

SIK inhibition in human myeloid cells modulates TLR and IL-1R signaling and induces an anti-inflammatory phenotype

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ABSTRACT

Macrophage polarization into a phenotype producing high levels of anti-inflammatory IL-10 and low levels of proinflammatory IL-12 and TNF- α cytokines plays a pivotal role in the resolution of inflammation. Salt-inducible kinases synergize with TLR signaling to restrict the formation of these macrophages. The expression and function of salt-inducible kinase in primary human myeloid cells are poorly characterized. Here, we demonstrated that the differentiation from peripheral blood monocytes to macrophages or dendritic cells induced a marked up-regulation of salt-inducible kinase protein expression. With the use of 2 structurally unrelated, selective salt-inducible kinase inhibitors, HG-9-91-01 and ARN-3236, we showed that salt-inducible kinase inhibition significantly decreased proinflammatory cytokines (TNF- α , IL-6, IL-1 β , and IL-12p40) and increased IL-10 secretion by human myeloid cells stimulated with TLR2 and-4 agonists. Differently than in mouse cells, salt-inducible kinase inhibition did not enhance IL-1Ra production in human macrophages. Salt-inducible kinase inhibition blocked several markers of proinflammatory (LPS + IFN- γ)-polarized macrophages [M(LPS + IFN- γ)] and induced a phenotype characterized by low TNF- α /IL-6/IL-12p70 and high IL-10. The downstream effects observed with salt-inducible kinase inhibitors on cytokine modulation correlated with direct salt-inducible kinase target (CREB-regulated transcription coactivator 3 and histone deacetylase 4) dephosphorylation in these cells. More importantly, we showed for the first time that salt-inducible kinase inhibition decreases proinflammatory cytokines in human myeloid cells upon IL-1R stimulation. Altogether, our results expand the potential therapeutic use of salt-inducible kinase

inhibitors in immune-mediated inflammatory diseases. *J. Leukoc. Biol.* 99: 711–721; 2016.

Introduction

Macrophages are innate immune cells that display a high degree of plasticity in their gene expression program, which enables them to perform multiple tasks (e.g., from the host defense to wound healing/tissue repair and resolution of inflammation). A peculiar characteristic of macrophages is their ability to “switch” from a phenotype to another in vitro and in vivo [1–3], suggesting that a given cell may participate sequentially in both the induction and the resolution of inflammation [4]. These functions are attributed to specific macrophage activation states [5]. Initially, a broad classification distinguished proinflammatory M1 (induced by IFN- γ) from the anti-inflammatory M2 (induced by IL-4) cells. However, it is now recognized that the M1 versus M2 dichotomy is far too reductive to encompass the full spectrum of macrophage activation. A recent revision of this model suggests that rather than defined subsets of macrophages, the complexity of the factors present in the systemic and local milieu will influence the kinetics, plasticity, and reversibility of macrophage responses leading to rather complex, even mixed, phenotypes [6, 7]. The idea of exploiting macrophage’s plasticity of responsiveness in therapeutic settings is intriguing and could represent an innovative approach for the treatment of a great variety of human diseases [5]. For example, it is increasingly appreciated that the sustained inflammation underlying the pathogenesis of chronic inflammatory diseases (e.g., RA, CD, and psoriasis) can be the result of an impaired resolution of inflammatory responses [8, 9] that is generally associated with a switch of macrophages from a proinflammatory to an anti-inflammatory phenotype (characterized by IL-10^{high} and IL-12^{low}) [10]. Transfer of such macrophages into mice is protective in models of experimental autoimmune encephalomyelitis [11] and

Abbreviations: $\Delta\Delta$ Ct = difference in comparative threshold, ACC = acetyl-CoA carboxylase, AMPK = AMP-activated protein kinase, BMDC = bone marrow-derived dendritic cell, BMDM = bone marrow-derived macrophage, CD = Crohn’s disease, CRT3 = CREB-regulated transcription coactivator 3, Ct = comparative threshold, DC = dendritic cell, FL2/9/10 = fluorescent 2/9/10, FSC = forward-scatter, HDAC = histone deacetylase, IL-1Ra = IL-1R

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endotoxic shock [12]. On the other hand, the direct use of anti-inflammatory molecules, e.g., rIL-10 in various clinical trials of RA or CD, gave disappointing results as a result of limited efficacy and/or development of side-effects following systemic administration, suggesting that elevated levels of IL-10 are rather required locally to exert their effect and/or because additional anti-inflammatory molecule(s) are also needed [13, 14]. Interestingly, RA patients with a polymorphism (-1082AA) in the IL-10 gene promoter, associated with higher IL-10 production detected in joint biopsies, showed less joint destruction [15]. There is need for alternative strategies to increase local levels of IL-10 and to combine it with neutralization of proinflammatory cytokines. Thus, it is of great interest that pharmacological inhibition of SIKs controls the switch from proinflammatory macrophages to a phenotype characterized by high levels of IL-10 and lower levels of proinflammatory cytokines [16, 17].

SIKs constitute a STK subfamily, belonging to the AMPK family. Three members (SIK1, -2, and -3) have been identified so far. Amino acid homology of SIK1 with SIK2 and SIK3 is 78% and 68%, respectively, in the kinase domain. The cloning of SIK1, abundantly expressed in the adrenal glands of high-salt, diet-fed rats, led to subsequent cloning of SIK2 (or QIK), mainly expressed in adipose tissues and the rather ubiquitous SIK3 (or QSK) [18]. TLR stimulation provides a first signal for the induction of NF- κ B, ERK, and p38 MAPK signaling pathways. Downstream secondary signals (e.g., mitogen- and stress-activated kinases 1 and 2, activated by p38 MAPK and ERKs, respectively) phosphorylate CREB and activate its function. CREB drives the transcription of anti-inflammatory molecules, including IL-10 [19, 20]. Mechanistic studies that use the selective SIK1-3 inhibitors HG-9-91-01 and KIN112 in mouse BMDMs revealed that SIK inhibition leads to dephosphorylation of CRTC3 and its subsequent translocation into the nucleus, where it associates with CREB to promote a strong up-regulation of IL-10 [16]. These roles of SIKs have been demonstrated mainly using the mouse cell line RAW264.7 and murine primary myeloid cells and only to a very limited extent, were confirmed in human myeloid cells [16, 21].

In this study, we analyzed the expression and function of SIK in human myeloid cells by use of 2 structurally unrelated SIK inhibitors (HG-9-91-01 and ARN-3236) and an RNAi approach. TLR and IL-1R share common intracellular signaling pathways [20]. Given the important role of IL-1 in inflammatory diseases [22, 23], we also examined whether SIK inhibition was able to impair IL-1 β -mediated cytokine production in human myeloid cells.

MATERIALS AND METHODS

Drugs and reagents

HG-9-91-01 was synthesized as described elsewhere [16] and purified to >96% purity by Syngene International (Bangalore, India). ARN-3236 (purity >98%,

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antagonist, LKB1 = liver kinase B1, M = macrophages, M0-M Φ = monocyte-derived macrophages, M1 = classically activated macrophages, M2 = alternatively activated macrophages, MFI = median fluorescence intensity, Mo-IDC = immature monocyte-derived dendritic cell, Pam₃CSK₄ = N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteiny]-[S]-seryl]-[S]-lysyl]-[S]-lysyl]-[S]-lysine, PKA = cAMP-dependent protein kinase, qPCR = quantitative PCR, RA = rheumatoid arthritis, rh = recombinant human, rm = recombinant murine, RNAi = RNA interference, SIK = salt-inducible kinase, siRNA = small interfering RNA, STK = serine/threonine kinase, TAK1 = TGF- β -activated kinase 1

U.S. Patent # US20140256704A1) was obtained by Arrien Pharmaceuticals (Salt Lake City, UT, USA). Powders were dissolved in DMSO (Hybri-Max; Sigma-Aldrich, St. Louis, MO, USA) as 10 mM stock solutions and stored at -20°C until use. Pam₃CSK₄ and LPS-EK-Ultrapur were from InvivoGen (San Diego, CA, USA), and PGE₂ was from Sigma-Aldrich. rhIFN- γ , rhIL-4, rhIL-10, and rhIL-1 β were from PeproTech (Rocky Hill, NJ, USA); rmGM-CSF, rhM-CSF, and rhGM-CSF were from ImmunoTools (Friesoythe, Germany).

