Salt-Inducible Kinases (SIK) inhibition in human myeloid cells modulates TLR and IL-1R signaling and induces an anti-inflammatory phenotype

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Background

Salt **I**nducible **K**inases (**SIKs**) constitute a serine/threonine kinase subfamily, belonging to the AMP-activated protein kinase (**AMPK**) family. Three members: SIK1, 2 and 3 have been identified so far. Experiments performed primarily in mouse cells showed that SIKs synergize with Toll-like receptors (**TLRs**) signaling to restrict the formation of regulatory macrophages involved in the resolution of inflammation via the production of high levels of anti-inflammatory **IL-10** and low levels of proinflammatory **IL-12** and tumor necrosis factor (**TNF-α**) cytokines. Mechanistically, pharmacological inhibition of SIKs or PGE_2/PKA-mediated effect leads to de-phosphorylation and nuclear translocation of **CREB transcriptional co-activator** (**CRTC3**) and **class II histone deacetylase HDAC4**, respectively. CRTC3 interacts with phospho-CREB to promote a gene expression program including strong up-regulation of IL-10, whereas HDAC4 deacetylates **p65-NF-κB** leading to repression of pro-inflammatory cytokines (*Clark K. et al PNAS, 2012 ; McKenzie KF. et al, JI, 2013 , Luan B. et al, Cell metabolism 2014)* (**Fig. 1**). However, the expression and function of SIKs in primary human myeloid cells are poorly characterized. It is critically important to test the influence of SIK inhibition in human cells to establish its clinical relevance. Both Toll-like receptors (TLRs) and interleukin-1 receptor (IL-1R) share common intracellular signaling pathways. Given the important role of IL-1 in inflammatory diseases, we also examined whether SIK inhibition was able to impair IL-1β-mediated cytokine production in human myeloid cells.

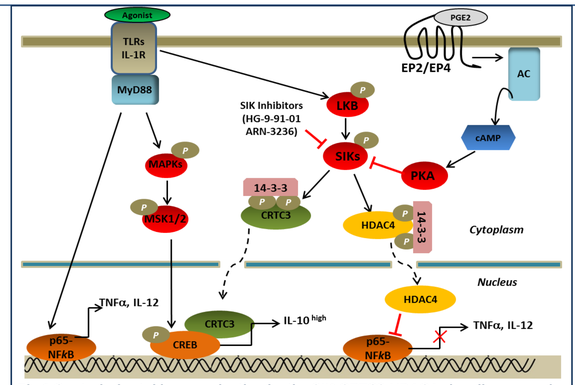


Fig.1 Control of cytokines production by the SIK-CRTC3-HDAC4 signaling cascade.

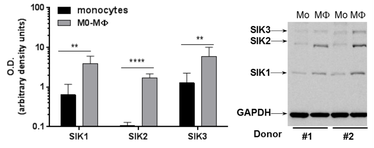
Objectives

- **To examine the regulation of expression and function of SIKs in human monocytes and monocyte-derived macrophages (MDM) and dendritic cells (MDC) in culture**
- **To profile small molecule SIK inhibitors in human myeloid cells and characterize the signaling pathways**

Methods

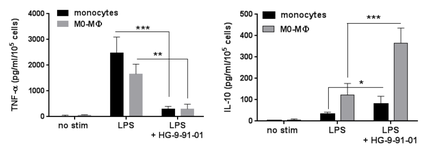
We used two structurally unrelated small molecule SIK inhibitors: HG-9-91-01 and ARN-3236 (Arrien Pharmaceuticals). Human monocytes were differentiated for 6 days with 100 ng/ml rhM-CSF to generate macrophages (M0-MΦ) or with 50 ng/ml GM-CSF+50ng/ml IL-4 to generate immature dendritic cells (iDC). Cells were pretreated one hour with SIK inhibitors or vehicle before challenge with TLR4 (LPS, 100 ng/ml) or IL-1β (10 ng/ml) for 3 to 24h. Total mRNA was isolated and gene expression determined by RT-qPCR and normalized using 18s RNA and calculated by $2^{-\Delta\Delta Ct}$ method. Cell lysates were separated by 4-12% PAGE-SDS gradient gel and analysed by immunoblotting for the expression of SIK kinases family members (SIK 1-3) or using specific phospho-antibodies for CRTC3, HDAC4, LKB1, AMPK and ACC. Cytokines secreted in the supernatants were determined by ELISA or multiplex immunoassays. Quantitative data are presented as the mean ±SD. Statistical differences were assessed by Student *t* test and considered significant if $p<0.05$.

