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

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Contributed by Philip Cohen, September 5, 2012 (sent for review August 20, 2012)

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-9-91-01

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 pathogen (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. S1*A*) 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. 1*A*). Similar results were obtained when macrophages were stimulated with ligands that activate other TLRs (Fig. 1*B*). 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. 2*A*). The

Author contributions: K.C., and K.F.M., designed research; K.C., K.F.M., K.P., Y.K., J.Z., and L.P. performed research; H.G.C., M.P., E.M., and N.S.G. contributed new reagents/analytic tools; P.G.A.P., and J.S.C.A. analyzed data; and K.C. and P.C. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1215450109/-/DCSupplemental.

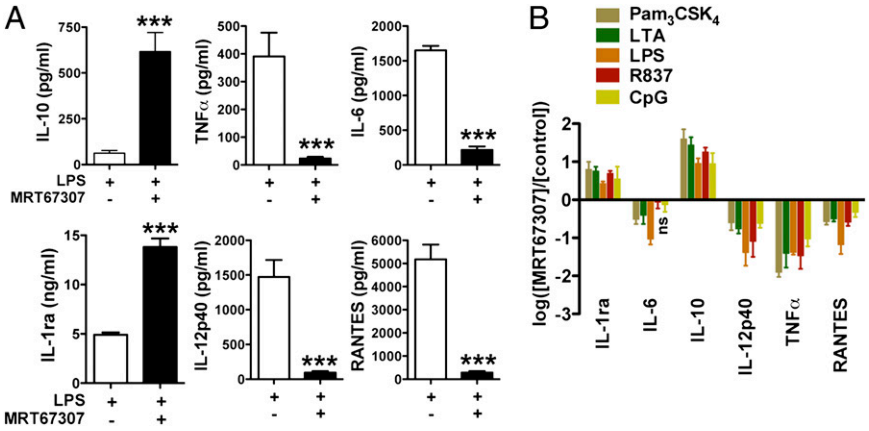

Fig. 1. MRT67307 increases IL-10 production and suppresses proinflammatory cytokine production in macrophages. (*A*) MRT67307 increases IL-10 and IL-1ra secretion while suppressing the release of proinflammatory cytokines in LPS-stimulated bone marrow-derived macrophages (BMDMs). In the absence of LPS, 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-Pam_3CSK_4, 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.

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. 2*B*). 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. 2*B*). Moreover, siRNA knockdown of CREB prevented MRT67307 from potentiating the induction of IL-10 mRNA by LPS (Fig. 2*C*). 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