Cell culture

Human PBMCs were isolated by density gradient centrifugation on Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). Monocytes were isolated from PBMCs by negative depletion by use of Monocyte Isolation Kit II and QuadroMACS. Cells were typically 80–90% CD14⁺, as assessed using FITC-conjugated CD14 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany).

Monocytes were seeded at 1.5×10^5 cells/well in 48-well plates or at $0.75-1 \times 10^6$ cells/well in 12-well plates (Corning, New York, NY, USA) in complete medium: RPMI 1640 supplemented with 2 mM glutamine, penicillin, and streptomycin (100 U/ml; all from Gibco, Grand Island, NY, USA) and 10% heat-inactivated FBS (PAA Laboratories, Pasching, Austria). For the generation M0-M Φ , monocytes were cultured with rhM-CSF (100 ng/ml) for 6 d. At d 2 and 4, half of the medium was replaced by fresh differentiation medium [24]. For polarization experiments, M0-M Φ were exposed for an additional 24 h to fresh complete medium containing LPS (100 ng/ml) + IFN- γ (20 ng/ml) for M(LPS + IFN- γ) or IL-4 (20 ng/ml) for M(IL-4) [25]. For M(LPS + IgG) polarization, M0-M Φ were washed once with $1 \times$ PBS, recovered with 2 mM EDTA, and plated at 2×10^5 cells/well in 96-well plates that were previously coated with 50 μ g/ml human purified IgG (Sigma-Aldrich) for 2 h at room temperature. LPS (100 ng/ml) was added for up to 24 h [24].

For the generation of Mo-IDCs, monocytes were seeded in 6-well plates at 2×10^6 cells/well in complete medium, supplemented with 50 ng/ml rhGM-CSF and 50 ng/ml rhIL-4 and for the next 7 d, with medium renewal at d 2 and 4. At d 7, Mo-IDCs were seeded in flat-bottom 96-well plates at a concentration of 1.25×10^5 cells/well or in 12-well plates at 1×10^6 cells/well and stimulated up to 48 h with LPS (100 ng/ml) or IL-1 β (10 ng/ml). RAW264.7 cells were cultured in DMEM, supplemented with antibiotics (all from Gibco) and 10% heat-inactivated FBS. Cell viability was measured by assessing lactate dehydrogenase release using the CytoTox-ONE kit (Promega, Madison, WI, USA), according to the manufacturer's instruction.

BMDMs were obtained by culturing bone marrow cells from 6- to 12-wk-old C57BL/6 mice (obtained from Charles River Laboratories, Wilmington, MA, USA) in bacterial culture-grade 10 mm Petri dishes in DMEM, supplemented with 2 mM glutamine and antibiotics (all from Gibco), 20% L929 conditioned medium, and 10% heat-inactivated FBS, for 7 d with medium renewal after 5 d. BMDMs were replated in 12-well tissue-culture-treated plates at 0.4×10^6 cells/well for 24 h before stimulation on d 8. BMDCs were generated from bone marrow as above and differentiated for 6 d in tissue-culture-treated plastic in RPMI 1640, supplemented with 20 ng/ml rmGM-CSF, 10% heat-inactivated FBS, 100 mM sodium pyruvate, 2 mM glutamine, antibiotics, and 50 μ M 2-ME (Sigma-Aldrich), with medium renewal every 2 d. BMDCs were replated in 12 wells at a density of 0.4×10^6 cells/well (without rmGM-CSF) before stimulation on d 8.

Cytokine measurements

IL-10, TNF- α , IL-6, IL-1 β , IL-1Ra- and IL-12p70 were measured using a custom Bio-Plex Pro human assay and the Bio-Plex MAGPIX Multiplex reader (Bio-Rad Laboratories, Hercules, CA, USA) or using ELISA Ready-SET-Go! kit from eBioscience (San Diego, CA, USA; for TNF- α , IL-10, and IL-6) or R&D Systems (Minneapolis, MN, USA; for CCL1), according to the manufacturers' protocols.

Flow cytometry

Phenotypic analysis of DCs was performed using flow cytometric direct immunofluorescence. After 48 h of stimulation, cells were recovered in FACS buffer (PBS 1% BSA, 10 mM EDTA). FcRs were blocked by incubation of

0.5×10^6 cells in 50 μ l 10% human serum in FACS buffer during 15 min at 4°C. After a wash in FACS buffer, cells were labeled or not with a cocktail of antibodies diluted in FACS buffer in a volume of 50 μ l for 30 min at 4°C. Cells were then washed twice with PBS and labeled with Zombie Yellow viability dye, diluted in PBS for 20 min at room temperature. After a final wash, cells were resuspended in 200 μ l FACS buffer, and data were acquired using Gallios 4 flow cytometer (Beckman Coulter, Brea, CA, USA). OneComp eBeads (01-1111-42; eBioscience) were used for compensation. The following antibodies and dyes were used: anti-CD14-FITC (Miltenyi Biotec), anti-CD86-PE (eBioscience), anti-CD209-PerCpCy5.5 (BD PharMingen, San Diego, CA, USA), and anti-CD83-BV421 (BioLegend, San Diego, CA, USA), all diluted 1/50; Zombie Yellow (BioLegend) was diluted 1/400. Kaluza software was used for analysis. In all conditions, total cells were gated on a FSC/side-scatter linear plot, and doublets and dead cells were excluded using, respectively, FSC-height versus FSC-area linear plot and FL10 (Zombie Yellow) histogram. This defined the “LiveCells” gate used for analysis and representing >80% of all cells in all conditions. MFI was calculated for FL2 (CD86) and FL9 (CD83) in all of the stained and unstained conditions, allowing the calculation of the difference in MFI (MFI stained – MFI unstained), representing the MFI normalized for the autofluorescence.

Immunoblotting

Cells were rinsed once in ice-cold 1 \times PBS and extracted in lysis buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100], supplemented with 1 \times cOmplete EDTA-free Protease Inhibitor mixture 1 \times PhosSTOP Phosphatase Inhibitor (Roche, Basel, Switzerland). Cell extracts were clarified by centrifugation at 14,000 g for 15 min at 4°C. Protein concentration was determined using the Bradford assay (Bio-Rad Laboratories), and 35–40 μ g cell extracts were separated by SDS-PAGE using a Novex 4–12% gradient gel (Life Technologies, Carlsbad, CA, USA) and transferred to nitrocellulose membranes. Immunoreactive bands were visualized by ECL reagents (Amersham Biosciences, Buckinghamshire, United Kingdom) or Radiance Plus (Axonlab, Baden, Switzerland) and signals acquired using a LAS 4000 mini imager (Fujifilm Life Science, Stamford, CT, USA) and quantified using ImageJ software 1.47v (NIH, Bethesda, MD, USA). Membranes were stripped in 1 \times ReBlot Plus Strong (EMD Millipore, Billerica, MA, USA).

Antibodies

The following antibodies were used for immunoblotting: anti-mouse, -rabbit, or -sheep HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA); anti-GAPDH clone 6C5 (EMD Millipore); anti-SIK1 (Proteintech, Chicago, IL, USA); anti-SIK2 (D28G3), anti-phospho-Ser246 HDAC4 (D27B5), anti-HDAC4, anti-phospho-Ser428-LKB1, anti-LKB1, anti-phospho-Thr172-AMPK- α , anti-AMPK- α , anti-phospho-Ser79-ACC, and anti-ACC (all from Cell Signaling Technology, Danvers, MA, USA); and anti-SIK3 and anti-CRTC3 EPR3440 (both from Abcam, Cambridge, United Kingdom). The antibody against the phospho-Ser370 peptide (S253C, bleed 2) of CRTC3 (Medical Research Council Protein Phosphorylation and Ubiquitylation Unit, Dundee, United Kingdom).

