of CRTC3 in macrophages.

Inhibition of SIKs by MRT67307, MRT199665, and HG-9-91-01 Increases IL-10 Production While Suppressing IL-6, IL-12, and TNF Secretion. In nonimmune cells, the phosphorylation of CRTC isoforms is catalyzed by members of the AMPK-related protein kinase (AMPK) family, including the microtubule affinity-regulating kinases (MARKs), the SIKs, and AMPK itself (12–14). These reports were intriguing because we had originally developed MRT67307 as an inhibitor of the 1εB kinase (IKK)-related kinases, TBK1 and IKKe (8, 9). However, we found that the concentration of MRT67307 required to enhance IL-10 secretion was 20-fold higher than that needed to block the TBK1/IKe-dependent production of IFNγ by LPS. Moreover, MRT67307 could still enhance transcription of the CREB-dependent Nur77 gene in TBK1/IKe−/− fibroblasts (Fig. S2). These observations suggested that inhibition of the IKK-related kinases did not underlie the effects of MRT67307 on CREB-dependent gene transcription and IL-10 production. We therefore examined whether members of the AMPK subfamily might be inhibited by MRT67307, which revealed that this compound inhibited the MARK, NUAK, and SIK isoforms with comparable potency to the IKK-related kinases (Fig. 3A). MRT67307 did not inhibit the brain-specific kinases (BRSKs) and only inhibited the maternal embryonic leucine zipper kinase (MELK) and AMPK itself more weakly.

To investigate whether and which AMPK family member might be regulating CREB-dependent gene transcription and IL-10 production, we exploited additional pharmacological inhibitors with specificities that were distinct from MRT67307 (Figs. S1 and S3). MRT199665 (Fig. S1B), a potent inhibitor of most AMPK-related kinases, which does not inhibit the IKK-related kinases (Fig. 3A), increased LPS-stimulated IL-10 mRNA and Nurr77 mRNA production, and IL-10 secretion (Fig. 3B), further supporting the view that inhibition of AMPK-related kinases, and not the IKK-related kinases, drives IL-10 production. The SIKs are unique among the AMPK-related kinases in possessing a small amino acid residue (threonine) at the “gatekeeper” site (15, 16). We therefore examined KIN112 (Fig. S1C) and, subsequently, a much more potent analog HG-9-91-01 (Fig. S1D), which not only targets the ATP-binding site, but also a small hydrophobic pocket adjacent to this site that is created by the presence of a small amino acid residue at this gatekeeper site. KIN112 and HG-9-91-01 inhibited a number of protein tyrosine kinases that possess a threonine residue at the gatekeeper site, such as Src family members (Src, Lck, and Yes), BTK, and the FGF and Ephrin receptors (Fig. S3). However, they also potently inhibited the SIKs and, crucially, did not inhibit any other member of the AMPK-related kinase subfamily (Fig. 3A), which all possess a large hydrophobic residue (Met or Leu) at the gatekeeper site. Like MRT67307 and MRT199665, HG-9-91-01 and KIN112 increased LPS-stimulated IL-10 production and greatly suppressed proinflammatory cytokine secretion (Fig. 3C and Fig. S4A and B), even when cells were costimulated with IFNγ to generate fully polarized classically activated (M1) macrophages (Fig. S4C). Moreover, CRTC3 was required for HG-9-91-01 to elevate IL-10 production in LPS-stimulated primary mouse macrophages (Fig. S5). SIK inhibitors also enhanced CREB-dependent gene transcription and IL-10 production in bone marrow-derived dendritic cells (Fig. S6 A and B), human THP-1 cells, and human primary macrophages (Fig. S6 C and D). Taken together, these results indicate that the SIK-CRTC3 signaling pathway and its role in regulating IL-10 production is present in different cells of the myeloid lineage and conserved in man.