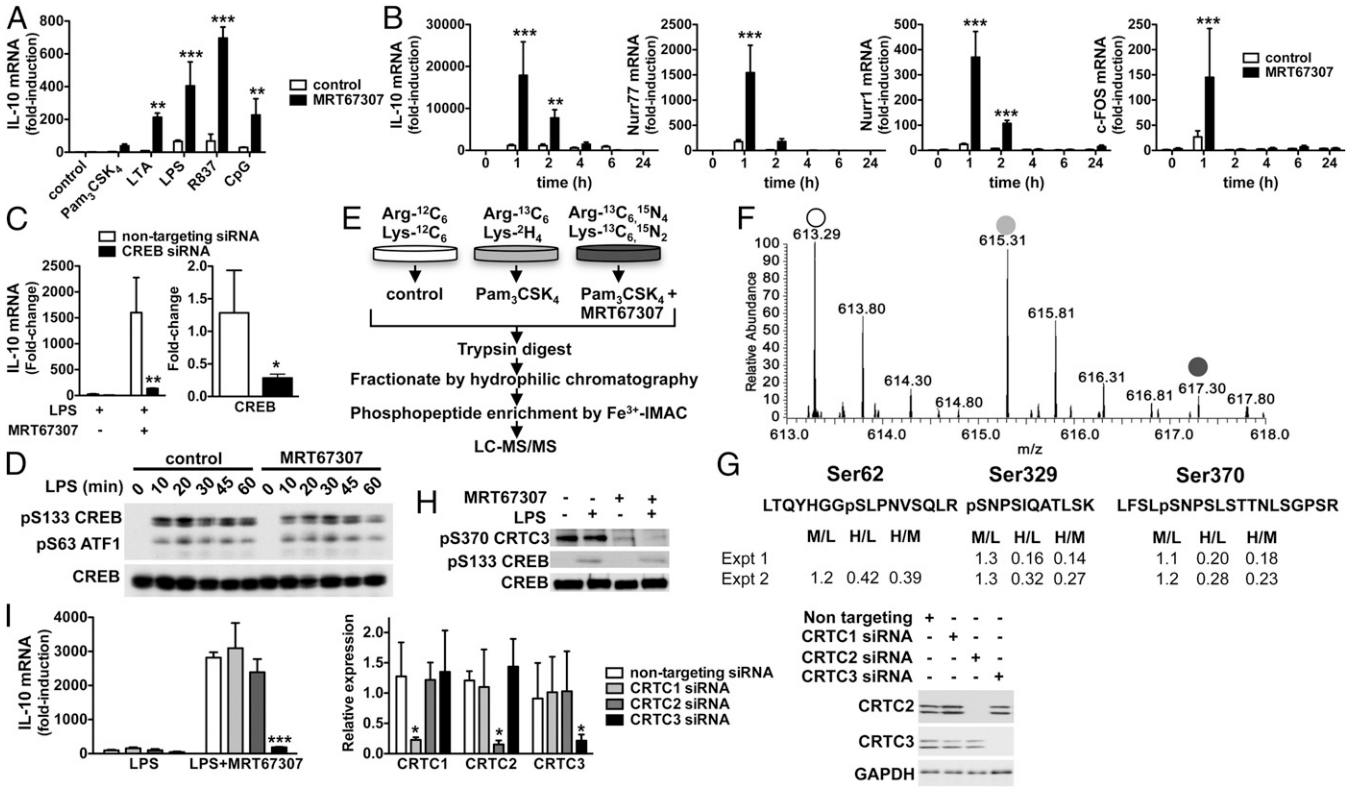

Fig. 2. MRT67307 increases CREB-dependent gene transcription by promoting the dephosphorylation of CRTC3. (*A*) Effect of MRT67307 on IL-10 mRNA levels in TLR-stimulated BMDMs ($n = 4$, mean ± SD). (*B*) Effect of MRT67307 on transcription of CREB-dependent genes in LPS-stimulated BMDMs ($n = 4$, mean ± 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 dephosphorylation 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 CRTCs. Studies in other mammalian cells, mainly based on experiments with CRTC2, have shown that the dephosphorylation of CRTCs 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. 2*D*). However, in a phosphoproteomic study to identify proteins whose phosphorylation was suppressed by MRT67307 (Fig. 2*E*), we found that the phosphorylation of CRTC3 was unaffected by stimulation with the TLR1/2 agonist Pam_3CSK_4, but nevertheless was robustly dephosphorylated at Ser62, Ser329, and Ser370 when macrophages were incubated with MRT67307 (Fig. 2 *F* and *G*). Similarly, phosphorylation of CRTC3 at Ser370 was unaffected by LPS stimulation but blocked by MRT67307 (Fig. 2*H*). siRNA knockdown of CRTC3, but not CRTC1 or CRTC2, suppressed the effects of MRT67307 on TLR-stimulated IL-10 production in macrophages (Fig. 2*I*). 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 AMP-activated 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 IκB kinase (IKK)-related kinases, TBK1 and IKKε (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/IKKε-dependent production of IFNβ by LPS. Moreover, MRT67307 could still enhance transcription of the CREB-dependent Nurr77 gene in TBK1/IKKε$^{-/-}$ 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 in vitro with comparable potency to the IKK-related kinases (Fig. 3*A*). 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. S1*B*), a potent inhibitor of most AMPK-related kinases, which does not inhibit the IKK-related kinases (Fig. 3*A*), increased LPS-stimulated IL-10 mRNA and Nurr77 mRNA production, and IL-10 secretion (Fig. 3*B*), 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. S1*C*) and, subsequently, a much more potent analog HG-9-91-01 (Fig. S1*D*), which not only target 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. 3*A*), 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. 3*C* and Fig. S4 *A* and *B*), even when cells were costimulated with IFNγ to generate fully polarized classically activated (M1) macrophages (Fig. S4*C*). 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. 2*G*), 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. 4*A*). This result