RNAi in human macrophages

Human monocytes were seeded in 24-well plates at 1.25×10^5 cells/well, and macrophages (M0-M Φ) were generated as described above. On d 7, macrophages were transfected in complete medium containing 5% FCS and 100 ng/ml M-CSF with siRNAs for SIK1, SIK2, and SIK3 (300 pM each) or scramble (900 pM) siRNA (Trilencer-27 siRNA; OriGene Technologies, Rockville, MD, USA) by use of INTERFERin (Polyplus-transfection, New York, NY, USA), according to the manufacturer’s instructions. After 48 h incubation at 37°C, medium was refreshed, and the macrophages were stimulated for 3 h with 100 ng/ml LPS in the absence or presence of 100 nM HG-9-91-01. Cytokine levels in cell supernatants were determined by ELISA, and knockdown of protein was checked in cell lysates by immunoblotting as described above.

qPCR

Total RNA was extracted by use of TRIzol (Ambion, Austin, TX, USA) or RNeasy Micro kit (Qiagen, Limburg, the Netherlands), following the manufacturers’ instructions. cDNA was generated from 0.5 to 1 μ g DNase RQ1-treated total RNA in a 20 μ l reaction with Superscript II RT (Invitrogen, Carlsbad, CA, USA), following the manufacturer’s instructions. Real-time qPCR (40 cycles, annealing temperature 60°C) was performed using a Master Mix (SYBR Green Supermix; Bio-Rad Laboratories) on a CFX96 real-time system (Bio-Rad Laboratories). Relative expression of each gene was calculated from Ct values by use of the Pfaffl method [26] and normalized against the mRNA levels of 18S RNA. Results are reported relative to untreated control cells, which were set to 1. When comparing relative expression levels in monocytes versus macrophages or Mo-iDC, the mean Ct values for each gene of interest in monocytes were used as calibrator and expression calculated by the $2^{-\Delta\Delta Ct}$ method [27]. The primers used for PCR are listed in Table 1.

Statistical analysis

Quantitative data are presented as the means \pm SD. Curve-fitting was obtained using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA). Statistical differences were assessed by 1-way ANOVA, followed by Tukey’s post-test or 2-tailed Student’s t test, and considered significant if $P < 0.05$.

RESULTS

Changes in SIK1–3 expression during monocytes differentiation to macrophages

We analyzed the relative mRNA expression of the SIK1–3 in freshly isolated human monocytes and in differentiated macrophages (M0-M Φ). SIK1 mRNA levels were \sim 25-fold higher, and SIK2 mRNA levels were 10-fold lower in monocytes than in macrophages, whereas SIK3 expression was not significantly different (Fig. 1A). However, the protein expression levels of

TABLE 1. Primers used in real-time PCR

Gene	Forward	Reverse
SIK1	5'-TCCAGACCATCTTGGGGCAG	5'-AAGGGGAAGGGGTTTGTGTTG
SIK2	5'-GGGTGGGGTCTACGACATC	5'-TATTGCCACCTCCGCTCTTG
SIK3	5'-CTCAGCCATCTCCACCTTCTCA	5'-GGCTGCCTGAAGAGATGGTTGT
CD80	5'-CTGCCTGACCTACTGCTTTG	5'-GGCGTACACTTTCCCTTCTC
CXCL9	5'-GTGGTGTCTTTTCTCTTG	5'-GTAGGTGGATAGTCCCTTG
CD200	5'-GAGCAATGGCACAGTACTGTT	5'-GTGGCAGGTACGGTAGACA
CCL22	5'-ATTACGTCCGTTACCGTCTG	5'-TAGGCTTTCATTGGCTCAG
18s	5'-GTAACCCGTTGAACCCATT	5'-CCATCCAATCGGTAGTAGCG

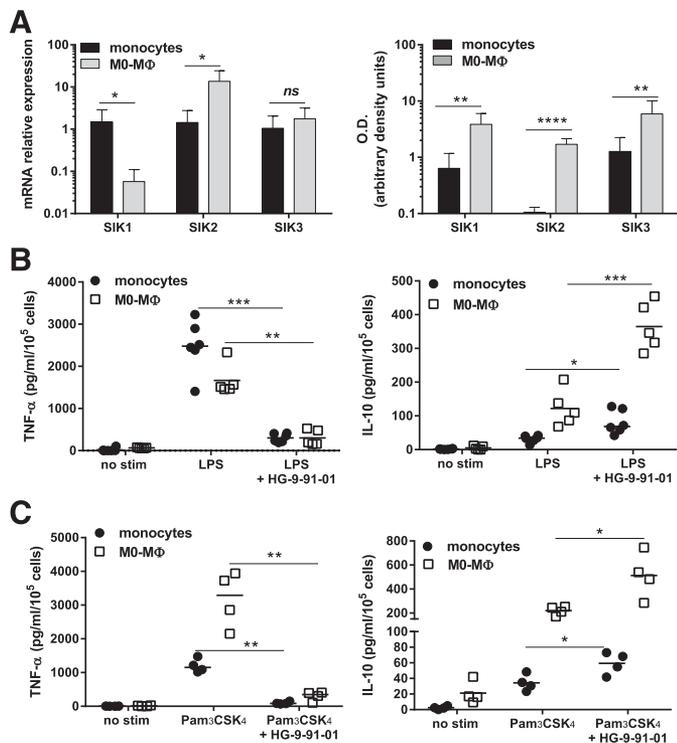


Figure 1. Expression and function of SIK1–3 in human monocytes and macrophages. Changes in SIK1–3 expression during monocytes differentiation to macrophages. (A) Relative SIK1, SIK2, and SIK3 mRNA levels in freshly isolated human monocytes ($n = 6$) and in macrophages ($n = 7$) differentiated by culturing monocytes for 6 d with 100 ng/ml rhM-CSF (M0-MΦ). Gene expression was measured by RT-qPCR using 18s RNA as normalization control and calculated by the $2^{-\Delta\Delta C_t}$ method. Protein expression levels of SIK1, SIK2, and SIK3 in cell lysates from freshly isolated human monocytes ($n = 8$) and in M0-MΦ ($n = 6$). (B) SIK inhibition down-regulates TNF- α and up-regulates IL-10 in human monocytes and macrophages stimulated with TLR agonists. TNF- α and IL-10 production measured by ELISA from freshly isolated human monocytes or macrophages differentiated as above and incubated 1 h with vehicle (0.03% DMSO) or 500 nM HG-9-91-01 and then stimulated for 3 h with (B) 100 ng/ml LPS or (C) 1 μ g/ml Pam₃CSK₄. Error bars = means \pm SD. For all graphs, statistical significance is reported as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

SIK1 and SIK3 were ~ 6 -fold and ~ 5 -fold higher in macrophages than in monocytes, respectively. The increase of SIK2 protein in macrophages was even more pronounced (~ 17 -fold; Fig. 1A). These results show that overall SIK protein levels are increased in *in vitro*-differentiated macrophages and suggest that post-translational mechanisms are likely operating to regulate SIK1 and to a lesser extent, SIK3 expression in macrophages.

SIK inhibition decreases TNF- α and increases IL-10 secretion by human monocytes and macrophages stimulated with TLR agonists

The SIK inhibitor HG-9-91-01 possesses a very good selectivity profile against the other members of the AMPK family and a good selectivity against the kinome [16]. We initially characterized the effect of the SIK inhibitor in the mouse RAW264.7 cells (Supplemental Fig. 2A). HG-9-91-01 inhibited LPS-induced secretion of TNF- α with an IC₅₀ of 267 nM, while showing no signs of

cellular toxicity up to a concentration of 10 μ M (Supplemental Fig. 2A). In all subsequent experiments, we used it at a concentration of 500 nM, unless specified otherwise. One hour pretreatment with HG-9-91-01 significantly blocked TLR4 (LPS)- and TLR2 (Pam₃CSK₄)-induced TNF- α production while increasing at the same time IL-10 secretion in BMDM (Supplemental Fig. 3A) and BMDC (Supplemental Fig. 3B). IL-1Ra was also increased in BMDM upon SIK inhibition (Supplemental Fig. 3A).

We next tested the effect of SIK inhibition on cytokine production by human monocytes and macrophages upon challenge with TLR4 or TLR2 agonists. Pretreatment with HG-9-91-01 markedly reduced TNF- α production in human monocytes and macrophages, stimulated with LPS (Fig. 1B) or Pam₃CSK₄ (Fig. 1C). Both stimuli induced IL-10 secretion, which was enhanced significantly by HG-9-91-01 in LPS (~ 3 -fold increase; Fig. 1B)- or in Pam₃CSK₄ (2.4-fold increase; Fig. 1C)-stimulated macrophages. In monocytes, we observed an ~ 2.6 -fold increase for LPS stimulation and 1.7-fold for PAM₃CSK₄ stimulation, respectively. The enhancement of IL-10 levels by the SIK inhibitor over LPS stimulation was very rapid (already evident after 2 h stimulation; data not shown) and was significant up to 4–6 h, whereas inhibition of TNF- α was more sustained and still highly significant up to 24 h (data not shown).