SIKs Phosphorylate CRTC3 at Ser62, Ser162, Ser329, and Ser370 to Regulate 14-3-3 Protein Binding, Nuclear Transport, and CREB Coactivator Function. To address the regulation of CRTC3 in macrophages, we mapped the key phosphorylation sites on this protein. Although we detected phosphorylation of CRTC3 at Ser62, Ser329, and Ser370 that was blocked by MRT67307 (Fig. 2G), and the mutation of these three sites to Ala increased CREB-dependent luciferase reporter gene expression, the inclusion of SIK inhibitors still produced a further increase in luciferase activity induced by the CRTC3[S62A/S329A/S370A] mutant (Fig. 4A). This result
suggested the existence of an additional phosphorylation site(s). Ser171 is thought to be a key regulatory phosphorylation site in CRTC2 (14) and corresponds to Ser162 in CRTC3. We found that Ser162 was phosphorylated when CRTC3 was overexpressed, which was lost when the cells were treated with SIK inhibitors (Fig. S7B). Moreover, the further mutation of Ser162 to Ala to create the CRTC3[S62A/S162A/S329A/S370A] mutant abolished the interaction of CRTC3 with 14-3-3 proteins, led to persistent nuclear localization and, as a consequence, induced maximal activation of CRTC3 in a CREB-luciferase assay, which was insensitive to SIK inhibitors (Fig. 4A–C). These results identify Ser62, Ser162, Ser329, and Ser370 as four of the key phosphorylation sites suppressing CRTC3 function.

We also found that purified preparations of SIK1, SIK2, and SIK3 phosphorylated CRTC3 at Ser162, Ser329, and Ser370 in vitro leading to an interaction with 14-3-3 proteins (Fig. 4D). Moreover, the phosphorylation of these sites and 14-3-3 binding to SIKs could be blocked and nuclear translocation enhanced by any of the SIK inhibitors (Fig. 4E and Fig. S7C). Finally, expression of the constitutively active mutant CRTC3[S62A/S162A/S329A/S370A] led to an increase in TLR-stimulated IL-10 production in macrophages that could not be increased further by SIK inhibition (Fig. 4F and G).

**Genetic Evidence That Inhibition of SIKs Induces IL-10 Production.** To obtain further evidence that inhibition of the SIKs underlies the ability of MRT67307, MRT199665, KIN112, and HG-9-91-01 to stimulate IL-10 production and suppress proinflammatory cytokine production, we investigated the effect of these compounds in LKB1−/− macrophages. All members of the AMPK subfamily, apart from MELK, are only active if they are phosphorylated by the protein kinase LKB1 (17). The activity of SIKs should therefore be low in LKB1−/− cells, and these cells should phenocopy the effects of SIK inhibitors. As predicted, CRTC3 phosphorylation at Ser370 was greatly reduced in LKB1−/− cells, indicating that the activity of SIKs was low (Fig. 5A). Moreover, LKB1−/− macrophages secreted higher levels of IL-10 and greatly reduced levels of IL-12 and TNFα in response to LPS, which were unaffected by HG-9-91-01 (Fig. 5B).

Quiescent macrophages express all three SIK isoforms with SIK2 and SIK3 mRNA being expressed at much higher levels than SIK1 mRNA (Fig. S8A). We used RNA interference to knock down the expression of all three SIK isoforms in macrophages (Fig. S8B). Reduced expression of SIK1, SIK2, and SIK3 consistently sensitized macrophages to HG-9-91-01 (Fig. S8C), with suboptimal concentrations of HG-9-91-01 inducing a four-fold higher expression of IL-10 mRNA in SIK-depleted macrophages compared with wild-type (WT) macrophages (Fig. S8D).

The most stringent experiment that can be carried out to establish that the effects of a pharmacological inhibitor are mediated via the inhibition of the presumed target, and not by an “off target” effect, is to show that the effects of the compound disappear when the WT enzyme is replaced by a drug-resistant mutant (15, 18, 19). As mentioned above, the compounds KIN112 and HG-9-91-01 inhibit SIKs and not other members of the AMPK subfamily because they target a hydrophobic pocket created by the presence of a small amino acid residue at the gatekeeper site (Fig. 5C). Mutation of the gatekeeper threonine to an amino acid residue with a larger side chain renders the SIKs 1000-fold less sensitive to KIN112 and HG-9-91-01, with mutation to glutamine generating the most drug-resistant mutant (Fig. 5D and E). When RAW264.7 macrophages were generated to inducibly express the SIK2[T96Q] mutant (Fig. 5F), the LPS-stimulated production of IL-10 mRNA could no longer be enhanced by HG-9-91-01 (Fig. 5G). In contrast, HG-9-91-01 continued to greatly enhance LPS-stimulated IL-10 mRNA production when WT SIK2 was expressed to the same level (Fig. 5F and G).