A

	IC50 values (nM)			
	MRT67307	MRT199665	KIN112	HG-9-91-01
LKB1	>10000	>10000	>10000	>10000
AMPKα1/α2	810	10	>10000	4500
MARK1	27	2	>10000	3300
MARK2	52	2	>10000	1600
MARK3	36	3	>10000	1900
MARK4	41	2	>10000	1300
SIK1	250	110	10	0.92
SIK2	67	12	22	6.6
SIK3	430	43	60	9.6
NUAK1	230	3	>10000	4280
NUAK2	2600	120	2100	145
BRSK1	>10000	>10000	>10000	>10000
BRSK2	10000	10000	>10000	8800
MELK	900	29	2000	2500
TBK1	19	5400	>10000	>10000
IKKε	160	7700	>10000	>10000

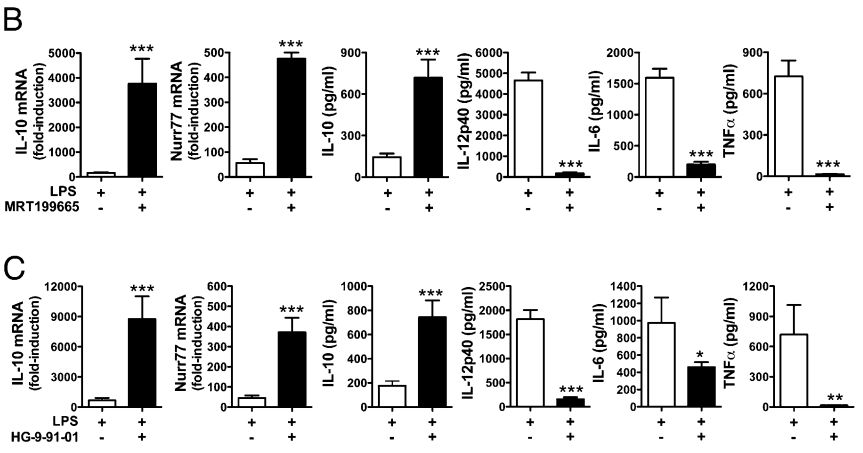

Fig. 3. Inhibition of SIKs simultaneously enhances IL-10 production and suppresses proinflammatory cytokine secretion. (*A*) IC_{50} (nanomolar) values for inhibition of the AMPK-related kinases and IKK-related kinases by MRT67307, MRT199665, KIN112, and HG-9-91-01 in vitro. (*B* and *C*) Effect of MRT199665 (*B*) and HG-9-91-01 (*C*) on IL-10 and Nurr77 mRNA formation and cytokine secretion in LPS-stimulated BMDMs ($n = 4$, mean ± SD, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

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. S7*B*). 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. 4 *A*–*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. 4*D*). 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. 4*E* and Fig. S7*C*). 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. 4 *F* 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. 5*A*). 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. 5*B*).

Quiescent macrophages express all three SIK isoforms with SIK2 and SIK3 mRNA being expressed at much higher levels than SIK1 mRNA (Fig. S8*A*). We used RNA interference to knock down the expression of all three SIK isoforms in macrophages (Fig. S8*B*). Reduced expression of SIK1, SIK2, and SIK3 consistently sensitized macrophages to HG-9-91-01 (Fig. S8*C*), with suboptimal concentrations of HG-9-91-01 inducing a fourfold higher expression of IL-10 mRNA in SIK-depleted macrophages compared with wild-type (WT) macrophages (Fig. S8*D*).

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. 5*C*). Mutation of the gatekeeper threonine to an amino acid residue with a larger side chain renders the SIKs 100–1,000-fold less sensitive to KIN112 and HG-9-91-01, with mutation to glutamine generating the most drug-resistant mutant (Fig. 5 *D* and *E*). When RAW264.7 macrophages were generated to inducibly express the SIK2[T96Q] mutant (Fig. 5*F*), the LPS-stimulated production of IL-10 mRNA could no longer be enhanced by HG-9-91-01 (Fig. 5*G*). 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. 5 *F* and *G*).