Profiling the effect of SIK inhibition in (LPS + IFN- γ)-polarized human macrophages

Human M0-MΦ can be polarized further toward a full proinflammatory (M1) phenotype following IFN- γ and LPS stimulation. We determined whether polarization toward an M(LPS + IFN- γ) phenotype would affect SIK1–3 protein expression. No significant changes were detected after 4 h of polarizing conditions (data not shown), whereas at 24 h, only SIK3 expression was increased by ~ 4 -fold. This increase was not significantly affected by HG-9-91-01 (Fig. 2A, inset).

Previous data in mouse BMDM stimulated by LPS or LPS + IFN- γ showed that SIK inhibition promoted the conversion to a phenotype characterized by higher levels of IL-10 and low IL-12 and mRNA up-regulation of other characteristic mouse markers [i.e., tumor necrosis factor superfamily member 14 (TNFSF14), sphingosine kinase 1, arginase 1], whereas classic M(IL-4) mouse markers were unaffected [16]. Thus, we examined whether HG-9-91-01 would also induce a similar switch in polarized human M(LPS + IFN- γ) macrophages. IFN- γ priming will induce expression of IL-12p40 [28]. Pretreatment with HG-9-91-01 almost completely blocked TNF- α and IL-12p70 production (Fig. 2B). The effect of HG-9-91-01 on TNF- α was already present at 4 h, whereas IL-12 production at this time point was negligible (data not shown). The effect of SIK inhibition on IL-6 and IL-1 β production was significant, albeit less pronounced ($\sim 36\%$ for IL-6 and $\sim 68\%$ for IL-1 β , respectively). SIK inhibition induced a significant increase in IL-10 production in M(LPS + IFN- γ), up to 4–6 h (Fig. 2B). M(LPS + IFN- γ) are also characterized by a significant up-regulation of the chemokine CXCL9 and the costimulatory molecule CD80 compared with M0-MΦ cells [25, 29]. HG-9-91-01 pretreatment significantly decreased CXCL9 and CD80 mRNA levels (Fig. 3A).

The IL-12^{low} and IL-10^{high} production as well as the chemokine CCL1/I309 are hallmarks of M(LPS + Ig)-polarized human macrophages [24, 30, 31]. Therefore, we assessed the levels of

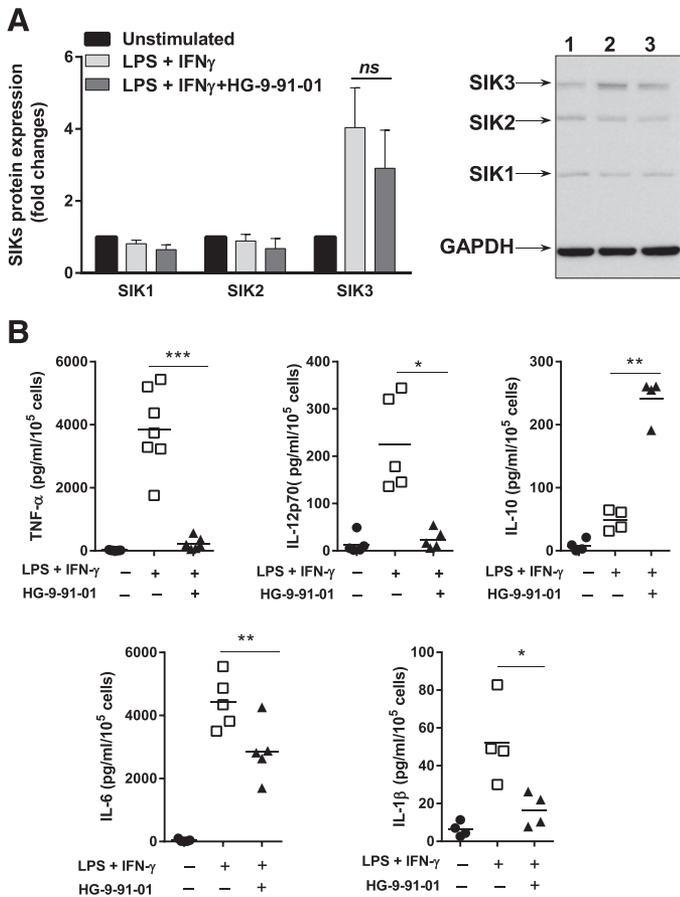


Figure 2. SIK inhibition down-regulates proinflammatory cytokines and up-regulates IL-10 in (LPS + IFN- γ)-polarized human macrophages.

Macrophages differentiated from peripheral blood monocytes for 6 d with 100 ng/ml rhM-CSF (M0-M Φ) were treated for 1 h with vehicle (0.01% DMSO) or 500 nM HG-9-91-01, followed by stimulation with LPS (100 ng/ml) and IFN- γ (20 ng/ml) to drive M1-like polarization (LPS + IFN- γ -M Φ). (A) Protein expression of SIK1–3 in cell lysates ($n = 3$), following LPS + IFN- γ -M Φ polarization and treatment with SIK inhibitor for 24 h, was assessed by Western blot analysis and normalized for GAPDH expression. Data are expressed as fold changes versus untreated (M0-M Φ) cells (set = 1). (Inset) Representative example from 1 donor depicting SIK1–3 and GAPDH expression in 15 μ g total cell lysate separated on a 4–12% SDS-PAGE gradient gel. Lane 1, Unstimulated; lane 2, LPS + IFN- γ ; lane 3, LPS + IFN- γ + HG-9-91-01. (B) Secreted cytokines TNF- α , IL-12p70, IL-6, and IL-1 β (24 h postpolarization) or IL-10 (4 h postpolarization) were quantified in the supernatants using multiplex immunoassay (Bio-Plex). For all graphs, statistical significance is reported as follows: *ns*, not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

CCL1 produced by M(LPS + IFN- γ) macrophages upon SIK inhibition. CCL1 was indeed markedly induced in macrophages cultured in the presence of IgG alone or LPS + IgG (Fig. 3B). In contrast, treatment with HG-9-91-01 was devoid of any effect on CCL1 production by LPS-IFN- γ -M Φ . As expected, CCL1 production was also not increased in M(IL-4), used as a negative control (Fig. 3B).

As there are no human homologs of the mouse M(IL-4) markers FIZZ(Found in Inflammatory Zone), YMI(chitinase 3-like 3), and macrophage galactose N-acetylgalactosamine-specific lectin 2 [32],

we measured the expression of CD200 and CCL22 mRNA, which have been more recently identified as markers of human M(IL-4) cells [25, 29]. SIK inhibition of LPS-IFN- γ -M Φ did not stimulate the expression of M(IL-4) markers, which were induced in IL-4-polarized macrophages (Fig. 3C).

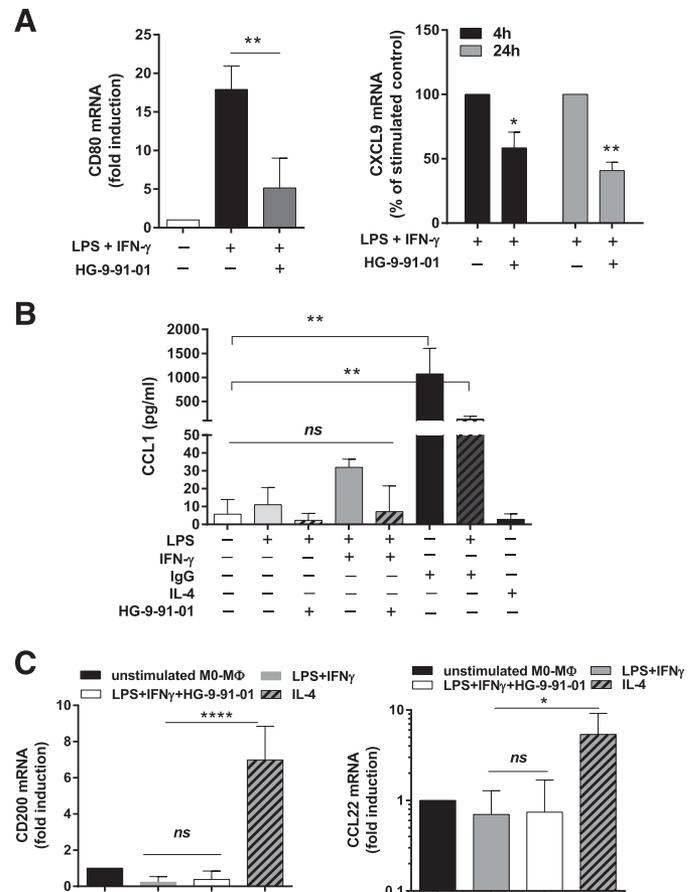


Figure 3. Profiling the effect of SIK inhibition in LPS + IFN- γ -polarized human macrophages.