**Fig. 4.** Dephosphorylation of CRTC3 at Ser62, Ser162, Ser329, and Ser370 leads to dissociation from 14-3-3 proteins, nuclear translocation, and activation of CREB-dependent gene transcription. (A) Coactivator function of CRTC3 becomes constitutive and insensitive to SIK inhibitors when S62, S162, S329, and S370 are mutated to Ala. CRTC3-3A is the mutant CRTC3[S62A/S162A/S329A/S370A], whereas CRTC3-4A is CRTC3-3A also carrying the S162A mutation. (mean ± SD, n = 3, **P < 0.001). (B) Mutation of CRTC3 at Ser62, Ser162, Ser329, and Ser370 to Ala abolishes interaction with 14-3-3 proteins. (C) WT CRTC3 translocates to the nucleus after treatment with MRT199665 or HG-9-91-01, whereas CRTC3-4A is localized to the nucleus even in absence of SIK inhibitors. (D) SIKs phosphorylate CRTC3 in vitro inducing interactions with 14-3-3 proteins. NK, no kinase. (E) Inhibition of SIKs promotes dephosphorylation of endogenous CRTC3 and loss of 14-3-3 interaction. (F) Expression of FLAG-CRTC3-WT and CRTC3-4A upon treatment of RAW264.7 macrophages with doxycyclin. (G) RAW264.7 macrophages expressing the phosphomutant CRTC3-4A produce elevated levels of IL-10 mRNA, which is not enhanced by SIK inhibition (n = 4, mean ± SD, **P < 0.001).
Inhibition of SIKs Induces the Expression of Regulatory Macrophage Markers via a CRTC3-IL-10 Pathway. Strikingly, we found that treatment of quiescent macrophages with SIK inhibitors did not just enhance TLR agonist-stimulated IL-10 production, but also switched their gene expression program from the classically activated (M1) route toward the regulatory (M2b) phenotype. In particular, production of other mRNAs including SPHK1, LIGHT, and Arginase 1 that encode characteristic markers of regulatory macrophages was increased (Fig. 6A). In contrast, inhibition of SIKs had no effect on the expression of FIZZ, Ym1, or Mgl2 (Fig. 6B), which are markers of wound-healing (M2a) macrophages.

Control experiments showed that, as expected, IL-4 induced the expression of FIZZ, Ym1, or Mgl2 but not LIGHT or SPHK1 (Fig. S9). Expression of the markers of regulatory macrophages was also enhanced in LKB1−/− macrophages after stimulation with LPS.

The same results were obtained when the mRNA encoding another CREB-dependent gene (Nurr77) was studied instead of IL-10 (Fig. 5G). In contrast to KIN112 and HG-9-91-01, MRT67307 potently inhibits members of the AMPK subfamily with bulky amino acids at the gatekeeper site. Notably, MRT67307 inhibited the SIK2[T96Q] mutant more potently than the WT enzyme (Fig. S5H) and, therefore, still increased Pam-CSK-stimulated IL-10 production in macrophages expressing the SIK2[T96Q] mutant (Fig. S5I). Collectively, these genetic studies prove that HG-9-91-01 exerts its effects by inhibiting SIKs and not another target, but further studies are needed to establish whether SIK2, or another SIK isoform(s), mediates these effects on macrophage biology.
The ability of HG-9.9-01-01 to increase the LPS-stimulated expression of SPHK1, LIGHT, and Arginase 1 did not occur in cells treated with CRTC3 siRNA oligonucleotides and was greatly reduced in IL-10−/− macrophages (Figs. 6 D and E). Thus, the IL-10 produced by inhibition of the LKB1-SIK-CRTC3 signaling axis reinforces the anti-inflammatory phenotype of macrophages by inducing a gene transcription program associated with regulatory macrophages. Finally, LPS-stimulated transcription and secretion of the anti-inflammatory molecule IL-10 was also increased by pharmacological inhibition of SIKs (Figs. 1 A and B and 6 D). Thus, inhibition of SIKs drives the gene expression program of regulatory macrophages.

Discussion

The results presented in this paper demonstrate that pharmacological or genetic inhibition of the SIKs leads to the dephosphorylation of CRTC3 at Ser2, Ser162, Ser239, and Ser370 in macrophages, stimulating the translocation of CRTC3 to the nucleus where it promotes CREB-dependent gene transcription, including IL-10 gene transcription in TLR-stimulated macrophages. IL-10 then signals in an autocrine manner (Fig. S10) and drives the anti-inflammatory state of macrophages by promoting the expression of markers of regulatory M2b macrophages, such as SPHK1, LIGHT, and Arg1. The present study identifies a key role for the SIK-CRTC3 signaling axis in the immune system, and it will be interesting in the future to identify physiological stimuli that inhibit SIKs to drive the formation of regulatory macrophages (Fig. 7).