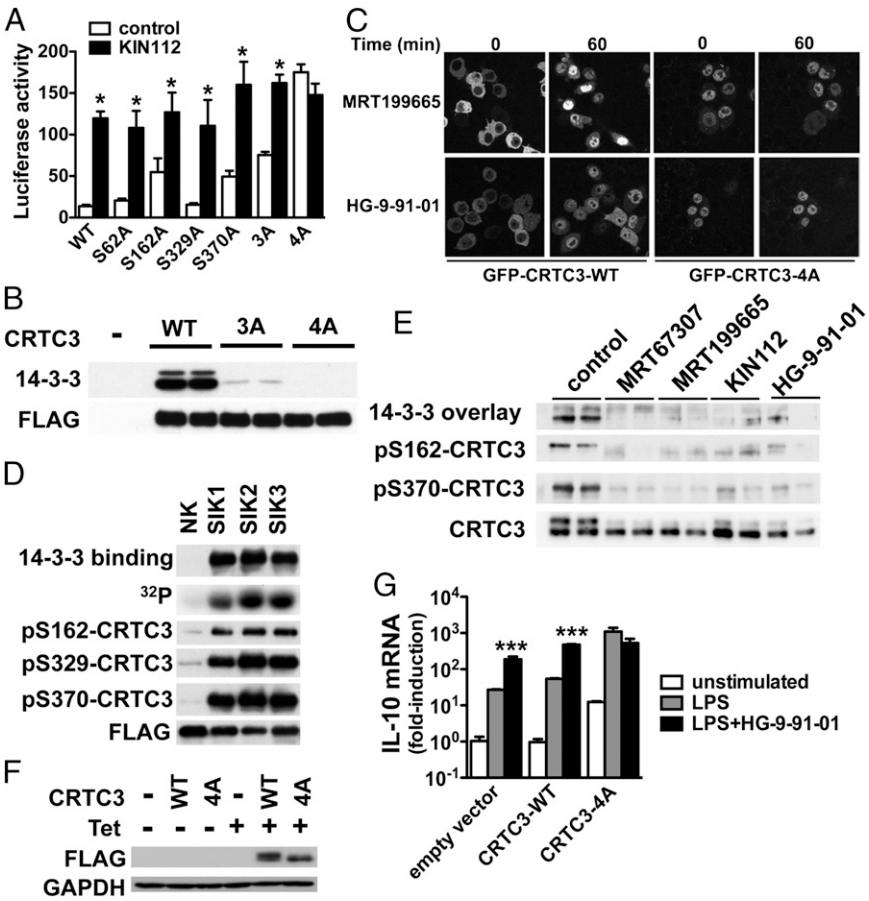

Fig. 4. Dephosphorylation of CRTC3 at Ser62, Ser162, Ser329, and Ser370 leads to dissociation from 14-3-3 proteins, nuclear translocation, and activation of CREB-dependent gene transcription. (*A*) Coactivator function of CRTC3 becomes constitutive and insensitive to SIK inhibitors when S62, S162, S329, and S370 are mutated to Ala. CRTC3-3A is the mutant CRTC3[S62A/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, $^{***}P < 0.001$).

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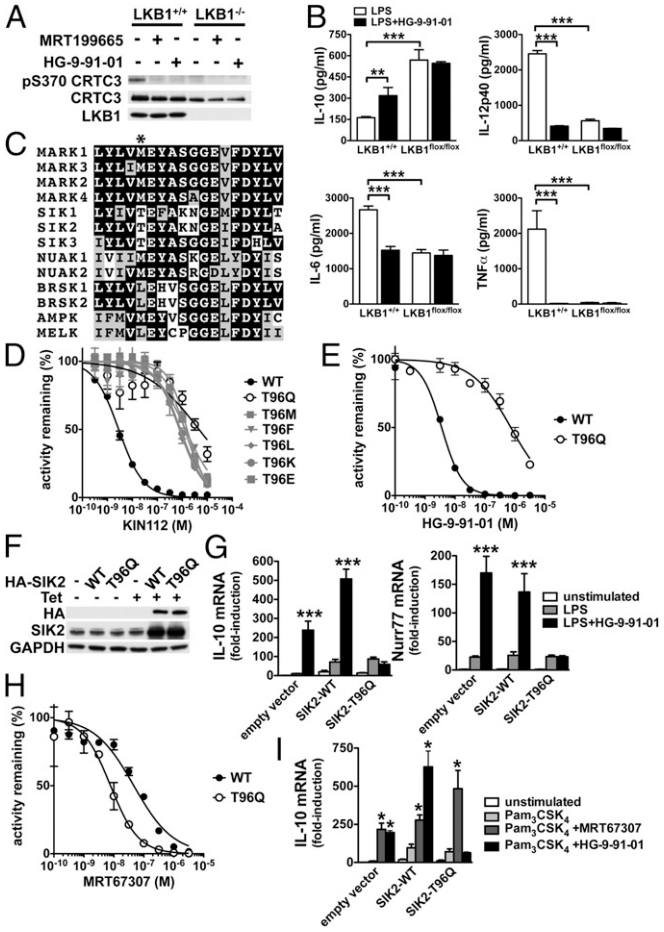