Macrophages differentiated from peripheral blood monocytes for 6 d with 100 ng/ml rhM-CSF (M0-M Φ) were treated for 1 h with vehicle (0.03% DMSO) or 500 nM HG-9-91-01, followed by stimulation with LPS (100 ng/ml) and IFN- γ (20 ng/ml) to drive M(LPS + IFN- γ) polarization; with 100 ng/ml LPS in wells precoated for 1 h with human IgG (50 μ g/ml), as detailed in Materials and Methods, to drive M(LPS + IgG); or with 20 ng/ml IL-4 to drive M(IL-4) for 4 or 24 h. (A) Following total RNA extraction, expression of the indicated transcript was determined by RT-qPCR and normalized using 18s RNA. CD80 mRNA levels were measured after 24 h and CXCL9 after 4 and 24 h of LPS + IFN- γ stimulation, respectively ($n = 4$). CD80 mRNA levels are expressed as fold changes versus untreated, unstimulated cells (set = 1). The fold changes in CXCL9 mRNA are expressed as percentage of stimulated condition (considered as 100%), and statistical significance was calculated by comparing each data set with “100” using 1 sample *t* test. (B) Secreted CCL1 ($n = 4$) was measured in the supernatants by ELISA after 24 h. (C) Human M(IL-4) macrophages markers are not induced by HG-9-91-01 treatment of M(LPS+ IFN- γ) macrophages. Cells were differentiated and treated as above for 24 h. Following total RNA extraction, expression of CD200 and CCL22 ($n = 4$) was determined by RT-qPCR and normalized using 18s RNA. mRNA levels are expressed as fold induction versus untreated, unstimulated cells (set = 1). For all graphs, statistical significance is reported as follows: *ns*, not significant, * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. Error bars = means \pm sd.

Inhibition of SIK blocks proinflammatory cytokines in human DCs

We analyzed the expression and function of SIK in human Mo-iDC. SIK1 mRNA levels were markedly lower (~65-fold) in Mo-iDCs than in monocytes, whereas SIK2 mRNA levels were increased (~5-fold), and SIK3 expression was not significantly different in Mo-iDC compared with monocytes. All SIK proteins were markedly increased in Mo-iDC compared with monocytes (Fig. 4A, inset). These results suggest that similar to macrophages, post-translational mechanisms regulate SIK1 and to a lesser extent, SIK3 protein expression in Mo-iDC.

We examined the effect of HG-9-91-01 in human Mo-iDC stimulated with LPS for up to 24 h. SIK inhibition induced a marked decrease in TNF- α and IL-12p70 levels, as well as a significant reduction of IL-1 β levels (~50%) but had no effect on IL-6 secretion. IL-10 levels were increased significantly after 2 h of stimulation (Fig. 4B) but had returned to baseline values at 24 h (data not shown).

We next examined whether SIK inhibition modulates Mo-iDC maturation and/or their ability to induce costimulatory molecules. LPS stimulation for 48 h significantly induced cell surface expression of CD83 and CD86 (Fig. 4C). Culture, in the presence of HG-9-91-01, did not modify the effect of LPS at 48 h on CD83 and CD86 expression, whereas the production of TNF- α was blocked completely by HG-9-91-01 at the same time point (data not shown).

Inhibition of SIK decreases proinflammatory cytokines in myeloid cells upon IL-1R stimulation

Given that the TLRs and IL-1R share common intracellular signaling pathways, we hypothesized that SIK inhibition would likely modulate IL-1R-mediated cytokine production. Human M0-M Φ were stimulated with IL-1 β following 1 h pretreatment with HG-9-91-01. TNF- α and IL-6 production, in response to IL-1 β , was markedly reduced in the presence of HG-9-91-01. In contrast, SIK inhibition did not affect the stimulatory effect of IL-1 β on IL-10 production (Fig. 5A). As in M0-M Φ , TNF- α and IL-6 production was also decreased by HG-9-91-01 in IL-1 β -stimulated Mo-iDC (Fig. 5B). However, in these cells, IL-10 and IL-12p70 levels were very modestly induced only in some donors by IL-1 β stimulation and were not significantly modified by SIK inhibition (data not shown).

SIK inhibition decreases CRT3 and HDAC4 phosphorylation in human macrophages stimulated with LPS or IL-1 β

Previous mechanistic studies in BMDM and RAW264.7 cells identified CRT3 and a class II HDAC4 as direct targets of SIK [16, 33]. Both proteins, when phosphorylated by SIK, are retained in the cytoplasm. Upon their dephosphorylation, they translocate into the nucleus, where CRT3 interacts with CREB to enhance CREB-dependent gene transcription [16], whereas HDAC4 deacetylates p65-NF- κ B, leading to repression of proinflammatory cytokines [33]. Phospho-Ser370 and phospho-Ser162 CRT3 and phospho-Ser246 HDAC4 were identified as SIK-phosphorylated critical residues in these proteins [16, 33]. To confirm that the downstream effects observed with HG-9-91-01

on cytokine modulation in human macrophages correlate with direct SIK target dephosphorylation in these cells, we examined the phosphorylation status of these proteins in LPS- or IL-1 β -stimulated macrophages, 1 h poststimulation (Fig. 6A, inset). Both CRT3 and HDAC4 are highly phosphorylated in basal conditions, as a consequence of high SIK activity in these cells [16, 34]. A modest but significant effect of LPS or IL-1 β stimulation was observed for CRT3. For HDAC4, LPS alone but not IL-1 β also induced a modest but significant dephosphorylation compared with basal condition. Of note, pretreatment with HG-9-91-01 before LPS or IL-1 β stimulation was associated with marked dephosphorylation of CRT3 and HDAC4. However, phospho-Ser370 CRT3 was significantly more dephosphorylated by HG-9-91-01 pretreatment upon LPS stimulation compared with IL-1 β stimulation ($97 \pm 2\%$ by LPS and $82 \pm 14\%$ by IL-1 β stimulation). Phospho-HDAC4 was decreased by $88 \pm 8\%$ upon LPS and by $68 \pm 27\%$ upon IL-1 β stimulation, but the difference was not significant (Fig. 6A, inset).

LPS or IL-1 β stimulation of human macrophages induces a rapid and transient LKB1 phosphorylation

LKB1, also known as STK11, is the master kinase that phosphorylates and activates SIK and all of the other members of the AMPK family except maternal embryonic leucine zipper kinase (MELK) [35]. LKB1 itself can be phosphorylated and activated at multiple sites by different kinases [36]. Thus, we examined in the same cell lysates (1 h poststimulation) whether LPS or IL-1 β stimulation will affect LKB1 phosphorylation. Interestingly, LPS induced a 2.4-fold increase and IL-1 β a 2-fold increase in phospho-Ser428-LKB1 compared with unstimulated control, respectively (Fig. 6B). To analyze this effect in more detail, we performed a time course upon LPS or IL-1 β stimulation. Both stimuli show a similar kinetic and induced a rapid increase in LKB1 phosphorylation, evident after 15 min, which peaked at 30 min and returned to basal levels by 90 min. This increase was not affected by the SIK inhibitor for LPS stimulation (Fig. 6C) or IL-1 β stimulation (data not shown).

It has been reported that when LKB1 is activated through phosphorylation at Ser428 in endothelial cells, this results in binding and phosphorylation of AMPK at Thr172 (the residue in the "T-activation loop," which is highly conserved among the AMPK family members) [37]. Activation of AMPK has also been shown to induce anti-inflammatory effects [38]. Thus, we tested whether LPS or IL-1 β stimulation would induce phospho-Thr172 AMPK and detected a modest increase in phospho-Thr172 AMPK, which was reflected in slightly increased phosphorylation of its substrate ACC (data not shown). The SIK inhibitor HG-9-91-01, in agreement with its *in vitro* kinase selectivity profile, did not significantly affect these phosphorylation/activation events, thus ruling out the possibility that the observed effect of SIK inhibition would be counter-regulated by AMPK signaling modulation (data not shown). Unfortunately, we could not presently address whether the modulation of LKB1 activation induced by LPS or IL-1 β might also have an influence on SIK1–3 activity/phosphorylation, as a result of lack of specific phospho-antibodies for the Thr residues in the T-activation loop of these kinases.

