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LACK OF PROTEIN PRENYLATION PROMOTES NLRP3 INFLAMMASOME ASSEMBLY IN HUMAN MONOCYTES

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Keywords: mevalonate kinase deficiency, HIDS, statin, prenylation, inflammasome, NLRP3, Pyrin, ASC, Caspase-1, IL-1 β , IL-18.

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Summary

We demonstrate that blocking the mevalonate pathway and protein prenylation in human monocytes leads to enhanced activity of an NLRP3-dependent, but not Pyrin-dependent, inflammasome, suggesting a prominent role for NLRP3 in the pathogenesis of mevalonate kinase deficiency.

To the Editor:

Mevalonate kinase deficiency (MKD, also known as hyper-IgD syndrome/HIDS) is a rare autoinflammatory disorder caused by recessive, hypomorphic mutations in the *MVK* gene¹. Impaired activity of mevalonate kinase, a crucial component of the mevalonate-cholesterol biosynthesis pathway, leads to decreased production of isoprenoid lipids, defective post-translational protein prenylation, and the cytosolic accumulation of unprenylated Rab proteins and other small GTPases in MKD patient cells². It has been proposed that excessive production of IL-1 β from monocytes of MKD patients is caused by loss of protein prenylation, specifically geranylgeranylation³. In this setting, defective protein prenylation promotes inflammasome activation and cleavage of pro-IL1 β , thereby triggering systemic inflammatory flares. However, the precise underlying mechanisms, including the type of inflammasome that is activated, remain unclear. Recently, two studies suggested that loss of protein prenylation in macrophages leads to activation of the Pyrin inflammasome, either via mechanisms involving loss of prenylated K-Ras⁴ or loss of prenylated Rho GTPase⁵. However, other inflammasomes could feasibly also play a role, since cholesterol homeostasis is linked to NLRP3 inflammasome activation⁶. To further clarify this issue we pharmacologically inhibited the mevalonate pathway in human THP-1 monocytic cells with simvastatin and then stimulated the cells with bacterial lipopolysaccharide (LPS), an archetypal inflammatory stimulus that acts via TLR4. In THP-1 cells and human peripheral blood mononuclear cells (PBMCs), blocking protein prenylation with statins or other pharmacologic agents such as GGTI-298 prior to LPS stimulation mimics the excessive release of IL-1 β from PBMCs of MKD patients^{3,7}.

First, we used an *in vitro* prenylation assay and western blotting to identify a concentration of simvastatin (SIM) that profoundly inhibited Rab and Rap1A prenylation in THP-1 cells without causing loss of cell viability (Supplementary Fig. 1A,B). Pretreatment with 5 μ M SIM for 24 hours was used thereafter and this substantially increased IL-1 β release after LPS stimulation (Fig. 1A). Cells with an ASC-containing inflammasome “speck” were virtually absent in THP-1 cells in the absence or presence of SIM alone (0.6% and 1.6% of cells, respectively). However,

SIM pretreatment significantly increased the proportion of speck-containing cells after 4 hours' LPS stimulation, from 30% to 45% (Fig. 1B). Similarly, SIM boosted LPS-induced Caspase-1 activity and pyroptotic cell death (Fig. 1C and Supplementary Fig. 1C). This increase in inflammasome formation, Caspase-1 activation and pyroptosis was accompanied by a quadrupling of LPS-induced IL-1 β (and a doubling of IL-18) release into the extracellular medium from SIM-pretreated cells (Fig. 1D).

SIM prevents protein prenylation by blocking the synthesis of essential isoprenoid lipids, particularly geranylgeranyldiphosphate. This can be overcome by addition of exogenous geranylgeraniol (GGOH) to allow normal protein geranylgeranylation. Supplementing THP-1 cells with 50 μ M GGOH completely abolished the synergistic effect of SIM on LPS-induced inflammasome formation, Caspase-1 activation, pyroptosis and IL-1 β and IL-18 release (Fig. 1B-D and Supplementary Fig. 1C). We confirmed that GGOH prevented the accumulation of unprenylated Rab and Rap1A proteins in SIM-treated THP-1 cells (Fig. 1E), supporting the concept that SIM exacerbates inflammasome activation as a result of loss of protein prenylation rather than via any off-target effects.

