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Genetic alterations that reduce the function of the immunoregulatory cytokine IL-10 contribute to colitis in mouse and man. Myeloid cells such as macrophages (MΦs) and dendritic cells (DCs) play an essential role in determining the relative abundance of IL-10 versus inflammatory cytokines in the gut. As such, using small molecules to boost IL-10 production by DCs–MΦs represents a promising approach to increase levels of this cytokine specifically in gut tissues. Toward this end, we screened a library of well-annotated kinase inhibitors for compounds that enhance production of IL-10 by murine bone-marrow–derived DCs stimulated with the yeast cell wall preparation zymosan. This approach identified a number of kinase inhibitors that robustly up-regulate IL-10 production including the Food and Drug Administration (FDA)-approved drugs dasatinib, bosutinib, and saracatinib that target ABL, SRC-family, and numerous other kinases. Correlating the kinase selectivity profiles of the active compounds with their effect on IL-10 production suggests that inhibition of salt-inducible kinases (SIKs) mediates the observed IL-10 increase. This was confirmed using the SIK-targeting inhibitor HG-9-91-01 and a series of structural analogs. The stimulatory effect of SIK inhibition on IL-10 is also associated with decreased production of the proinflammatory cytokines IL-1β, IL-6, IL-12, and TNF-α, and these coordinated effects are observed in human DCs–MΦs and anti-inflammatory CD11c^{+} CX$_3$CR1^{hi} cells isolated from murine gut tissue. Collectively, these studies demonstrate that SIK inhibition promotes an anti-inflammatory phenotype in activated myeloid cells marked by robust IL-10 production and establish these effects as a previously unidentified activity associated with several FDA-approved multikinase inhibitors.

Crohn's disease and ulcerative colitis are the most common forms of the chronic relapsing inflammatory disorders known as inflammatory bowel disease (IBD). Although the etiology of IBD is complex, impaired function of anti-inflammatory immune mechanisms is observed in many patients (1). For instance, single-nucleotide polymorphisms (SNPs) in genetic loci containing *IL10* or its receptor (*IL10RA*) are associated with increased risk of Crohn's disease and ulcerative colitis (2), and rare loss-of-function mutations in the coding regions of *IL10* or *IL10RA* lead to severe, pediatric-onset enterocolitis (3). The link between gut inflammation and defective IL-10 signaling in humans is recapitulated in *Il10*$^{-/-}$ and *Il10ra*$^{-/-}$ mice, which both develop spontaneous colitis (4, 5). Conversely, prophylactic administration of recombinant IL-10 partially alleviates gut inflammation and weight loss in murine models of chemically induced colitis (6). Unfortunately, well-tolerated doses of recombinant IL-10 therapy did not show efficacy in clinical trials for Crohn's disease (7). Potential explanations of this lack of efficacy include insufficient delivery recombinant IL-10 to the gut mucosa by systemic administration and/or the need to combine IL-10 supplementation with neutralization of inflammatory cytokines (8). Of note, disease activity is reduced in Crohn's disease patients following oral administration of *Lactococcus lactis* engineered to express IL-10 (9), which suggests that specifically increasing IL-10 levels in the gut microenvironment can be therapeutically beneficial in the absence of toxicities (e.g., headache, anemia, and thrombocytopenia) that limit systemic treatment.

Circulating monocytes continuously seed tissues where they differentiate into dendritic cells (DCs) and macrophages (MΦs) that play important roles in both homeostatic and inflammatory conditions (10). Monocyte-derived inflammatory myeloid cells are recruited to gut tissues in murine models of colitis and IBD patients and up-regulate expression of inflammatory cytokines (11, 12). In contrast, a highly abundant population of monocyte-derived cells expressing CD11c and the CX_3CL1–fractalkine receptor (CD11c^{+} CX$_3$CR1^{hi}) play an essential role in maintaining gut immune homeostasis due, in part, to robust production of IL-10 (13). CD11c^{+} CX$_3$CR1^{hi} myeloid cells, which display characteristics of both DCs and MΦs, are closely associated with the intestinal epithelium enabling these cells to

Significance

IL-10 plays an essential role in maintaining gut immune homeostasis as evidenced by the link between genetic perturbation of this anti-inflammatory cytokine and inflammatory bowel disease (IBD). Here, we describe a small-molecule screen that identified inhibition of salt-inducible kinases (SIKs) as a strategy to enhance IL-10 production by macrophages and dendritic cells. Significantly, the IL-10–potentiating effects of SIK inhibition are associated with reduced secretion of the inflammatory cytokines IL-1β, IL-6, IL-12, and TNF-α, and these coordinated effects are observed in cells relevant to IBD including anti-inflammatory CD11c^{+} CX$_3$CR1^{hi} cells from murine gut tissue and in human dendritic cells and macrophages. Collectively, these results identify SIK inhibition as a promising approach to treat IBD by increasing gut IL-10 levels.