Fig. 5. Genetic evidence using LKB1-deficient macrophages and a drug-resistant mutant of SIK2 to establish that inhibition of SIKs induces IL-10 production. (*A*) Loss of CRTC3 phosphorylation in LKB1$^{-/-}$ MEFs. (*B*) Enhanced IL-10 production and reduced secretion of proinflammatory cytokines in LysM-Cre-LKB1$^{flox/flox}$ macrophages ($n = 3$, mean ± SD, $^{**}P < 0.01$, $^{***}P < 0.001$). (*C*) Sequence alignment of AMPK-related kinases. The gatekeeper site is indicated by using an asterisk. (*D*) IC_{50} curves of KIN112 against different SIK2 mutants. (*E*) IC_{50} curve of HG-9-91-01 against WT SIK2 and the SIK2[T96Q] mutant. (*F*) Induction of HA-SIK2 (WT and [T96Q]) expression in RAW264.7 macrophages. (*G*) HG-9-91-01 fails to induce IL-10 and Nurr77 mRNA production in cells expressing SIK2[T96Q]. RAW264.7 cells were induced to express HA-SIK2-WT or HA-SIK2[T96] using doxycyclin. As a further control, cells transduced with the empty vector and which only express endogenous SIK isoforms were also used. Subsequently, cells were treated without or with 500 nM HG-9-91-01 and stimulated for 1 h with 100 ng/mL LPS. mRNA levels were measured by qPCR (mean ± SD, $n = 3$, $^{***}P < 0.001$). (*H*) IC_{50} curve of MRT67307 against WT SIK2 and the SIK2[T96Q] mutant. (*I*) MRT67307 but not HG-9-91-01 can potentiate the production of IL-10 in macrophages expressing SIK2[T96Q]. Experiment was performed as in *E*, but cells were treated with 2 μM MRT67307 or 500 nM HG-9-91-01 before stimulation for 1 h with Pam_3CSK_4 (mean ± SD, $n = 3$) ($^{*}P < 0.001$ compared with cells stimulated with Pam_3CSK_4 in the absence of inhibitors).

The same results were obtained when the mRNA encoding another CREB-dependent gene (Nurr77) was studied instead of IL-10 (Fig. 5*G*). 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. 5*H*) and, therefore, still increased Pam_3CSK_4-stimulated IL-10 production in macrophages expressing the SIK2[T96Q] mutant (Fig. 5*I*). 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.

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. 6*A*). In contrast, inhibition of SIKs had no effect on the expression of FIZZ, Ym1, or Mgl2 (Fig. 6*B*), 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