To determine the requirement for NLRP3 or Pyrin in SIM-enhanced inflammasome formation we used THP-1 cells in which the *NLRP3*, *MEFV* or *CASP1* genes were disabled by doxycycline-inducible CRISPR/Cas9 gene editing⁸ (Fig. 1F). NLRP3 or Caspase-1 deficiency abolished LPS-induced IL-1 β release and completely prevented the synergistic effect of SIM pretreatment, but Pyrin deficiency did not affect IL-1 β production under any conditions (Fig. 1G). Furthermore, NLRP3 deficiency in THP-1 cells also ablated LPS-induced ASC speck formation and Caspase-1 activity, with and without SIM pretreatment (Supplementary Fig. 1D,E). Unlike Caspase-1 deficiency (Fig. 1G), lack of Caspase-4/5 had no effect on IL-1 β release (Supplementary Fig. 1F), indicating that SIM did not promote a non-canonical inflammasome pathway. Consistent with a requirement for the NLRP3 inflammasome, we found that preventing K⁺ efflux by using high extracellular [K⁺], or neutralising ROS, completely blocked the synergistic effect of SIM (Supplementary Fig. 1G,H). Together, these observations clearly demonstrate that loss of prenylation in human THP-1 monocytes enhances the LPS-induced formation of an NLRP3-dependent, and Pyrin-independent, inflammasome.

We next examined whether pharmacological inhibition of NLRP3 could prevent the excessive inflammasome activation caused by defective protein prenylation in THP-1 cells. Treatment with the small-molecule NLRP3 inhibitor MMCC950⁹, completely blocked LPS-induced Caspase-1 activation (Fig. 1H) and IL-1 β release (Fig. 1I) and pyroptosis (Supplementary Fig. 1C) in the

presence or absence of SIM. Similarly, MCC950 significantly inhibited the elevated IL-1 β release from THP-1 cells pretreated with GGTI-298 (Supplementary Fig. 1I). Finally, we tested the effect of MCC950 on PBMCs isolated from a patient with a mild form of MKD/HIDS (compound heterozygous *MVK*^{V377I/H20N} mutations) and from both parents (heterozygous for either *MVK*^{V377I+} or *MVK*^{H20N/+}). We have previously shown that protein prenylation is defective in PBMCs from this patient but not in either parent². Stimulation with LPS and the NLRP3 activator nigericin elicited much greater IL-1 β release in cells from the MKD patient than from either parent, and this response was abolished by MCC950 (Fig. 1J).

Our observations clearly demonstrate that lack of prenylation in monocytic cells (using statin treatment - a pharmacologic model of MKD - or using authentic MKD cells from a patient) leads to enhanced formation of an NLRP3-dependent, Pyrin-independent, inflammasome upon LPS stimulation. Whilst loss of prenylation has been suggested to activate the pyrin inflammasome in mouse bone marrow-derived macrophages^{4,5}, it is possible that defective prenylation promotes activity of distinct inflammasomes in different cell types. In monocytes, loss of protein prenylation caused by SIM treatment was alone insufficient for inducing NLRP3 inflammasome formation or Caspase-1 activation (Fig. 1B,C) but substantially boosted LPS-induced inflammasome activity (speck formation, Caspase-1 activation, IL-1 β and IL-18 release, and pyroptosis) upon stimulation with LPS (Fig. 1B-D, Supplementary Fig. 1C). The underlying mechanism remains to be clarified but did not involve an enhancement of LPS-induced changes in mRNA levels (Supplementary Fig. 2A) or changes in protein levels (Supplementary Fig. 2B) of pro-IL-1 β , pro-IL-18, NLRP3, ASC or pro-Caspase-1. Enhanced inflammasome formation in prenylation-deficient THP-1 cells also occurred after stimulation with the TLR2 agonist Pam3CSK4 (Fig. 1K), consistent with the enhanced sensitivity of MKD patient PBMCs to both TLR2 and TLR4 agonists¹.