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sample gut microbes and suppress immune responses to commensal species (14). The anti-inflammatory function of $CD11c^+$ CX_3CR1^{hi} myeloid cells is highlighted in adoptive transfer experiments where coadministration of these cells suppresses colitis induced by $CD45RB^{hi}$ $CD4^+$ T cells in lymphopenic hosts (15). Given the central role of myeloid cells in shaping gut immunity, using small molecules to enhance IL-10 production by DCs–MΦs represents a potentially promising approach to increase levels of IL-10 specifically in this tissue microenvironment. Additionally, autocrine IL-10 signaling biases DCs–MΦs toward anti-inflammatory phenotypes (16), which suggests that elevating IL-10 may also reduce inflammatory cytokines levels in the gut, thereby overcoming two potential limitations of systemic IL-10 supplementation.

The relative levels of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) versus cAMP response element-binding protein (CREB) gene-expression programs represent a key regulatory node governing IL-10 production by myeloid cells (16). For instance, disruption of signaling pathways linking microbial recognition receptors to NF-κB activation with small-molecule inhibitors of protein kinase C (PKC) or glycogen synthase kinase-3β (GSK-3β) up-regulates IL-10 production by lipopolysaccharide (LPS)-stimulated MΦs (17, 18). Conversely, CREB activation by EP2/EP4 prostanoid receptor agonists like prostaglandin E_2 (PGE_2) promotes IL-10 production by myeloid cells (19). Recently, salt-inducible kinases (SIKs), which are members of the AMP-activated protein kinase family, have been identified as a key component of the CREB activation cascade (20, 21). In quiescent myeloid cells, SIKs phosphorylate CREB-regulated transcriptional coactivator-3 (CRTC3), which results in its sequestration by cytoplasmic 14–3-3 proteins. Following EP2–EP4 stimulation, inhibitory phosphorylation of SIKs by protein kinase A (PKA) leads to decreased CRTC3 phosphorylation, allowing it to enter the nucleus and enhance transcription of CREB target genes including IL-10, and this effect can be recapitulated with small-molecule SIK inhibitors (21). The central role of PKC, GSK-3β, and SIKs in IL-10 production suggests that additional kinase inhibitors are likely to modulate pathways that regulate IL-10 production by DCs–MΦs.

In this study, we screened a library of >150 kinase inhibitors comprising Food and Drug Administration (FDA)-approved drugs and well-annotated probes (Dataset S1) for compounds that enhance IL-10 production by activated murine bone-marrow–derived dendritic cells (BMDCs), which are ex vivo differentiated primary cells bearing a functional resemblance to monocyte-derived myeloid cells recruited to inflamed gut tissues (10). This screen identified SIK inhibition as a common feature of several kinase inhibitors that enhanced IL-10 production, including FDA-approved drugs developed to target other kinases. Using a selective SIK-targeting inhibitor we demonstrate that, along with up-regulating IL-10, SIK inhibition converts human and mouse DCs–MΦs to an anti-inflammatory phenotype marked by reduced production of inflammatory cytokines. We also show that these coordinated effects are observed in $CD11c^+$ CX_3CR1^{hi} myeloid cells isolated from the small intestine.

Results

Focused High-Throughput Screen Identifies Kinase Inhibitors That Enhance Il-10 Production by Activated Dendritic Cells. To enable measurement of IL-10 production by myeloid cells in high throughput, we established an AlphaLISA (amplified luminescent proximity homogeneous assay linked immunosorbent assay)-based protocol to detect IL-10 released by BMDCs in 384-well plate format (Fig. 1*A*). Consistent with the established role of EP2/EP4 prostanoid receptor signaling in promoting IL-10 expression in myeloid cells (19), differentiation of BMDCs in the presence of PGE_2 increased levels of IL-10 detectable in the culture media by ELISA following stimulation with a variety of microbial-derived ligands (Fig. S1*A*). The robust IL-10–potentiating effect of differentiating BMDCs in the presence of PGE_2 was recapitulated in fully differentiated BMDCs treated with