The treatment of inflammatory diseases still relies heavily on the use of glucocorticoids, or broad-spectrum immunosuppressants, but these therapies have multiple side effects due to the nonselective nature of these treatments, while many patients become resistant to glucocorticoids (20, 21). Neutralizing antibodies that inhibit the actions of particular proinflammatory cytokines, such as the anti-TNFα Humira, are having a major impact on the treatment of rheumatoid arthritis, Crohn’s, and other inflammatory diseases, but these therapies are very expensive and only about half of the patients are good responders (22). The anti-inflammatory properties of IL-10 are being exploited in clinical trials to develop therapeutics for several inflammatory diseases, but results in the clinic using recombinant IL-10 have been disappointing, perhaps because elevated levels of IL-10 are required locally rather than systemically and/or because additional anti-inflammatory molecules are also needed (23, 24). For these reasons, there is diminished interest in developing orally available drugs to improve the treatment of chronic inflammatory and autoimmune diseases. By simultaneously activating several anti-inflammatory pathways and by inhibiting proinflammatory pathways, drugs that inhibit SIKs may provide an advantage over current therapies and improve the treatment of these disorders.

Materials and Methods

Macrophages were treated for 1 h with inhibitors (2 μM MRT67307, 1 μM MRT2199665, 500 nM HG-9.9-01-01, and 10 μM KIN112), 1 μM DMSO for control incubations then stimulated for up to 24 h with 1 μg/mL Pam3CSK4, 2 μg/mL lipopolysaccharide (LTA), 100 ng/mL LPS, 1 μg/mL R837, or 2 μM CpG. Proteins were extracted and immunoblotted as described (8) by using the indicated antibodies. RNA was extracted by using the RNeasy Micro Kit (Qiagen). cDNA was generated by using the iScript cDNA synthesis kit and quantified by qPCR using the SoFast EvaGreen Supermix (Bio-Rad Laboratories). The relative expression of each gene was calculated from Ct values by using the Pfaffl method (25) and was normalized against the mRNA levels of 18S or GAPDH RNA. Fold induction for each gene was reported relative to untreated control cells, which was set to 1. The concentrations of TNFα, IL-6, IL-10, IL-12p40, and RANTES in culture supernatants were measured by using the Bio-Plex Pro Assay system from Bio-Rad. Further details are in SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Shizuo Akira (University of Osaka) for generously providing the TBK-1/IKKε−/− MEFs; Tomi Makela (University of Helsinki) for the LKB1−/− MEFs; Alan Ashworth (Institute of Cancer Research) for the LKB1−/− fibroblast mouse; Denise Harding who synthesized MRT99665 under the supervision of E.M.; George Allen and Alan Prescott for help with light microscopy; Catherine Johnson for providing 14-3-3 reagents; Julia Carr and Gail Fraser for genotyping; the University of Dundee Resource Centre (coordinated by Don Tennant and Lorraine Malone) for housing the mice; the International Centre for Kinase Profiling (www.kinase-screen.mrc.ac.uk) for establishing the potency and selectivity of the inhibitors; and the Medical Research Council-Protein Phosphorylation Unit’s DNA Sequencing Service (coordinated by Nicholas Holmes) and the Antibody Production Team (coordinated by Hilary McLau- chlan and James Hastie) for outstanding technical support. The work was supported by the UK Medical Research Council, AstaR’D (to R.W.), Springer Institute, GlaxoSmithKline, Janssen Pharmaceutica, Merck-Serono, and Pfizer.

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 MRT199665, 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) 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 = 0.035% 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 RediSepRf 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 = 2.50) 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-dimethoxaniline (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 (1:9:3, ethyl acetate/hexane) to afford 6-chloro-N-[2-(4,6-dimethoxyphenyl)pyrrolo[2,3-d]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), 8.25 (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].
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 (1:99–3:79, 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. 

The residue was purified by column chromatography on silica gel (3:79–7:93, methanol/dichloromethane) and additionally purified by HPLC to afford 1-(2,4-dimethoxyphenyl)-3-(2,6-dimethylphenyl)urea, were generated in a ratio of 4:1 by HPLC analysis.