Fig.2 Changes in SIK 1-3 expression during monocytes differentiation to macrophages



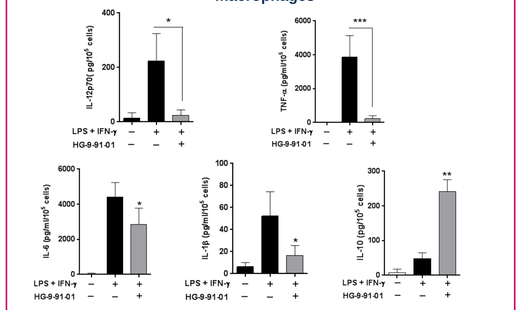
Protein expression levels of SIK1-3 in cell lysates from freshly isolated human monocytes (Mo, n=8) and in macrophages (M0-MΦ, n=6). Inset, representative example from two donors depicting SIK1-3 and GAPDH expression in 35 μg of total cell lysate

Fig.3 SIK inhibition decreases TNF-α and increases IL-10 secretion by human monocytes and macrophages stimulated with TLR4 agonist



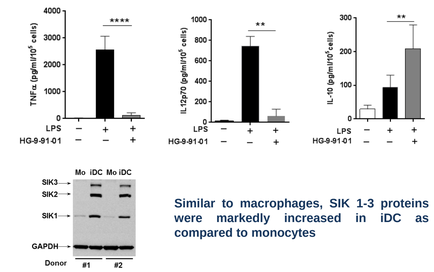
Cytokines production measured from freshly isolated human monocytes or macrophages differentiated for 6 days with 100ng/ml M-CSF (M0-MΦ, n=5) and incubated 1h with vehicle (DMSO) or 500nM HG-9-91-01 and then stimulated for 3 h with 100 ng/ml LPS.

Fig.4 SIK inhibition down-regulates proinflammatory cytokines and up-regulates IL-10 in M1 (LPS+IFN-γ)-polarized human macrophages



M0-MΦ were differentiated from monocytes as described in Methods. M0-MΦ were treated for 1h with vehicle (DMSO) or 500nM HG-9-91-01 followed by stimulation for 24h with LPS (100 ng/ml) and IFN-γ (20ng/ml) to drive M1-like polarization (LPS+IFNγ-MΦ). Cytokines were measured in supernatants.

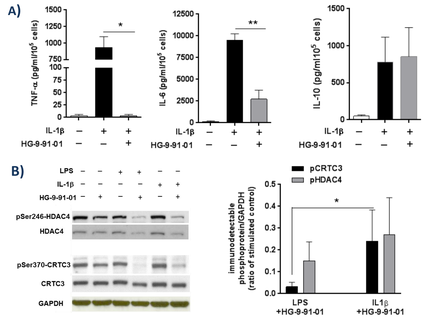
Fig. 5 SIK inhibition down-regulates proinflammatory cytokines and up-regulates IL-10 in human dendritic cells (iDC)



Similar to macrophages, SIK 1-3 proteins were markedly increased in iDC as compared to monocytes

iDC were differentiated from monocytes as described in Methods and were treated for 1 h with vehicle (DMSO) or 500 nM HG-9-91-01 followed by stimulation for LPS (100 ng/ml). Cytokines were measured in supernatants. Inset, representative example from two donors depicting SIK1-3 and GAPDH protein expression in total cell lysate from monocytes (Mo) vs. iDC (n=4).

Fig. 6 Effect of SIK inhibition on cytokine production and SIK targets phosphorylation in human macrophages stimulated with IL-1β or LPS

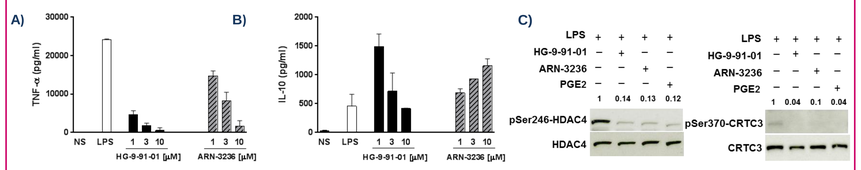


In response to IL-1β stimulation, HG-91-91-01 markedly reduced TNF-α and IL-6 production but did not affect IL-10 production

Downstream effects observed with HG-9-91-01 on cytokine modulation in human macrophages correlate with direct SIK targets dephosphorylation in these cells.