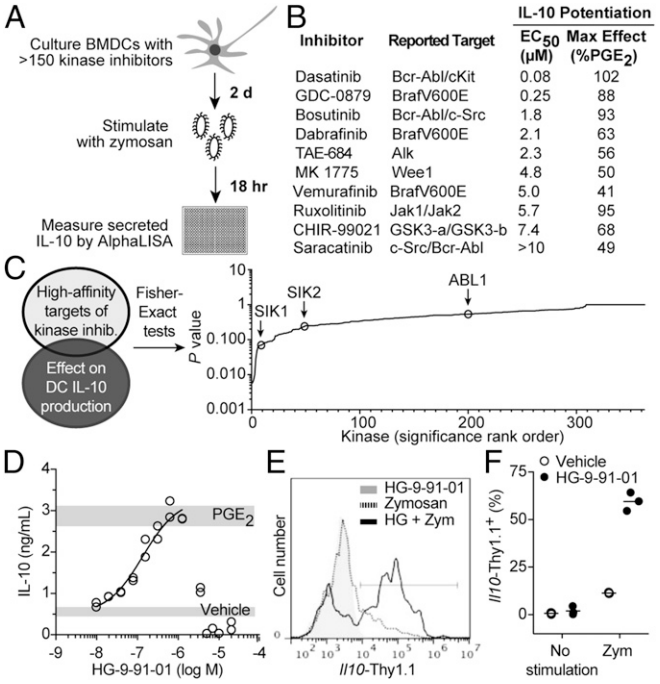

Fig. 1. Targeted small-molecule screen identifies SIK inhibition as a common activity of multikinase inhibitors that up-regulate IL-10 production by activated DCs. (*A*) Screening strategy for identification of small-molecule enhancers of IL-10 production. (*B*) IL-10–potentiating activity and reported targets of hit compounds. Data are mean of two independent experiments. (*C*) Correlation of IL-10–potentiating activity of kinase inhibitors in this screen with their high-affinity targets (K_d < 500 nM) identifies common targets of active compounds potentially accounting for the observed effects on IL-10. (*D*) BMDCs treated with DMSO, PGE_2 (5 μM, gray bars = mean ± SD, n = 12 from one independent experiment) or the indicated concentrations of the SIK inhibitor HG-9-91-01 in duplicate for 2 d followed by stimulation with zymosan. After 18 h, secreted IL-10 was detected by ELISA. Data are representative of >5 independent experiments. (*E*) Cell surface abundance of *Il10*-Thy1.1 reporter gene in 10BiT BMDCs treated with DMSO or HG-9-91-01 (0.5 μM) for 2 d before stimulation with zymosan for 18 h. (*F*) Black bars are mean frequency of *Il10*-$Thy1.1^+$ cells from 10BiT BMDCs from three individual mice treated and analyzed as in *E*.

PGE_2 for 2 d before stimulation with the yeast cell wall preparation zymosan (Fig. S1*B*). Moreover, the stimulatory effects of treatment with PGE_2 for 2 d on zymosan-induced IL-10 production were observed with BMDCs cultured in 384-well plates using AlphaLISA-based IL-10 detection (Fig. S1*C*). Together, these results establish zymosan-stimulated BMDCs as a robust, reproducible assay system to identify small molecules that enhance IL-10 production by activated myeloid cells.

To discover small-molecule enhancers of IL-10 production by inflammatory DCs, we screened a collection of >150 kinase inhibitors comprising FDA-approved drugs and well-annotated probe compounds for their effects on zymosan-induced IL-10 production in BMDCs (Fig. S1*D*). Ten kinase inhibitors were classified as hits based on their ability to dose-dependently potentiate IL-10 production with a maximum effect of >30% of the PGE_2 response (Fig. 1*B* and Fig. S2). These hits include the GSK-3β inhibitor CHIR-99021, which further validates the screening strategy because pharmacological or genetic disruption of GSK-3β has been previously reported to enhance IL-10 production by LPS-stimulated MΦs (17). Several of the IL-10-enhancing compounds reduced IL-10 at concentrations greater than 1 μM, an effect that correlates with reduced viability in the case of bosutinib, saracatinib, TAE-684, and CHIR-99021 (Fig. S2). Alternatively, inhibition of kinases (e.g., Src and Syk) that convey proximal signals downstream of the zymosan receptor dectin-1 may account for the inhibitory effect on

IL-10 production at high concentrations of compound. In these cases, EC_{50} values were calculated solely using IL-10-potentiating concentrations for each inhibitor.

Analysis of Shared Targets of Hit Compounds Suggests Role for Inhibition of SIKs in Il-10 Potentiation. Dasatinib, bosutinib, and saracatinib, which are used clinically to inhibit BCR-Abl, c-Kit, and several other kinases for treatment of chronic myeloid leukemia (CML) (22), were among the most potent and active (i.e., large maximum effect) IL-10 enhancers identified in this screen. However, the BCR-Abl–targeting CML drug imatinib was nontoxic in BMDCs, but did not up-regulate IL-10 production (Fig. S2). This contrast suggests that the observed IL-10-potentiating effects of the hit compounds might arise from shared "off-target" inhibition of kinases other than Abl1. This possibility is supported by kinase-profiling data indicating that dasatinib and bosutinib modulate the activity of a large number of kinases (23). To investigate this possibility, we correlated the IL-10–potentiating activity of 38 of the screened kinase inhibitors, including six of the hit compounds, with publically available binding affinities (K_ds) for a panel of >400 recombinant kinases (23). Fisher-Exact tests were used to determine whether high-affinity ligands of a particular kinase, defined as compounds with $K_d < 500$ nM, were enriched among the hit compounds. This analysis identified statistically significant associations ($P < 0.05$) between IL-10 up-regulation and binding to several kinases (Fig. 1*C* and Dataset S2). However, due to the small number of compounds with available kinase profiling data and large number of tests, none of the associations remained significant after correction for multiple hypothesis testing.