These findings are of clinical relevance because they demonstrate that targeting the Pyrin inflammasome in isolation may not be sufficient to resolve all the pathology associated with MKD. Rather, approaches to overcome the metabolic defect in the mevalonate pathway and restore normal protein prenylation could be more effective at preventing broader inflammasome activation.

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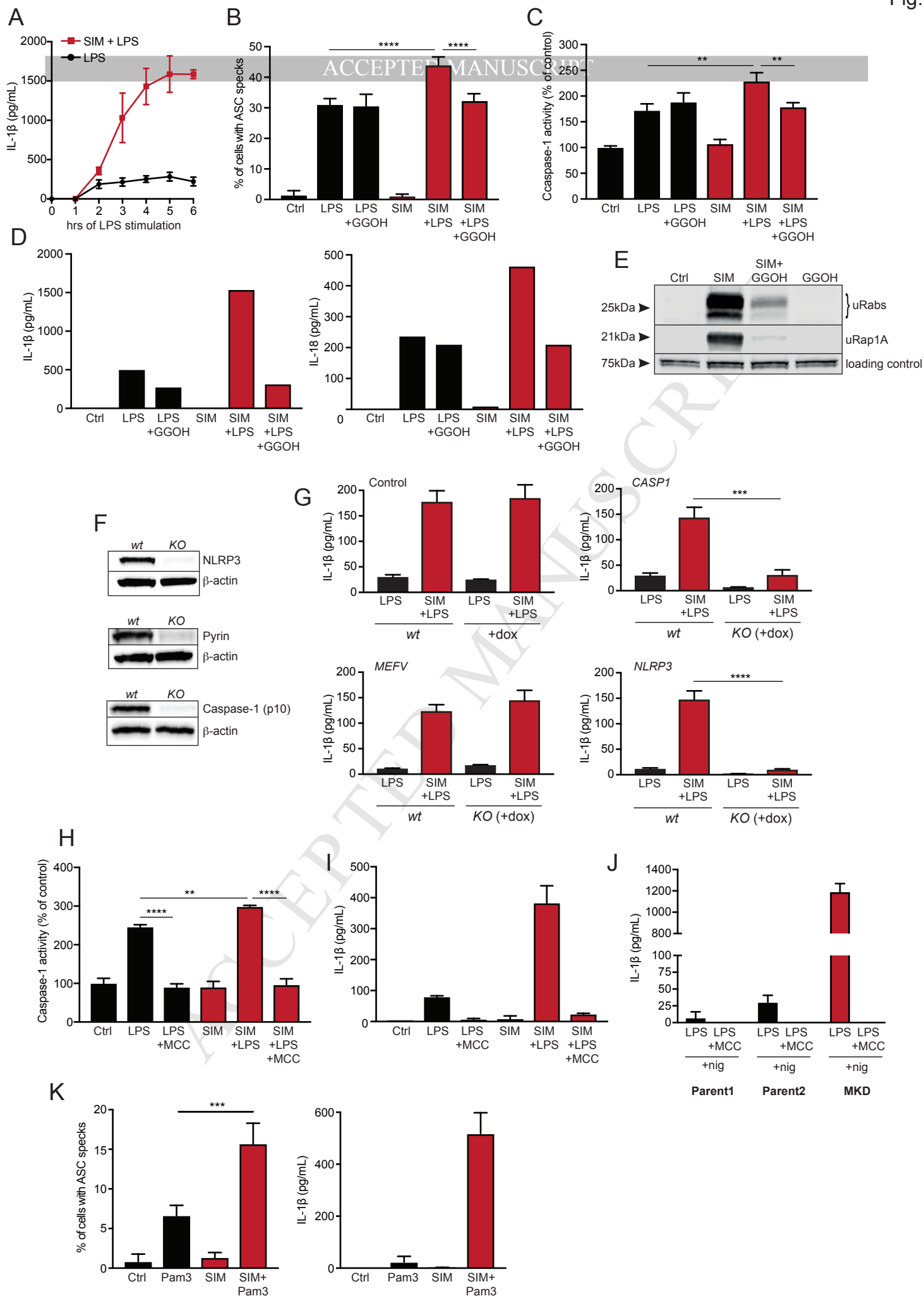
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Figure Legends