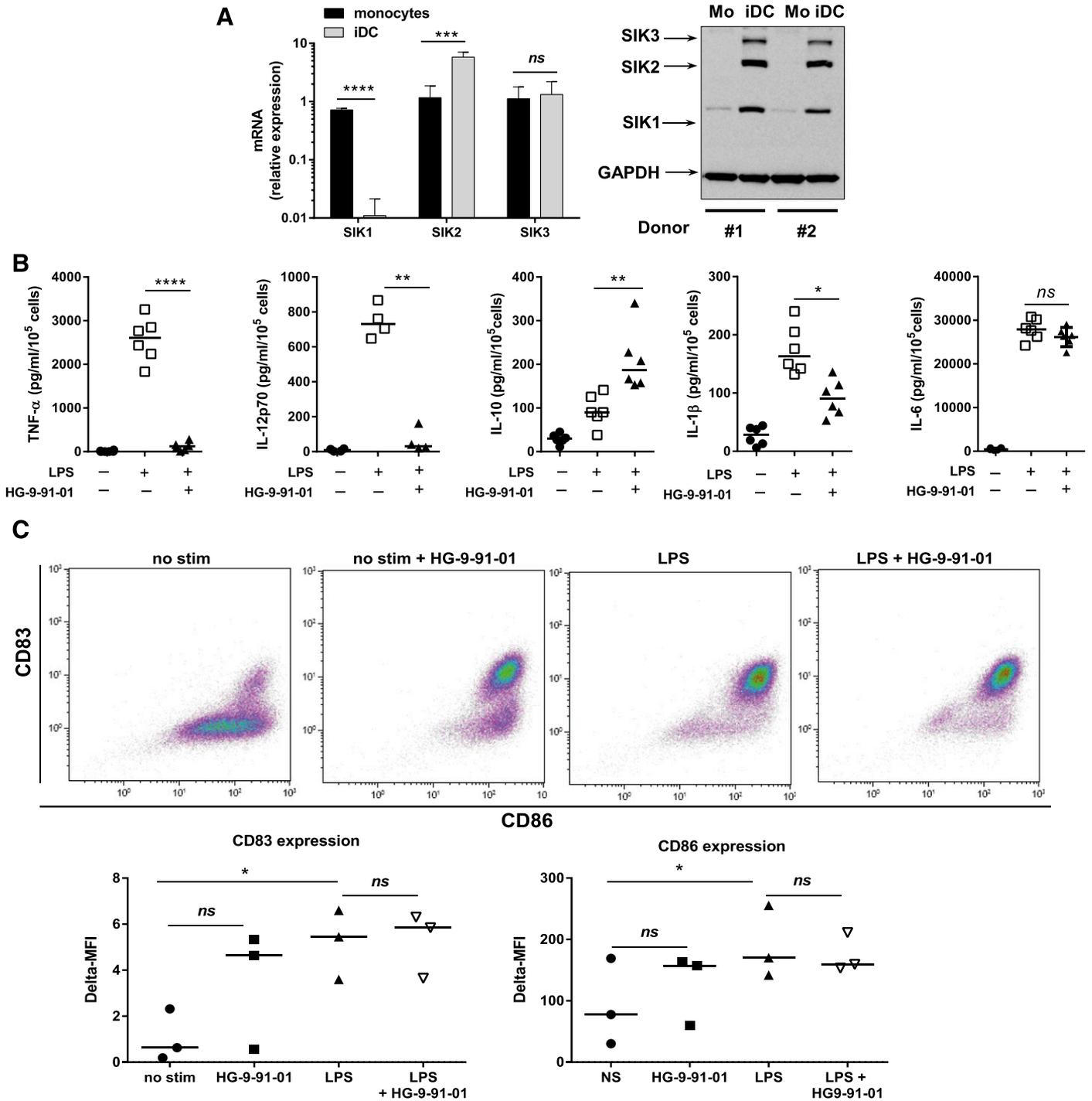


Figure 4. Expression of SIK1–3 and effect of SIK inhibition in human Mo-iDCs. (A) Relative SIK1, SIK2, and SIK3 mRNA levels in freshly isolated human peripheral blood monocytes ($n = 4$) and Mo-iDC differentiated from monocytes for 7 d in the presence of 50 ng/ml each of rhGM-CSF and rhIL-4 ($n = 4$). Gene expression was measured by RT-qPCR using 18s RNA as normalization control and calculated by $2^{-\Delta\Delta C_t}$ method. (Inset) SIK1–3 and GAPDH protein expression in 35 μ g total cell lysate from freshly isolated monocytes (Mo) and immature-DCs (iDC) from 2 donors separated on a 4–12% SDS-PAGE gradient gel. (B) Mo-iDCs were treated for 1 h with vehicle (0.03% DMSO) or 500 nM HG-9-91-01, followed by stimulation with LPS (100 ng/ml). Secreted TNF- α , IL-6, IL-1 β , and IL-12p70 were measured in the supernatants using multiplex immunoassay after 24 h. IL-10 was measured at 2 h postincubation. (C) Mo-iDCs were treated for 1 h with vehicle (0.01% DMSO) or 500 nM HG-9-91-01, followed by stimulation with 100 ng/ml LPS for 48 h, and phenotype was determined by flow cytometry. Delta-MFI, Difference in MFI. One representative donor is shown ($n = 3$). Expression of CD86 and CD83 was measured as detailed in Materials and Methods. For all graphs, statistical significance is reported as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

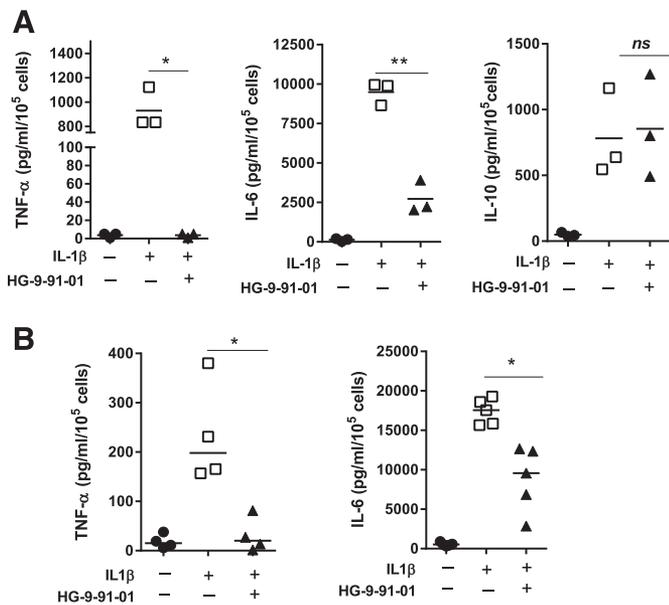


Figure 5. Effect of SIK inhibition on cytokine production and SIK targets phosphorylation in human myeloid cells stimulated with IL-1 β or LPS. (A) Macrophages differentiated from peripheral blood monocytes for 6 d with 100 ng/ml rhM-CSF (M0-M Φ) or (B) Mo-iDC were treated for 1 h with vehicle (0.03% DMSO) or 500 nM HG-9-91-01, followed by stimulation with 10 ng/ml IL-1 β for 6 h. Secreted cytokines were measured in the supernatants using multiplex immunoassay. For all graphs, statistical significance is reported as follows: * $P < 0.05$, ** $P < 0.01$.

Comparison of the effects of SIK inhibition by HG-9-91-01 and ARN-3236 on cytokine levels and SIK targets dephosphorylation

Some experiments were conducted by use of ARN-3236, another pan-SIK inhibitor (U.S. Patent #US20140256704A1), belonging to a different chemical series than HG-9-91-01. In vitro enzymatic potency assays of ARN-3236 toward the 3 SIKs showed that it is a potent inhibitor of SIK2 ($IC_{50} < 1$ nM) and inhibits SIK1 and -3 with IC_{50} 21.63 and 6.63 nM, respectively. The overall selectivity profile of ARN-3236 over 74 kinases is reported in Supplemental Fig. 1A. We tested ARN-3236 in parallel with HG-9-91-01 in RAW264.7 cells stimulated with LPS. ARN-3236 blocked TNF- α secretion with IC_{50} of ~ 2.5 μ M compared with 0.26 μ M IC_{50} and showed by HG-9-91-01, whereas signs of cell toxicity with ARN-3236 ($\sim 30\%$ of remaining viable cells) were present at concentration of 30 μ M (Supplemental Fig. 2A). We cannot exclude that the 20-fold difference in enzymatic potency toward SIK1 inhibition could contribute to the 1-log IC_{50} difference observed between the 2 compounds when tested in parallel (see Supplemental Fig. 2A) in cellular assays. The effect of ARN-3236 was confirmed in human macrophages where a preincubation with 3 μ M ARN-3236 significantly blocked TNF and induced IL-10 upon LPS stimulation ($n = 4$; Supplemental Fig. 2B). In addition, ARN-3236 decreased the production of IL-1 β upon activation of TLR4 and TLR2 signaling (data not shown).