The reaction mixture was stirred at 100 °C for 24 h, and the solvent concentrated under reduced pressure.

The residue was purified by column chromatography on silica gel (1:99–7:93, methanol/dichloromethane) and additionally purified with HPLC to afford 1-(2,4-dimethoxyphenyl)-3-(2,6-dimethylphenyl)-1-(6-((4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)urea (90 mg, 34% yield) as an off-white solid. RT = 3.30 min; 1H-NMR 600 MHz (DMSO-d6) δ 11.54 (s, 1H), 8.07 (s, 1H), 8.31 (s, 1H), 7.25 (bs, 1H), 7.29 (m, 2H), 7.15 (d, J = 9.0 Hz, 1H), 7.07 (m, 3H), 6.85 (d, J = 8.5 Hz, 2H), 6.74 (d, J = 7.8 Hz, 2H), 6.63 (d, J = 7.8 Hz, 1H), 7.80 (d, J = 7.8 Hz, 2H), 6.63 (d, J = 3.0 Hz, 1H), 3.82 (s, 3H), 3.74 (s, 3H), 3.05 (m, 4H), 2.44 (m, 4H), 2.23 (s, 6H), 2.22 (s, 3H); 13C NMR 125 MHz (DMSO-d6) δ 161.87, 160.80, 157.45, 157.31, 157.76, 146.20, 132.56, 126.40, 121.45, 129.03, 116.07, 104.38, 99.17, 83.76, 73.71, 55.56, 55.32, 54.26, 48.36, 45.13; MS m/z: 421.45 [M+1].

Retroviral 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 (Clontech). 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 then 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.

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 and immortalized mouse embryonic fibroblasts (MEFs) from TBK1/IKKe−/−, TBK1/IKKe−/−, LKB1−/−, and LKB1−/− were cultured in DMEM containing 10% (vol/vol) FCS, 2 mM glutamine and penicillin, and streptomycin, whereas THP-1 monocytes were grown in RPMI medium 1640 supplemented with 10% (vol/vol) FCS and antibiotics. For the culture of primary human macrophages, PBMCs were isolated from human blood by using Ficoll and the monocytes were purified by using anti-CD14 magnetic beads (Miltenyi Biotec). Monocytes were differentiated into macrophages for 6 d in the presence of 100 ng/ml M-CSF. Cells were cultured for 7 d in DMEM supplemented with 5% FCS and antibiotics. For the culture of macrophages, PBMCs were isolated from human blood by using Ficoll and the monocytes were purified by using anti-CD14 magnetic beads (Miltenyi Biotec). Monocytes were differentiated into macrophages for 6 d in the presence of 100 ng/ml M-CSF. Cells were cultured for 7 d in DMEM supplemented with 5% FCS and antibiotics.
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:

- Human β-actin-R, TTGACAACACTTCAG; human β-actin-F, CTACCAAACCTTGAGAGAG;
- Human GAPDH-R, ACTTGATTTTGGAGGGAT; human GAPDH-F, GCCTTCCGTGTTCCTACCC;
- Human CRTC3-R, ATTCCCATCAAACTGTCTCC; human CRTC3-F, AGCCATCACTTCATCAAGC;
- Human CRTC2-R, GAGTGCTCCGAGATGAATCC; human CRTC2-F, ACTCAAAGAAGGCGGGTTCC;
- Human SIK3-R, ATAGCCAAGAGGACATCA; human SIK3-F, ACAGCACCACTCTTCTACCGC;
- Human YM1-R, GTCTTGCTCATGTGTGTAAGTGA; human YM1-F, AGAAGGGAGTTTCAAACCTGGT;
- Human Mgl2-R, GGCCTCCAATTCTTGAAACCT; human Mgl2-F, TTAGCCAATGTGCTTAGCTGG;
- Human FIZZ-F, CCCAGGATGCCAACTTTGAA; human FIZZ-R, CCCAGGATGCCAACTTTGAA;
- Human CREB, pSer171; human CRTC2, total CRTC2, GAPDH, total STAT3, and pTyr705 STAT3 were purchased from Cell Signaling Technology; 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 Santa Cruz; and antibodies against pSer329 (S256d bleed 2) and pSer370 (S253d bleed 2) of CRTC3 were raised against the peptide CWKEEEKHPGR (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 Santa Cruz; and antibodies against pSer329 (S256d bleed 2) and pSer370 (S253d bleed 2) of CRTC3 were raised against the phosphopeptides GLOSSRpsNP5Q and RLSFLspNP5SLST in sheep and purified by affinity chromatography in the Division of Signal Transduction Therapy, University of Dundee.