Figure 1. SIM pre-treatment enhanced LPS-induced IL-1 β release (A), ASC-speck formation (B), Caspase-1 activity (C) and IL-18 release (D), all of which were restored to control levels in the presence of GGOH (A-D). Data in (D) are from one representative experiment of n>4 experiments (IL-1 β) or n=2 experiments (IL-18). (E) GGOH prevented SIM-induced accumulation of unprenylated Rab (uRabs) and unprenylated Rap1A (uRap1A) proteins. (F) Doxycyclin-inducible knockout of NLRP3, Pyrin or Caspase-1 proteins in Cas9-expressing THP-1 cells. (G) Lack of *NLRP3* or *CASP1*, but not *MEFV* (Pyrin) abolished LPS-induced IL-1 β release and prevented the enhancing effect of SIM. MCC950 inhibited LPS-induced Caspase-1 activity (H) and IL-1 β release (I), and blocked the enhancing effect of SIM in THP-1 cells. (J) Stimulation with LPS/nigericin caused elevated IL-1 β release in a MKD patient's PBMCs compared to PBMCs from each unaffected parent, and MCC950 completely abolished IL-1 β production in all PBMC cultures. (K) SIM pre-treatment enhanced the formation of ASC specks and IL-1 β release following Pam3CSK4 stimulation in THP-1 cells. Values represent either the mean $\pm$ SD from 3 independent experiments (**p<0.01, ***p<0.001, ****p<0.0001), or are representative of at least 3 separate experiments.



METHODS

Cell culture and cytokine measurement

THP-1 cells (obtained from ATCC, confirmed mycoplasma-free throughout the studies) were seeded into 25cm³ flasks (5-8x10⁵ cells/mL in RPMI/10% heat-inactivated fetal calf serum) and cultured $\pm$ 5 μ M simvastatin (SIM, Sigma-Aldrich) or 10 μ M GGTI-298 (Sigma-Aldrich), $\pm$ 50 μ M geranylgeraniol (GGOH; Sigma-Aldrich) for 24 hours. Cells were then replated into 96-well plates in triplicate wells (1x10⁶ cells/mL in 200 μ L) and stimulated with 200ng/mL LPS (*E.coli* O111:B4, Sigma-Aldrich) for 4 hours $\pm$ 10 μ M MCC950, 50mM KCl or 100 μ M butylated hydroxyanisole (BHA, Sigma-Aldrich), or stimulated for 4 hours with 1 μ g/mL Pam3CSK4 (R&D Systems). Conditioned media were then collected for measurement of human IL-1 β and IL-18 (Instant ELISA, eBioscience). Unless stated otherwise, values shown of IL-1 β or IL-18 release are from one experiment and representative of at least 3 independent experiments (IL-1 β) or n=2 experiments (IL-18).

For time-course studies, THP-1 cells were seeded into 6-well plates (10⁶ cells in 1 mL) and pretreated with 5 μ M SIM for 24 hours followed by addition of 200ng/mL LPS. Samples of conditioned media for IL-1 β ELISA were then taken at 1 hour intervals up to 6 hours and cells were collected for quantitative PCR analysis. Values of IL-1 β are the mean $\pm$ SD from three independent experiments.

Fresh blood samples from a MKD patient and both unaffected parents were obtained after informed consent. PBMCs were isolated from buffy coat preparations and seeded in duplicate at 3x10⁶ cells per well (96 well-plate) $\pm$ 50 μ M MCC950, then stimulated with 1000ng/ml LPS for 3 hours then 10 μ M nigericin for 30 minutes. IL-1 β release into condition media was assayed by ELISA (Instant ELISA, eBioscience). Values shown are the mean $\pm$ SD from one experiment and representative of two independent experiments.