SIKs (SIK1 and SIK2) were enriched among the high-affinity targets of the IL-10–potentiating kinase inhibitors (Fig. 1*C*), which is consistent with the IL-10–potentiating effects of SIK inhibition in activated MΦs (20, 21). In agreement with these reports, pretreating BMDCs with HG-9-91-01, a recently described inhibitor of SIK1-3, along with several other kinases (21), results in concentration-dependent potentiation of zymosan-induced IL-10 production with an $EC_{50} \sim 200$ nM and a maximum effect similar to that observed with PGE_2 (Fig. 1*D*). Similar to our observations with dasatinib and bosutinib, concentrations of HG-9-91-01 greater than 1 μM suppressed zymosan-induced IL-10 production. This decrease in IL-10 secretion does not correlate with reduced viability (Fig. 2*A*), suggesting that it may instead result from inhibition of additional kinase targets mediating the effects of zymosan stimulation. For example, HG-9-91-01 potently inhibits Src with less than 4% activity remaining at 1 μM (21). As with PGE_2, extended preincubation of BMDCs with HG-9-91-01 was required for robust up-regulation of IL-10 in response to stimulation with zymosan, LPS, or the viral RNA mimetic R848 (Fig. S3). To determine if SIK inhibition increases the fraction of cells producing IL-10, BMDCs derived from transgenic mice in which the *Il10* promoter drives expression of Thy1.1 ["10BiT" reporter (24)] were treated with HG-9-91-01 for 2 d before stimulation with zymosan for 18 h and immunostaining for cell surface Thy1.1 (Fig. 1*E*). In this assay system, SIK inhibition enhanced the fraction of BMDCs expressing *Il10* following zymosan stimulation more than fivefold (Fig. 1*F*). Collectively, these results suggest that SIK inhibition underlies the IL-10–potentiating activity of several of the hit compounds identified in this screen by enhancing the fraction of activated dendritic cells that produce IL-10 in response to microbial stimulation.

FDA-Approved CML Drugs Up-Regulate Il-10 Production by a Mechanism Involving Enhanced CRTC3/CREB Signaling. Potent binding to SIK1 and SIK2 by dasatinib, bosutinib, and the annotated Alk inhibitor TAE-684 [$K_d < 30$ nM in all cases (23)] contributes to the positive correlation between SIK inhibition and IL-10 potentiation. Based on the kinase profiling data, we then studied whether dasatinib and bosutinib, as representative hits, induced cellular effects consistent with up-regulation of IL-10 production via the SIK-CRTC3-CREB pathway. In support of this hypothesis, preincubation of BMDCs with dasatinib or bosutinib resulted in a fourfold induction of a CREB-dependent luciferase reporter construct following zymosan stimulation (Fig. S4*A*). In addition, as reported for HG-9-91-01 (21), bosutinib treatment is associated with a decrease in SIK-specific phosphorylation of CRTC3 at S370 with an EC_{50} similar to that observed for up-regulation of CREB transcriptional activity and IL-10 production (Fig. S4*B*). Finally, abundance of CRTC3 protein was reduced to nearly undetectable levels in BMDCs stably transduced with shRNAs targeting its transcript to test whether the IL-10–potentiating effects of dasatinib and bosutinib require functional CREB–CRTC3 complexes (Fig. S4*C*). In support of this hypothesis, CRTC3 knockdown significantly reduced the maximum IL-10 enhancement induced by both dasatinib and bosutinib relative to control shRNAs targeting RFP or *LacZ* (Fig. S4 *D* and *E*). Together, these biochemical and cellular data support a model where enhancement of CREB–CRTC3 signaling following SIK inhibition mediates the IL-10–potentiating activity of dasatinib, bosutinib, and potentially other FDA-approved multikinase inhibitors.

Activity of HG-9-91-01 Analogs Suggests Specific Role of SIK2 Inhibition in IL-10 Potentiation. HG-9-91-01 potently inhibits SIK1-3 [$IC_{50} < 20$ nM (21)], making it challenging to determine how inhibiting particular SIK isoforms contributes to IL-10 up-regulation in activated DCs. This question was addressed by determining how modifying the structure of HG-9-91-01 affects IL-10 potentiation relative to inhibition of SIK1-3. To do so, we first developed a succinct, modular synthesis of HG-9-91-01 and related analogs with varying side chains and/or alterations to the central heteroaromatic core (Fig. S5*A* and *Schemes* S1–S6). This series of compounds was then tested for their effects on SIK1-3 activity as well as IL-10 production by and viability of zymosan-stimulated DCs (Fig. 2*A* and Fig. S5*B*). Despite displaying a range of EC_{50}s for IL-10 up-regulation, these compounds were all potent inhibitors of SIK1 such that the two activities appear to be uncorrelated (Fig. 2*B*). In contrast, compounds lacking the 2,4-dimethoxy aniline moieties and/or bearing alterations to the central heteroaromatic core of HG-9-91-01 had differentially reduced potencies of SIK2 and SIK3 inhibition (Fig. 2*B*). Comparing the EC_{50} values for IL-10 induction versus the IC_{50}s

A

Analog	IC_{50} (nM)			IL-10 potentiation		Viability loss	
	SIK1	SIK2	SIK3	EC_{50} (μM)	Max Effect (%PGE_2)	EC_{50} (μM)	Max Effect (%DMSO)
HG-10-11-01	N.D.	10.1	17.4	0.30	66	>10	-59
HG-9-91-01	0.92	6.5	19.4	0.43	87	>10	-55
HG-10-7-01	36.2	65.1	120	1.5	35	1.0	-60
HG-9-148-01	13.2	73.3	49.3	1.7	22	>10	-57
HG-9-150-01	9.2	78.3	85.8	2.4	94	7.6	-76
HG-9-88-01	13.1	20.5	64.1	5.3	47	>25	No change