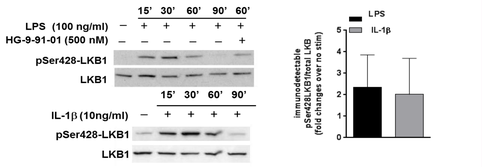
A) *M0-MΦ were treated for 1h with vehicle (DMSO) or 500nM HG-9-91-01 followed by stimulation with 10 ng/ml of IL-1β for 6h. Secreted cytokines were measured in the supernatants.* ***B)*** *M0-MΦ were treated as indicated for 1h. Phospho-Ser370 CRTC3 and phospho-Ser246 HDAC4 were assessed by immunoblotting and quantified relative to GAPDH and ratio calculated relative to stimulated controls is represented as bar graph.*

Fig. 7 Comparison of the effects of SIK inhibition by HG-9-91-01 and ARN-3236 on cytokines and SIK substrates phosphorylation



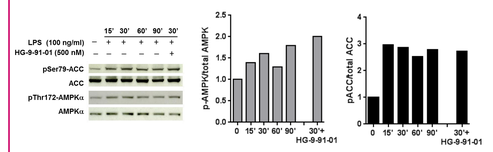
A) *Levels of TNF-α and IL-10 in human M0-MΦ preincubated with vehicle (DMSO), increasing concentrations of HG-9-91-01 or ARN-3236, and stimulated with 100 ng/ml of LPS for 3h.* ***B)*** *M0-MΦ were preincubated with vehicle or with 0.5 μM HG-9-91-01 or 10 μM ARN-3236 for 1h or with 10 μM PGE2 (for 5 min) followed by 1h stimulation with 100 ng/ml LPS.* ***C)*** *pSer370 CRTC3 or pS246 HDAC4 assessed by immunoblotting with specific phosphoantibodies. Phosphoprotein were quantified relative to total CRTC3 or HDAC4 and represented as ratio of their respective stimulated controls.*

Fig. 8 LPS or IL-1β stimulation of human macrophages induces a rapid and transient LKB1 phosphorylation



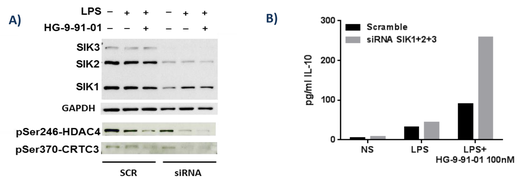
Time course of LKB1 phosphorylation induced by LPS or IL-1β stimulation of human macrophages. Cells were treated and stimulated for the indicated time points in absence or presence of SIK inhibitor (1h preincubation before stimulation). Inset depicts one representative donor (n=4).

Fig. 9 HG-9-91-01 does not affect AMPK activity



Cells were stimulated for the indicated time points in absence or presence of SIK inhibitor (1h preincubation before stimulation). Inset depicts one representative donor (n=4). Levels of phospho-AMPK and phospho-ACC were determined using specific phosphoantibodies, quantified relative to total AMPK or ACC and ratio calculated relative to unstimulated control is represented as bar graph.

Fig. 10 siRNA knockdown of SIKs sensitizes macrophages to a suboptimal concentration of HG-9-91-01



300pM each of scramble (SCR) or SIK1, 2 and 3 siRNA were used for RNAi. 48h later, cells were incubated for 1h with 100nM of HG-9-91-01 and stimulated for 3h with 100ng/ml LPS. ***A)*** *Protein abundance of SIK1, SIK2, SIK3, pSer370-CRTC3 and pSer246-HDAC4 were assessed by immunoblotting.* ***B)*** *Levels of IL-10 were measured in the supernatants. Depicted is one representative donor (n=4).*

Summary

- **Differentiation from peripheral blood monocytes to macrophages or iDCs, induces overall increase of SIK1-3 protein expression.**
- **SIK inhibition synergizes with TLRs signaling to block pro-inflammatory cytokines production and increase IL-10 secretion in human monocytes, macrophages and dendritic cells.**
- **The effect of SIK inhibition on human M1-(LPS-+IFN-γ) polarized macrophages is to convert them to anti-inflammatory phenotype characterized by TNF-α^{low}, IL-12^{low}, IL-10^{high} and modest expression of IL-6 and IL-1β, which is reminiscent of the "regulatory" macrophages phenotype.**
- **We demonstrate for the first time that SIK inhibition significantly reduces IL-1β-mediated production of pro-inflammatory cytokines by macrophages and dendritic cells.**
- **Two structurally unrelated pan-SIK inhibitors, HG-9-91-01 and ARN-3236, are able to dephosphorylate direct SIK substrates (CRTC3 and HDAC4) in human macrophages in a way comparable to that observed with a physiological stimulus (e.g. PGE_2) and have an impact on cytokines production.**

Conclusion & Next Steps

- **Overall, our analysis on the effects of SIK inhibitors on cytokines production in human myeloid cells are in line with those obtained in mouse cells.**
- **Our demonstration that SIK inhibition impacts not only TLR-, but also IL-1R-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.**
- **Our validation of ARN-3236 in these settings supports its use in *in vivo* disease models of inflammatory diseases.**

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NOVARTIS Novartis Stiftung für medizinisch-biologische Forschung

iAR Arthritis Research