Analysis of gene expression

RNA was extracted from cell pellets for quantitative RT-PCR analysis as described previously⁷. mRNA expression of the following genes was assessed using Taqman probes: *IL-1 β* , *IL-18*, *NLRP3*, *MEFV*, *ASC*, *CASP1* and *B2M* (beta-2-microglobulin) (Taqman probes from Thermo Fisher Scientific; Hs01555410_m1, Hs01038788_m1, Hs00918082_m1, Hs00925524_m1, Hs01547324_gH, Hs00354836_m1 and Hs99999907_m1 respectively). Values

are expressed relative to β 2M as control and are the mean $\pm$ SD from three independent experiments.

CRISPR/Cas9 knockout

THP-1 cells expressing Cas9 that had been lentivirally transduced with doxycycline-inducible sgRNA to *NLRP3*, *MEFV*, *CASP1*, and *CASP4* and *CASP5*⁸ (or un-transduced control cells), were cultured with 1 μ g/mL doxycycline for 72 hours and then without doxycycline for a further 24 hours. Loss of protein expression was confirmed by western blotting (analysis of cleaved Caspase-1 (p10) was performed in LPS-treated cells). After doxycycline induction, cells were treated with SIM for 24 hours before 4 hours' LPS stimulation, as described above. Unless stated otherwise, values of IL-1 β release are the mean $\pm$ SD from three independent experiments.

Western blotting and protein prenylation assays

Cell pellets were extracted using 66mM Tris-Cl (pH7.4), 2% SDS, 1x EDTA-free Complete Protease Inhibitor (Roche) and 30 μ g of lysate were analysed by western blotting for pro-IL-1 β (R&D Systems AF-201-NA, 1:000), Caspase-1 (Cell Signalling D7F10, 1:000), NLRP3 (Adipogen Cryo-2, 1:1000), Pyrin (Adipogen AL196, 1:000), Caspase-4 (MBL M029-3, 1:1000) and β -actin (Sigma-Aldrich A1978, 1:1000) using peroxidase-labelled secondary antibodies and chemiluminescence detection using SuperSignal West Pico and Femto reagent (Pierce). Unprenylated Rap1A or unprenylated Rab proteins were detected in 30 μ g of cell lysate by western blotting for unprenylated Rap1A or using an *in vitro* prenylation assay to detect unprenylated Rab GTPases, as described previously⁷. All blots shown are representative of at least 3 separate experiments.

Detection of inflammasomes by ASC immunostaining

THP-1 cells were cultured for 24 hours $\pm$ 5 μ M SIM before seeding at 1 $\times$ 10⁶ cells/mL in 96 well plates. Caspase 1 inhibitor VX-765 (50 μ M; Merck) was added prior to 4 hour stimulation with 200ng/mL LPS or 1 μ g/mL Pam3CSK4. Cells were cytospun onto glass slides, fixed with 10% formalin and immunostained for ASC (anti-ASC antibody N-15, Santa Cruz sc-22514-R; Alexa647 secondary antibody and DAPI nuclear counterstain), before imaging using a Leica DM 5500 fluorescence microscope. Quantification of ASC specks was performed using ImageJ image analysis software, using 6 fields of view at x20 magnification per sample. Each field of view had

100-600 cells. Unless stated otherwise, values of percentage of cells with ASC specks are the mean $\pm$ SD from 3 independent experiments.

Measurement of Caspase-1 activity

THP-1 cells were cultured for 24 hours $\pm$ 5 μ M SIM and re-seeded in white, clear-bottom 96 well plates as described above, before 1 hour stimulation with 200ng/mL LPS. As a positive control, cells were treated with 10 μ M nigericin (Sigma-Aldrich) for 45 min. 100 μ L of Caspase-Glo 1 reagent (Promega) was added to wells at room temperature before measuring luminescence after 1 hour on a Clariostar (BMG Labtech) plate reader. Values were expressed as a percentage of Caspase-1 activity in control cells and, unless stated otherwise, are the mean $\pm$ SD from 3 independent experiments.

Measurement of cell viability/pyroptosis

Cell viability was assessed by Trypan Blue exclusion on THP-1 cultures treated with various concentrations of SIM (1, 2, 5, 10, 20 μ M) for 24 hours. Pyroptotic cell death was measured by propidium iodide (PI) uptake (50 μ g/mL; Sigma-Aldrich) and flow cytometric analysis using a CytoFLEX (Beckman Coulter) flow cytometer.