B

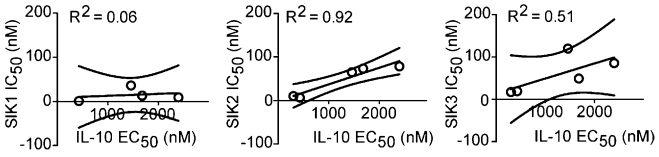

Fig. 2. Activity of HG-9-91-01 analogs suggests specific role for SIK2 inhibition in IL-10 up-regulation. (*A*) Inhibitory activity of HG-9-91-01 analogs on recombinant SIKs as well as their effects on IL-10 production and viability of zymosan-stimulated BMDCs. N.D., not determined. Data are mean of two independent experiments. (*B*) Comparison of IL-10–potentiating activity versus SIK inhibitory potencies of HG-9-91-01 analogs retaining the phenyl piperazine moiety. Curved lines indicate 95% confidence intervals for linear regression.

for SIK2 or SIK3 inhibition for the five compounds retaining the phenyl piperazine moiety revealed a significant (R^2 = 0.92) positive correlation between the potency of SIK2 inhibition and IL-10 induction, whereas this trend was less pronounced for SIK3 (Fig. 2*B*). Several compounds in this series displayed toxicity at concentrations greater than 1 μM (Fig. 2*A* and Fig. S5*B*). However, consistent with data indicating that reduction of *Sik2* expression by RNAi is well tolerated in macrophages (21), the toxicity of HG-9-91-01 analogs did not correlate with the IC_{50}s for SIK1-3 inhibition. The close correlation between the potencies of SIK2 inhibition and IL-10 up-regulation suggests that inhibition of this SIK isoform is primarily responsible for the IL-10–potentiating activity of HG-9-91-01 and structurally related compounds in activated dendritic cells.

SIK Inhibition Converts Activated Myeloid Cells to an Anti-Inflammatory State. To test whether SIK inhibition promotes anti-inflammatory phenotypes beyond enhanced IL-10 production, we pretreated BMDCs with concentrations of HG-9-91-01 or PGE_2 that equivalently up-regulate IL-10, and measured the abundance of ∼100 transcripts encoding key myeloid cytokines and transcriptional regulators after 4-h stimulation with LPS, R848, or zymosan (Dataset S3). Transcriptional responses to PGE_2 or HG-9-91-01 were normalized to vehicle-treated samples for each activating stimulus, and significantly induced or repressed genes ($P < 0.05$) were clustered using One-minus Pearson's correlations (Fig. 3*A*). Whereas IL-10 mRNA levels were robustly enhanced by both PGE_2 and HG-9-91-01 (Fig. S6*A*), notable differences are observed in the transcriptional response to these two CREB activating stimuli. Expression of the proinflammatory cytokines *Il12b* and *Il1b* was significantly elevated in PGE_2- versus HG-9-91-01–treated BMDCs (Fig. S6 *B* and *C*) and similar trends were observed with *Il23a* and *Il33* (Fig. S6 *D* and *E*). Similarly, abundance of *Arg1* and *Nos2* transcripts, which encode genes essential arginine catabolism and nitric oxide (NO) production, respectively, was highly induced by PGE_2, whereas these changes were significantly blunted in BMDCs pretreated with HG-9-91-01 (Fig. S6 *F* and *G*).

Next, we asked how the differential transcriptional responses activated by PGE_2 versus SIK inhibition translate into production NO and inflammatory cytokines. Whereas IL-10 secretion was similarly induced in BMDCs pretreated with PGE_2 or HG-9-91-01 before stimulation with LPS, R848, or zymosan, production of several proinflammatory mediators was significantly different (Fig. 3*B*). For instance, production of IL-12p40 and TNF-α was suppressed to a significantly greater extent by treatment with HG-9-91-01 rather than PGE_2 regardless of activating stimuli. More strikingly, IL-1β production was enhanced by PGE_2 following stimulation with R848 or zymosan, whereas production of this proinflammatory cytokine was suppressed in the presence of the SIK inhibitor. Lastly, elevated expression of *Nos2* in PGE_2-treated BMDCs relative to HG-9-91-01–treated BMDCs correlated with increased NO production following zymosan stimulation. Consistent with the effects of SIK inhibition in BMDCs, preincubating human MΦs or DCs with HG-9-91-01 enhanced IL-10 production while dramatically reducing IL-12p70 secretion in response to zymosan stimulation (Fig. S7 *A* and *B*). Hence, SIK inhibition converts human and murine myeloid cells to an anti-inflammatory phenotype in which up-regulation of IL-10 is accompanied by reduced secretion of inflammatory cytokines. Moreover, the stimulatory effect of SIK inhibition on several inflammatory mediators is substantially blunted relative to the responses induced by PGE_2.

SIK Inhibition Does Not Enhance IL-10 Production by Regulatory T-Cell Subsets. Regulatory $CD4^+$ T cells expressing the master transcription factor FoxP3 (i.e., T_{reg}s) play an essential role in maintaining tolerance to self- and commensal antigens (25). In addition, a subset of $FoxP3^-$ $CD4^+$ T cells that produce high levels of IL-10, termed type 1 regulatory (Tr1) cells, have been implicated in gut immune homeostasis (26). To determine if SIK inhibition affects differentiation and/or IL-10 production by either regulatory T-cell subset, we treated naive splenic $CD4^+$ T cells with HG-9-91-01 in the presence of culture conditions that promote T_{reg} or Tr1 differentiation for 4 d. In contrast with myeloid cells, concentrations of HG-9-91-01 greater than 0.5 μM were toxic in both T_{reg}s and Tr1 cells (Figs. S8*A* and S9*A*). Differentiation of T_{reg}s in the presence of concentrations of HG-9-91-01 sufficient to potently inhibit SIKs and increase IL-10 in BMDCs did not increase the fraction of $Foxp3^+$ cells or promote IL-10 secretion (Fig. S8 *B* and *C*). Similarly, although Tr1 cells abundantly secreted IL-10, this activity was not further enhanced by nontoxic concentrations of HG-9-91-01 (Fig. S9*B*). Together, these data suggest that SIK–CRTC–CREB signaling may play a greater role in regulating *Il10* expression in myeloid cells than T lymphocytes.