SIK2 activity can be also suppressed by PKA phosphorylation following incubation of macrophages with ligands that elevate the intracellular concentration of cAMP, such as PGE $_2$ [34], thus mimicking the effect of the inhibitors. We compared in

LPS-stimulated human macrophages of the same donor the effect of the 2 SIK inhibitors and of PGE $_2$ stimulation on CRTC3 and HDAC4 phosphorylation. Supplemental Fig. 2C shows that HG-9-91-01 and ARN-3236 or PGE $_2$ induced a robust dephosphorylation of CRTC3 and HDAC4.

siRNA-mediated SIK1–3 knockdown sensitizes human macrophages to HG-9-91-01

To complement the observations obtained using SIK inhibitors, we studied the effect of RNAi of SIK1–3 on cytokine production. Transient transfection of human M0-M Φ with siRNA for SIK1, SIK2, and SIK3 showed that we could achieve an almost complete knockdown of protein levels of SIK3 ($\geq 90\%$ reduction) and SIK2 ($\geq 80\%$ reduction), whereas SIK1 was only partially affected by $\sim 20\%$ at 48 h post-transfection (Supplemental Fig. 4B). The latter can be the consequence of the observed discrepancy between mRNA and protein SIK1 levels in these cells (Fig. 1A), suggesting that SIK1 protein expression is likely more difficult to knock down in a transient transfection setting. Nevertheless, we observed that reduced SIK protein expression could sensitize human macrophages to a suboptimal concentration of HG-9-91-01 (now used at 100 nM) by inducing, respectively, a 2-fold increase of IL-10 and a 40% decrease of TNF- α secretion over LPS-stimulated macrophages in SIK1–3 siRNA-treated cells compared with scramble siRNA (Supplemental Fig. 4A). The reduction of SIK expression also decreased the level of phospho-HDAC4 and phospho-CRTC3 in the absence of inhibitor (Supplemental Fig. 4B, lanes 1 vs. 4 and lanes 2 vs. 5).

DISCUSSION

It is critically important to test the influence of SIK inhibition in human cells to establish its clinical relevance. In this study, we provided a detailed characterization of the expression and the function of SIK in human primary myeloid cells. We expanded previous literature data, which were mainly obtained in mouse cells, by showing that SIK inhibition synergizes with TLR signaling to block proinflammatory cytokine production and increase IL-10 secretion in human monocytes, macrophages, and DCs. Moreover, we demonstrate for the first time that SIK inhibition significantly reduced IL-1 β -mediated production of proinflammatory cytokines by macrophages and DCs.

The importance of the function of SIK in myeloid cells is underlined by the fact that differentiation from peripheral blood monocytes to macrophages or DCs induces an overall marked up-regulation of SIK protein expression. Of note, human monocytes are still significantly affected by SIK inhibitors, albeit to a lesser extent for IL-10 production following TLR stimulation. This finding likely reflects the relatively lower expression of SIK in monocytes.

Overall, the effects of SIK inhibitors on cytokines production in human myeloid cells are in line with those obtained in mouse cells [16, 21]. However, we detected some differences between the 2 species. SIK inhibitors induce an increase in IL-1Ra mRNA production in BMDMs but not in human macrophages.

Taken together, our results suggest that the effect of SIK inhibition on human M(LPS + IFN- γ)-polarized macrophages is

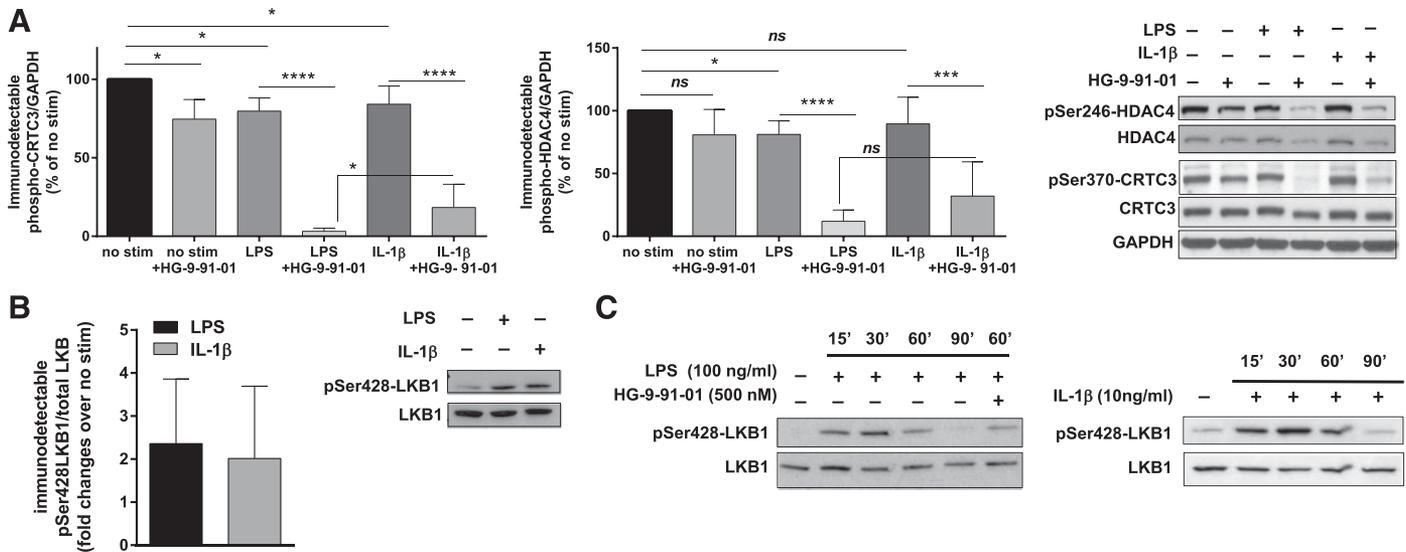


Figure 6. LPS or IL-1 β stimulation of human macrophages induces a rapid and transient LKB1 phosphorylation. (A) Comparison of the effect of SIK inhibitor HG-9-91-01 on CRTC3 and HDAC4 phosphorylation upon LPS (100 ng/ml) or IL-1 (10 ng/ml) stimulation. Human macrophages (M0-M Φ) were preincubated with vehicle (0.006% DMSO) or with 500 nM HG-9-91-01 for 1 h, followed by 1 h stimulation with 100 ng/ml LPS or 10 ng/ml IL-1 β . Phospho-Ser370 CRTC3 and phospho-Ser246 HDAC4 were assessed in 35–40 μ g cell lysates, separated by SDS/PAGE, followed by immunoblotting with specific phosphoantibodies or GAPDH. Membranes were stripped and reprobed with anti-CRTC3 and anti-HDAC4, respectively. Phosphoprotein levels were quantified relative to GAPDH and represented as a ratio of their respective unstimulated controls. Inset depicted is 1 representative donor ($n = 6$). (B) Human macrophages (M0-M Φ) were stimulated with 100 ng/ml LPS or 10 ng/ml IL-1 for 1 h ($n = 5$). Error bars = means \pm SD. Phospho-Ser428-LKB1 levels were assessed in 35–40 μ g cell lysates, separated by SDS/PAGE, followed by immunoblotting. Membranes were stripped and reprobed with anti-LKB1. The phosphoprotein levels were quantified relative to total LKB1 and represented as a ratio of the unstimulated control. (Inset) Depicted is 1 representative donor. (C) Time course of LKB1 phosphorylation induced by LPS or IL-1 β stimulation of human macrophages. Cells were treated as above and stimulated for the indicated time points in the absence or presence of the SIK inhibitor (500 nM, 1 h preincubation before stimulation). (Inset) Depicted is 1 representative donor ($n = 4$). * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

to convert them to an anti-inflammatory phenotype characterized by TNF^{low}, IL-12^{low}, IL-10^{high}, and modest expression of IL-6 and IL-1 β , which is reminiscent of the M(LPS + IgG)-stimulated macrophages. This conclusion is supported by the fact these macrophages are typically IL-10^{high}/IL-12^{low} but still retain some level of production of proinflammatory cytokines (e.g., IL-6 and IL-1 β) [32]. Our analysis of 2 human M(IL-4) markers (i.e., CD200 and CCL22) shows that consistent with findings in the mouse system, SIK inhibition is not associated with this phenotype.