Immunofluorescence. RAW264.7 cells were grown on glass coverslips and treated with inhibitors for 1 h. Cells were then fixed for 10 min in 3.7% paraformaldehyde, permeabilized for 5 min by using 0.1% Triton X-100 in PBS, and blocked for 45 min by using 3% BSA in PBS. Cells were stained with anti-CRTC3 (Abcam; 1:200) and anti–α-tubulin (Santa Cruz; 1:500) followed by anti-rabbit-Alexa488 and anti–mouse-Alexa594 (Invitrogen; 1:500), respectively. Antibodies were diluted in blocking buffer and incubated with the cells for 45 min. Cells were mounted in Prolong Gold Antifade Reagent containing DAPI (Invitrogen) and visualized under a Nikon Eclipse Ti-S microscope equipped with a 60x/1.4 oil-immersion lens. For live imaging, RAW264.7 cells were transduced with MMLV-based retroviral vectors encoding GFP-CRTC3 or GFP-CRTC3[S62A/S162A/S329A/S370A] and plated onto glass-bottom dishes. Cells were imaged on a Zeiss LSM 700 confocal microscope equipped with a 100x Plan Apochromat N.A. 1.46 lens. Images were recorded every 30 s for 1.5 min before the addition of drug, after which cells were imaged for an additional 60 min.

Kinase Assays. Recombinant SIK1, SIK2, and SIK3 were expressed as GST-fusion proteins in HEK293 cells and purified on a glutathione-Sepharose column. To purify dephosphorylated CRTC3, FLAG-CRTC3 was coexpressed with lambda phosphatase in HEK293 cells that were also treated for 1 h with 2 μM MRT67307 before cell lysis. FLAG-CRTC3 was immunoprecipitated by using anti-FLAG M2 agarose, washed in kinase buffer (50 mM Tris-HCl at pH 7.4, 1 mM Mg acetate, 0.1 mM EGTA, and 2 mM DTT), and reactions were initiated by adding 4 U/mL recombinant kinase with 0.1 mM ATP (specific activity ~3,000 cpm/pmol). Phosphorylation of CRTC3 was monitored by autoradiography and immunoblotting by using phosphospecific antibodies raised against Ser162, Ser329, and Ser370. For the IC50 curve measurements,
kinase assays were performed as described by Hastie et al. (6) but using a peptide derived from sequences surrounding Ser171 of CRTC2 (ALNRRTSSDHALRRR) 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 digoxygenin (DIG) (1 μg/mL) overnight. An interaction with 14-3-3 was detected by subsequent 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 apronin, and 1 μg/mL leupeptin) containing 5 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 1:1:1 ratio and then alkylated for 30 min with 50 milligrams of iodoacetamide. The sample was diluted with 0.1 M ammonium bicarbonate to a concentration of 80% buffer A containing 0.1% (vol/vol) formic acid, 2% (vol/vol) acetonitrile. The digests were acidified, desalted by using C18 Sep-Pak cartridges (Waters), and the peptides were dried by using a SpeedVac. The digests were acetylated, desalted by using C18 Sep-Pak cartridges, and the peptides were dried by using a SpeedVac.

The digests were mixed in a 1:1:1 ratio and then alkylated for 30 min with 50 milligrams of iodoacetamide. The sample was diluted with 0.1 M ammonium bicarbonate to a final urea concentration of 1.5 M, and the samples 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. These peptides were dissolved in 750 μL of 80% (vol/vol) ACN/0.1% (vol/vol) trifluoroacetic acid and fractionated by hydrophilic interaction chromatography (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 Fe3+-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 (Mircrom Bioresources) and the following gradient: (i) 0–170 min from 2 to 35% buffer B [0.08% (vol/vol) formic acid, 90% (vol/vol) acetonitrile in water]; (ii) 170–187 min from 35 to 80% buffer B; (iii) 187–198 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 < 0.01, ***P < 0.001). (C) Effect of HG-9-91-01 on cytokine production in macrophages costimulated with LPS and IFN-γ. BMDMs were incubated for 1 h with 500 nM HG-9-91-01, then stimulated with 10 ng/mL IFN-γ 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.