SIK Inhibition Enhances IL-10 Production by Immunoregulatory Gut Myeloid Cells. To determine if SIK inhibition enhances IL-10 production by primary myeloid cells from gut tissues, we isolated immunoregulatory $CD11c^+$ CX_3CR1^{hi} cells and a control

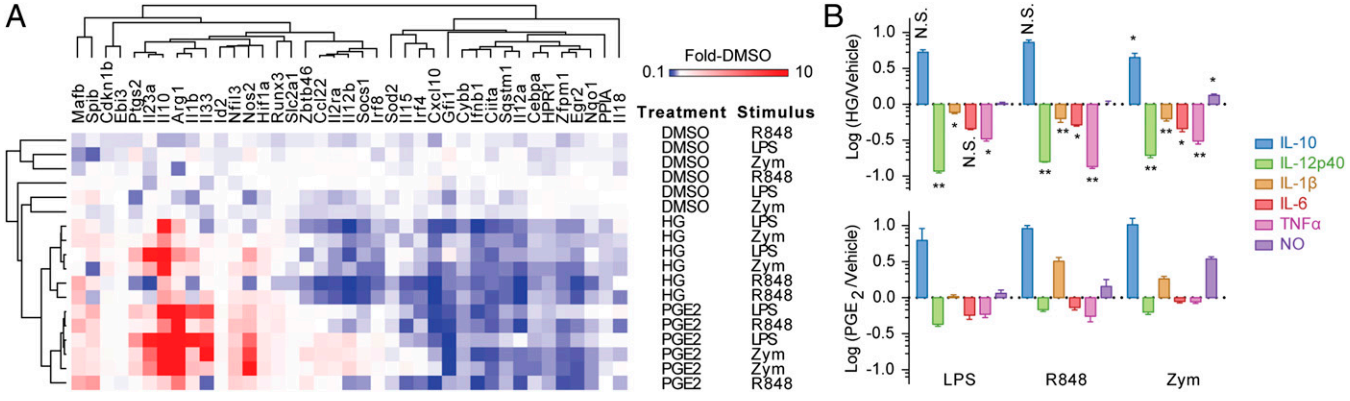

Fig. 3. SIK inhibition converts activated BMDCs to an anti-inflammatory phenotype distinct from that induced by PGE_2. (*A*) BMDCs were treated with DMSO, PGE_2 (5 μM), or HG-9-91-01 (0.5 μM) for 2 d, followed by stimulation with LPS, R848, or zymosan for 4 h. Abundance of actin-normalized transcripts in PGE_2- or HG-9-91-01–treated samples is expressed relative to the mean of the DMSO-treated samples for each microbial stimulus and clustered using One-minus Pearson's correlation. $n = 2$, data are representative of two independent experiments. (*B*) BMDCs were treated with DMSO, HG-9-91-01 (0.5 μM), or PGE_2 (5 μM) for 2 d, followed by stimulation with LPS, R848, or zymosan for 18 h. Effect of HG-9-91-01 (*Upper*) or PGE_2 (*Lower*) on cytokine (CBA assays) and NO production (Greiss assay) is expressed relative to DMSO-treated samples for each microbial stimulus. Error bars = mean ± SD, $n = 3$ from one independent experiment. Data are representative of two independent experiments. *$P < 0.05$, **$P < 0.01$, N.S. = not significant, unpaired Student *t* test (HG-9-91-01 versus PGE_2).

population of CD11c$^+$ CX$_3$CR1$^-$ DCs from the small intestine of *Cx3cr1*$^{eGFP/+}$ mice (Fig. 4*A*). Because ex vivo survival of primary gut cells is limited, both subsets were pretreated with HG-9-91-01 for 30 min before 18-h stimulation with LPS or zymosan. Despite the limited pretreatment, HG-9-91-01 enhanced IL-10 production by CD11c$^+$ CX$_3$CR1^{hi} cells activated with LPS or zymosan (Fig. 4*B*). Consistent with our results in BMDCs and human DCs–MΦs, up-regulation of IL-10 was accompanied by reduced secretion of TNF-α in both gut myeloid cell subsets. SIK inhibition does not appear to nonspecifically disrupt function of gut DCs–MΦs because production of IL-6 or the IFNγ-responsive chemokine RANTES was not affected by HG-9-91-01 pretreatment in either subset (Fig. 4*B*). Thus, the IL-10–potentiating activity of HG-9-91-01 in CD11c$^+$ CX$_3$CR1^{hi} myeloid cells isolated from the small intestine provides experimental evidence supporting SIKs as potential targets to enhance gut IL-10 levels.