Statistical analysis

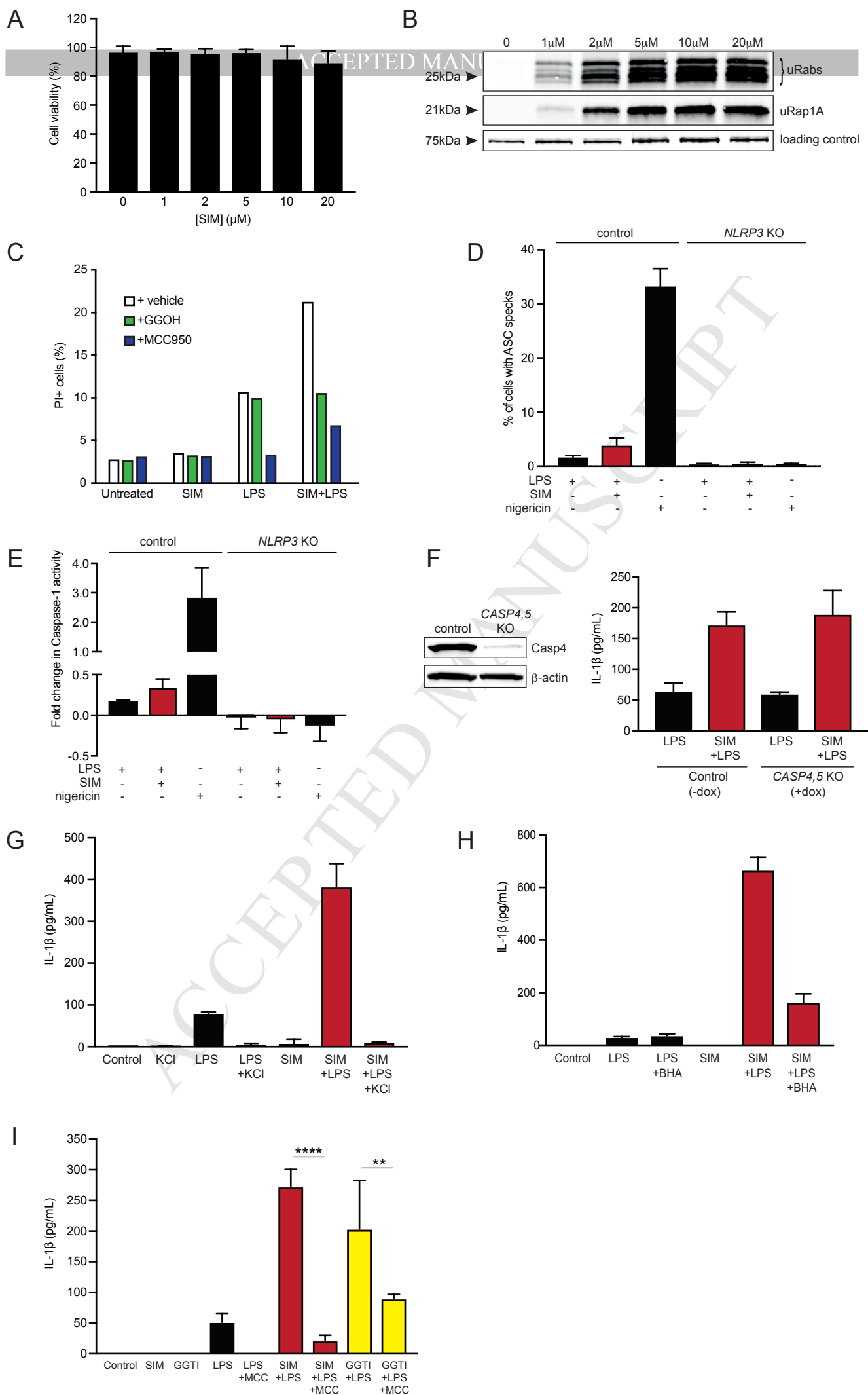
One-way ANOVA with Tukey's multiple comparisons post-hoc test was performed using GraphPad Prism (v7).