Human DCs (Mo-iDC) are also significantly affected by SIK inhibition, showing an anti-inflammatory phenotype characterized by profound and sustained down-regulation of IL-12p70 and TNF- α and a more transient increase in IL-10. However, these changes do not impact the ability of iDC to mature and to express costimulatory molecules and thus, do not represent a nonspecific disruption of DC function.

The effect of SIK inhibition on production of proinflammatory cytokines was robust and sustained (still highly significant at 24 h), whereas the increase in IL-10 production in macrophages was very rapid and more transient (significant up to 4–6 h). The latter likely reflects the fact that SIK inhibition induces a very rapid and robust increase in IL-10 mRNA levels, which return to basal levels by 4 h [16]. IL-10 production is induced by several transcription factors and is tightly regulated at multiple checkpoints [39]. Previous genetic analysis that uses siRNA for CRTC3 or constitutive-active CRTC3 mutants demonstrated its

requirement for up-regulation of IL-10 and other markers in mouse macrophages upon its interaction with phosphorylated CREB in the nucleus [16, 19]. It is noteworthy that the NF- κ B p65 subunit binds a site in the IL-10 locus (4.5 kb upstream of the IL-10 start site) and induces IL-10 expression in mouse macrophages [40]. The latter could also contribute to the production of IL-10 upon LPS stimulation in the absence of SIK inhibitors. SIK inhibition leads to the dephosphorylation of HDAC4 and its translocation to the nucleus where it deacetylates NF- κ B p65, leading to repression of inflammatory genes (i.e., TNF- α and IL-12), as was demonstrated by chromatin immunoprecipitation assays in mouse BMDM [33]. It is tempting to suggest that the same mechanism responsible for proinflammatory cytokine repression could also later impact IL-10 transcription.

IL-1 β -mediated production of proinflammatory cytokines is significantly impacted by SIK inhibition. Whether SIK inhibitors similarly inhibit the effect of other cytokines (i.e., IL-18 and IL-33) of the IL-1 family, which signals through the MyD88 pathway is an intriguing possibility that remains to be investigated. Of note, whereas SIK inhibition efficiently blocked proinflammatory cytokines (i.e., TNF- α and IL-6), there was no significant increase of IL-10 by the SIK inhibitor as it occurs for TLR-mediated responses. A possible explanation may reside in the different extent of dephosphorylation of SIK substrates following pre-treatment with the SIK inhibitor, which was more pronounced for CRTC3 upon TLR4 compared with IL-1R stimulation. This would suggest that a full dephosphorylation and maximal translocation of

CRTC3 are needed to induce the further up-regulation of IL-10, whereas even a partial dephosphorylation/translocation of HDAC4 is sufficient to impact proinflammatory cytokine repression.

According to the current accepted model, SIKs are highly active enzymes in resting macrophages, as they are activated by the constitutively active “master kinase” LKB1, which phosphorylates them at a conserved Thr residue [19, 35]. LKB1 itself can be phosphorylated and activated at multiple sites by different kinases, and this could potentially alter its *de novo* activity and ability to interact with its substrates [36]. We demonstrated that LPS and IL-1 β induced a rapid and transient LKB1 phosphorylation at Ser428. Although, the precise mechanism(s) and molecular intermediate(s) for increased LKB1 activation in this setting remain to be elucidated, it suggests that the LKB1–SIK pathway is stimulated by LPS or IL-1 β treatment.

Our RNAi results recapitulate the effect observed with SIK inhibitors, confirming the specificity of the effect of these small molecules. However, whereas the siRNA-mediated SIK knock-down was affecting the extent of phosphorylation of direct SIK substrates compared with scramble siRNA, the effect on cytokine production in the same samples was not significant, and the use of a suboptimal dose of compound was required to achieve significant changes in cytokines levels. The latter was also true, not only in our transient RNAi settings but also with the use of short hairpin RNA in mouse cells [16]. A possible explanation of this effect is the kinase-independent role of SIK3 and SIK1, which has been shown to regulate TLR4 receptor signaling negatively via interaction with the TAK1–TAK1-binding protein 2–TNFR-associated factor 6 complex. This work suggested that knockdown of SIK1 and/or -3 protein but not SIK2 would actually increase proinflammatory cytokines [41]. In line with this, a recent paper demonstrated that SIK3 protein deficiency results in elevated levels of proinflammatory cytokine production by macrophages, whereas SIK3 overexpression would decrease proinflammatory cytokines. In addition, a prolonged (16 h) LPS stimulation of RAW264.7 cells results in a significant increase in SIK3 but not SIK1 or -2 mRNA [42]. Our results in M(LPS + IFN- γ)-polarized cells suggest indeed that SIK3 protein levels and not SIK1 and -2 are modulated by LPS + IFN- γ stimulation. Thus, it is likely that in the initial phase of the innate immune response, higher LKB1–SIK activity favors the increase of proinflammatory mediators required for optimal defense against pathogens. In a later phase, the production of other mediators (e.g., PGE₂) would inhibit SIK activity, and the increase of SIK3 expression via its kinase-independent effects would repress the production of proinflammatory cytokines and induce IL-10. In line with this hypothesis, it was suggested that 2 stimuli are needed to induce the anti-inflammatory activity of macrophages. The first signal (e.g., PGE₂, immune complexes, apoptotic cells) has little anti-inflammatory function on its own [34]; however, when combined with a second stimulus, such as a TLR ligand, these 2 signals would “reprogram” the macrophages to produce increased levels of IL-10 [31, 34].

To demonstrate the effect of SIK inhibitors on the direct kinase targets, CRTC3 and HDAC4, we used 2 structurally unrelated pan-SIK inhibitors (HG-9-91-01 and ARN-3236). We showed that both molecules are able to dephosphorylate these

substrates in human macrophages in a way comparable with that observed with a physiologic stimulus, such as PGE₂. The effect of PGE₂ stimulation suggests that SIK inhibition is a crucial checkpoint in the TLR–G protein-coupled receptor crosstalk during the modulation of the innate immune response. It is likely that other receptors (e.g., adenosine, β -adrenergic receptors) or cAMP-inducing agents (i.e., phosphodiesterase inhibitors), which also signal through cAMP/PKA, exert their known anti-inflammatory effect, at least partially, by inhibiting SIK. In line with this, 1 of the proposed mechanisms of anti-inflammatory action of methotrexate is to increase local extracellular adenosine levels [43]. Salbutamol (a β ₂-adrenergic receptor agonist) is a potent suppressor of established collagen-induced arthritis [44], and Apremilast (a phosphodiesterase inhibitor) has recently been approved for the treatment of psoriatic arthritis [45]. If proven, this link will further, strongly support the use of selective SIK inhibitors as therapeutic molecules. In this respect, our demonstration that SIK inhibition impacts not only TLR- but also IL-1-mediated signaling in human myeloid cells further expands the potential therapeutic implications of the use of SIK inhibitors for the treatment of immune-mediated inflammatory diseases. It cannot be excluded that as observed during anti-cytokine therapy (e.g., anti-TNF- α), an increase of IL-10 associated with a decrease in TNF- α and other proinflammatory cytokines following SIK inhibition will increase the susceptibility to infectious complications. However, our data suggest that the macrophage phenotype after SIK inhibition is not completely immunosuppressive, as some levels of IL-6 and IL-1 β are maintained, as shown for “regulatory-like macrophages” [32]. Interestingly, regulatory macrophages have been shown to produce NO [31], suggesting that they can retain their capacity to limit the development of intracellular infections.

AUTHORSHIP

M.S.L. designed the research, performed experiments, supervised the project, and wrote the manuscript. C. Gilliéron performed the experiments and analyzed the data. D.D. performed the FACS experiments and analyzed the data. C. Gabay supervised the project and contributed to writing the manuscript.

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DISCLOSURES

All of the authors declare no conflicts of interest.

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