Discussion

In this study, we describe a small-molecule screen that identified SIK inhibition as a common mechanism by which several multikinase inhibitors, including FDA-approved drugs for CML, enhance IL-10 production by activated BMDCs. Evaluation of a series of structural analogs of the selective SIK targeting inhibitor HG-9-91-01 demonstrates a strong correlation between the potency of SIK2 inhibition and enhanced IL-10 production. These results, together with data indicating that expression of an HG-9-91-01–resistant SIK2 allele suppresses the IL-10-potentiating effects of this compound in RAW264.7 murine macrophages (21), suggest that SIK2 plays a primary role in restraining IL-10 production in activated myeloid cells. Along with enhanced IL-10 secretion, SIK inhibition converted activated BMDCs to an anti-inflammatory phenotype marked by reduced secretion of the inflammatory cytokines IL-1β, IL-6, IL-12, and TNF-α, and these coordinated effects of SIK inhibition were conserved in human DCs–MΦs. In addition, we evaluated whether SIK inhibition enhanced IL-10 production in other immune cell types known to produce IL-10 such as FoxP3$^+$ T$_{reg}$s or Tr1 cells. In contrast with our observations in myeloid cells, treatment with HG-9-91-01 did not increase IL-10 production during ex vivo differentiation of either regulatory T-cell subset, which is consistent with studies indicating that c-MAF and mitogen-activated protein (MAP) kinase signaling play a central role in IL-10 regulation in T lymphocytes (16).

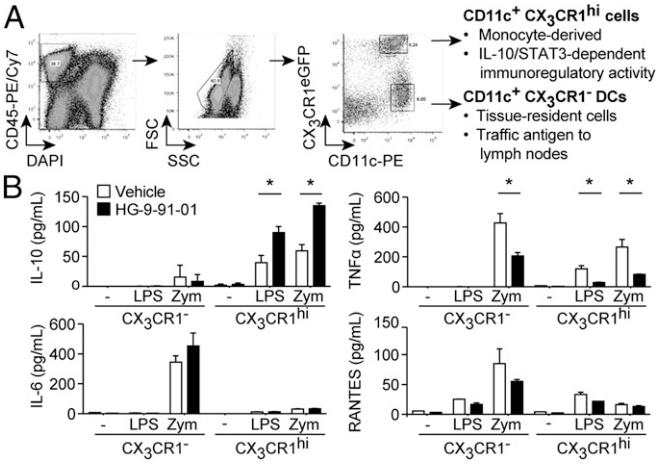

Fig. 4. SIK inhibition enhances IL-10 production by gut myeloid cells. (*A*) FACS-based isolation of CD11c$^+$ CX$_3$CR1^{hi} myeloid cells and CD11c$^+$ CX$_3$CR1$^-$ DCs from the small intestine of *Cx3cr1*$^{eGFP/+}$ mice. (*B*) CD11c$^+$ CX$_3$CR1^{hi} myeloid cells and CD11c$^+$ CX$_3$CR1$^-$ DCs were treated with vehicle or HG-9-91-01 (0.3 μM) for 30 min before stimulation with LPS or zymosan. After 18 h, abundance of the indicated cytokines in the resulting supernatants was quantified by cytokine bead array. Error bars = mean ± SD, $n = 3$ from one independent experiment. Data are representative of three independent experiments. *$P < 0.05$, unpaired Student *t* test.

Our results indicate that dasatinib and bosutinib induce effects consistent with SIK inhibition in DCs including activation of a CREB-dependent luciferase reporter construct and reduction of SIK-specific phosphorylation of CRTC3 at S370. Significantly, the IL-10–enhancing activity of both dasatinib and bosutinib in activated BMDCs was blunted by knockdown of CRTC3. Together, these results support a model where SIK inhibition underlies the immunomodulatory effects of these FDA-approved CML drugs in myeloid cells and provide mechanistic insight into the recent report that dasatinib treatment increased serum IL-10 levels, while decreasing TNF-α, following systemic LPS challenge (27). These findings have several potential implications for therapeutic use of dasatinib and related compounds. Dasatinib doses administered to CML patients achieve serum concentrations sufficient to inhibit SIKs and to up-regulate IL-10 by activated DCs (28), which suggests that SIK inhibition is unlikely to be toxic and that the IL-10–potentiating activity of FDA-approved drugs targeting these kinases might be explored as candidate treatments for IBD. In the context of cancer, it is possible that elevated serum IL-10 levels mediated by SIK inhibition could modulate the chemotherapeutic activity of dasatinib given that T-cell–mediated immune responses targeting tumor antigens are a critical component of dasatinib's mode-of-action (29). Therefore, combining dasatinib or related kinase inhibitors with an IL-10 neutralizing strategy might be a rational approach to enhance the chemotherapeutic activity of these compounds.