Supplementary Figure 1

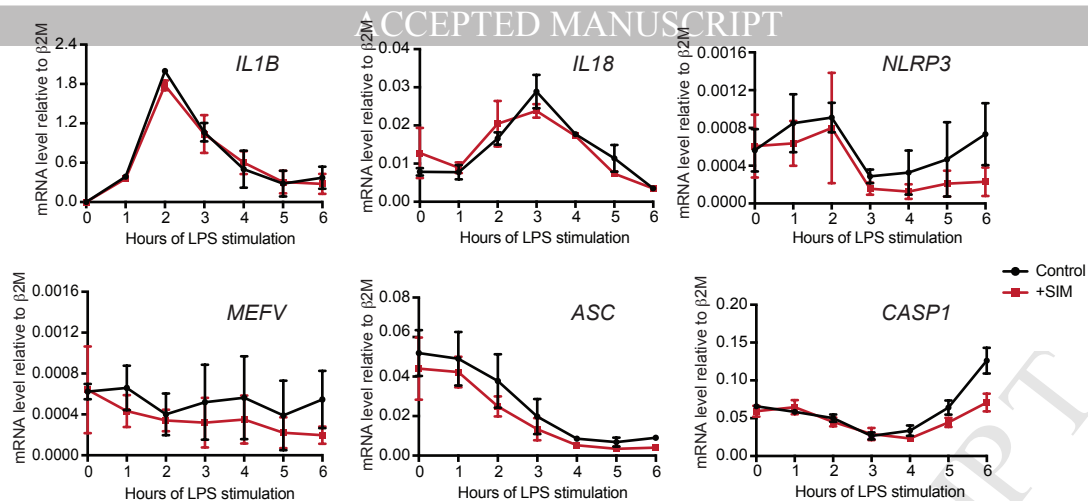
(A) SIM treatment did not affect THP-1 cell viability (trypan blue exclusion), but caused a concentration-dependent accumulation of unprenylated Rab (uRabs) and Rap1A (uRap1A) GTPases (B). Endogenous 75kDa biotinylated protein was used as a loading control. (C) SIM treatment enhanced LPS-induced pyroptosis (propidium iodide/PI uptake), and pyroptosis was restored to normal levels by GGOH addition and abolished by MCC950. Doxycycline-induced *NLRP3* deletion abolished ASC speck formation (D) and Caspase-1 activity (E) upon LPS stimulation (nigericin treatment was used as a positive control). (F) *CASP4* and *CASP5* were not required for the enhancing effects of SIM on IL-1 β release after LPS stimulation. Loss of Caspase-4 was confirmed by western blotting. (G) Blocking [K⁺] efflux with 50mM extracellular [K⁺] abolished LPS-induced IL-1 β release in the presence or absence of SIM. (H) The ROS scavenger butylated hydroxyanisole (BHA) prevented SIM-enhanced IL-1 β release after LPS stimulation. (I) MCC950 inhibited LPS-stimulated IL-1 β release from cells pre-treated with SIM or GGTI-298 (**p<0.01, ****p<0.0001). Values in all figures are the mean $\pm$ SD of triplicate wells, with the exception of (H) showing mean of duplicate wells. All data shown are representative of three independent experiments.

Supplementary Figure 2

Pre-treatment of THP-1 cells with SIM did not enhance mRNA or protein expression of inflammasome components/substrates. (A) Quantitative PCR analysis of mRNA expression of genes encoding IL-1 β , IL-18, *NLRP3*, *Pyrin* (*MEFV*), *ASC* and *Caspase-1* in control cells (black lines) or SIM-pre-treated cells (red lines), for up to 6 hours after LPS stimulation. Values are the mean $\pm$ SD from 3 independent experiments. (B) Western blots showing protein levels of pro-IL-1 β , *NLRP3*, *Pyrin* and pro-Caspase-1 in control or SIM-pre-treated cells for up to 4 hours after LPS stimulation. 0h represents control or SIM pre-treatment prior to LPS stimulation; β -actin was used as loading control. Blots are representative of 3 independent experiments.



A



B