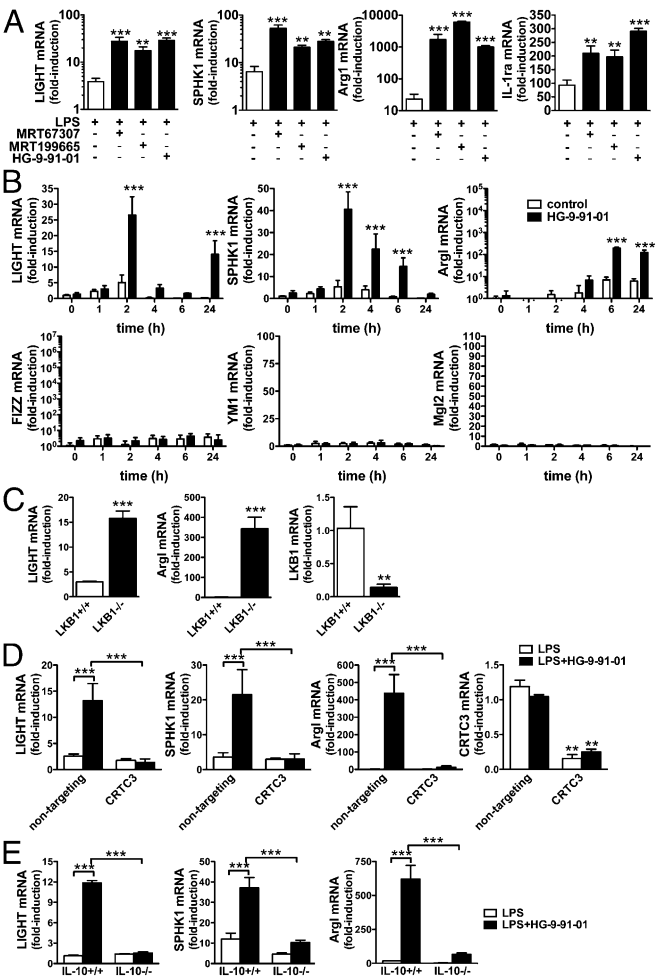

Fig. 6. Inhibition of the LKB1-SIK-CRTC3-IL10 signaling axis drives the expression of markers of regulatory macrophages. (*A*) Effect of SIK inhibitors on the LPS-stimulated expression of LIGHT, SPHK1, Arg1, and IL-1ra ($n = 4$, mean ± SD). (*B*) Effect of HG-9-91-01 on macrophage marker expression in LPS-stimulated BMDMs. mRNA levels were normalized to 1 in unstimulated cells not treated with HG-9-91-01 (mean ± SD, $n = 4$). (*C*) Elevated expression of markers of regulatory macrophage in LKB1$^{-/-}$ macrophages. BMDMs were generated from LysM-Cre/LKB1$^{+/+}$ and LysM-Cre/LKB1$^{flox/flox}$ mice and stimulated with 100 ng/mL LPS (mean ± SD, $n = 3$). (*D*) CRTC3 is required for the induction of markers of regulatory macrophages. BMDMs were transfected with control or CRTC3 siRNA oligonucleotides, treated without or with 500 nM HG-9-91-01 and then stimulated with 100 ng/mL LPS. LIGHT and SPHK1 mRNA was measured at 2 h and Arg1 at 8 h after LPS stimulation (mean ± SD, $n = 4$). (*E*) Regulatory macrophage markers are not induced by HG-9-91-01 in IL-10$^{-/-}$ macrophages. The experiment was carried out as in *D* except that BMDMs were generated from IL-10$^{+/+}$ and IL-10$^{-/-}$ mice (mean ± SD, $n = 4$). For all graphs, statistical significance is reported as follows: $^{**}P < 0.01$, $^{***}P < 0.001$.

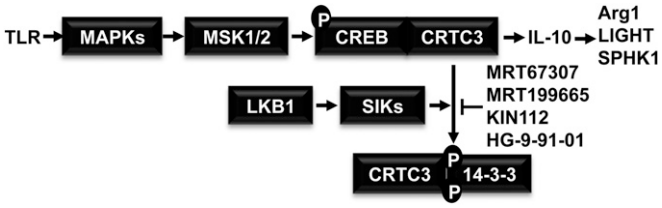

Fig. 7. Model for regulation of IL-10 production by the LKB1-SIK-CRTC3 signaling axis. TLR agonists lead to the activation of MSK1 and MSK2, which phosphorylate CREB at Ser133, whereas inhibition of SIKs leads to dephosphorylation of CRTC3. The CREB-CRTC3 complex leads to maximal transcription of the IL-10 gene which signals in an autocrine fashion to induce other anti-inflammatory molecules such as Arg1, LIGHT, and SPHK1.

(Fig. 6*C*). The ability of HG-9-91-01 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 (Fig. 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-1 receptor antagonist (IL-1ra) was also increased by pharmacological inhibition of SIKs (Figs. 1 *A* and *B* and 6*A*). Thus, inhibition of the 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 Ser62, Ser162, Ser329, 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 undiminished 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 MRT199665, 500 nM HG-9-91-01, 10 μM KIN112), or an equivalent volume of DMSO for control incubations then stimulated for up to 24 h with 1 μg/mL Pam_3CSK_4, 2 μg/mL lipoteichoic acid (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 SsoFast 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 TBK1/IKKε$^{-/-}$ MEFs; Tomi Makela (University of Helsinki) for the LKB1$^{-/-}$ MEFs; Alan Ashworth (Institute of Cancer Research) for the LKB1$^{flox/flox}$ mice; Denise Harding who synthesized MRT199665 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 Helps) and the Antibody Production Team (coordinated by Hilary McLauchlan and James Hastie) for outstanding technical support. The work was supported by the UK Medical Research Council, AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, Janssen Pharmaceutica, Merck-Serono, and Pfizer.

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