The results described here provide additional mechanistic insight into the ability of SIK inhibitors to enhance selective IL-10 production by activated myeloid cells. For instance, key differences in the expression of genes encoding inflammatory cytokines (e.g., *Il1b*, *Il12b*, and *Il23a*) or NO production (*Nos2*) were observed in the transcriptional profiles of BMDCs treated with concentrations of PGE_2 or HG-9-91-01 that up-regulate IL-10 to a similar extent. Significantly, these differential transcriptional responses were mirrored by greater suppression of IL-12p40 (i.e., the cytokine encoded by *Il12b*) secretion and a lack of increased NO production in HG-9-91-01- versus PGE_2-treated BMDCs. Reduced induction of *Il12b* and *Il23a* expression with HG-9-91-01 is significant because these genes encode the two subunits of IL-23, a heterodimeric cytokine that promotes expansion of the inflammatory T$_H$17 T-cell lineage (30). Similarly, whereas NO plays an essential role in the antimicrobial activity of myeloid cells, aberrantly high production of this inflammatory mediator is thought to contribute to IBD pathogenesis (31). Both SIK inhibition and PGE_2 enhance IL-10 transcription via SIK–CRTC–CREB signaling. However, in the case of EP2/EP4 prostanoid receptor agonists like PGE_2, these effects are mediated by activation of PKA following an intracellular cAMP flux. In addition to the PKA–CREB cascade, cAMP activates scaffolding proteins, called exchange proteins activated by cAMP (EPACs), that nucleate MAP kinase and cytoskeletal signaling components (32). Of note, the stimulatory effects of PGE_2 on IL-23 and NO production have both been linked to the cAMP–EPAC pathways in myeloid cells (19). Hence, our findings extend previous studies of SIK inhibitors by suggesting a model in which SIK inhibition promotes selective enhancement of CREB-dependent *Il10* expression in the absence of pleiotropic effects of elevated intracellular cAMP (Fig. S10).

In addition to IL-10 and its receptor, SNPs near the genetic loci for *PTGER4*, which encodes the EP4 prostanoid receptor, and *CRTC3* confer increased risk for Crohn's disease and ulcerative colitis (2). In addition, an intronic SNP in *SIK2* confers susceptibility to primary sclerosing cholangitis, a degenerative liver disease that shares significant comorbidity with IBD (33). These human genetic data potentially implicate EP4–SIK2–CRTC3–CREB signaling as a contributor to IBD (patho)physiology via regulation of gut IL-10 levels, and make it an intriguing target for therapeutic manipulation. This hypothesis is supported by the efficacy of phosphodiesterase (PDE) inhibitors, which suppress

cAMP hydrolysis, and gut-restricted delivery of EP4-selective agonists in murine models of colitis (34, 35). Unfortunately, potent emetic effects have hampered therapeutic development of PDE inhibitors (36), whereas gut-specific activation of prostanoid receptors remains an unproven therapeutic strategy. Specific experimental support for SIK inhibition as a potential IBD treatment comes from our observation that the IL-10–potentiating activity of HG-9-91-01 is maintained in murine $CD11c^+$ CX_3CR1^{hi} cells, a highly abundant subset of myeloid cells that play a key role in maintaining gut immune homeostasis (13, 14). The anti-inflammatory activity of $CD11c^+$ CX_3CR1^{hi} myeloid cells in the $CD45RB^{hi}$ T-cell transfer colitis model requires intact IL-10/STAT3 signaling (15), which suggests that SIK inhibition will enhance the T-cell suppressive activity of these cells and, in turn, suppress pathogenic auto-inflammation characteristic of IBD.

In summary, our findings demonstrate the SIK inhibition by small molecules converts activated DCs–MΦs to an anti-inflammatory phenotype characterized by enhanced IL-10 production coupled with reduced secretion of inflammatory cytokines. Integrating genetic analysis of IBD susceptibility with the IL-10–potentiating activity of HG-9-91-01 in primary gut myeloid cells supports SIKs as a potential new target for treatment of these disorders. However, because aberrant recruitment–activation of inflammatory myeloid cells also contributes to the pathogenesis of type-1 diabetes, rheumatoid arthritis, and systemic lupus erythematosus (10), SIK inhibition may be a more broadly applicable therapeutic strategy for treatment of autoimmune–auto-inflammatory disorders.

Materials and Methods

Bone marrow-derived DCs were differentiated from C57BL/6 bone marrow in the presence of GM-CSF–conditioned media. $CD11c^+$ CX_3CR1^{hi} myeloid cells and $CD11c^+$ CX_3CR1^- DCs were isolated from the small intestine lamina propria of *Cx3cr1*$^{eGFP/+}$ mice (12). Detection of cell-surface Thy1.1 expression on BMDCs from 10BiT reporter mice was conducted as described previously (24). Cell viability was estimated by change in total cellular ATP levels using CellTiterGlo assays (Promega). Preparation of recombinant SIK proteins and IC_{50} measurements were conducted as described previously (21). Total RNA was extracted using a NucleoSpin 96 RNA isolation kit (Macherey-Nagel) followed by cDNA synthesis and multiplex RT-PCR using a microfluidic dynamic array (Fluidigm). The concentrations of TNF-α, IL-6, IL-10, IL-12p40, IL-1β, and RANTES in culture supernatants were detected using a FlexSet Cytometric bead array (BD Biosciences). Alternatively, IL-10 secretion was quantified using ELISA (BD Biosciences) or AlphaLISA assays (PerkinElmer). NO production was estimated based on nitrate content in culture medium using the Greiss reagent. Details on reagents, chemical synthesis, and experimental protocols are available in *SI Materials and Methods*.

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