MCC950 closes the active conformation of NLRP3 to an inactive state

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NLRP3 (NOD-like receptor pyrin domain-containing protein 3) is an innate immune sensor that contributes to the development of different diseases, including monogenic autoinflammatory syndromes, gout, atherosclerosis, and Alzheimer’s disease. The molecule sulfonylurea MCC950 is a NLRP3 inflammasome inhibitor with potential clinical utility. However, the mechanism of action of MCC950 remains unknown. Here, we characterize the mechanism of action of MCC950 in both wild-type and autoinflammatory-related NLRP3 mutants, and demonstrate that MCC950 closes the ‘open’ conformation of active NLRP3.

The activation of the NOD-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome is involved in the pathophysiology of numerous non-communicable diseases through the development of sterile inflammation1–3. NLRP3 senses different host-derived sterile signals and oligomerizes with the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), forming intracellular signaling hubs that activate caspase-1 (refs. 4, 5). Caspase-1 proteolytically generates the active forms of a number of pro-inflammatory cytokines from the interleukin-1 (IL-1) family, and induces pyroptotic cell death by gasdermin D cleavage, resulting in the release of sterile intracellular content, which amplifies host-derived danger signaling6–8. The development of compounds that inhibit IL-1 release started with the identification of sulfonylurea CP-456,773/CRID3, recently renamed MCC950, a compound with proven specificity in inhibiting the NLRP3 inflammasome9–11. Different compounds that target NLRP3 have demonstrated beneficial effects in several murine models of disease, and MCC950 has shown a strong inhibitory effect with good pharmacokinetic and pharmacodynamic properties12–15. Despite the selectivity of MCC950 for inhibiting NLRP3 activation, its mechanism of action has not yet been elucidated. In this study, we found that MCC950 is able to modify the active conformation of NLRP3, and that it blocks NLRP3 oligomerization in response to external stimulation or when NLRP3 contains gain-of-function mutations.

We developed a method to study the molecular conformation of NLRP3 by using bioluminescence resonance energy transfer (BRET)16,17. We found that compared to wild-type NLRP3, the disease-associated mutant p.D305N NLRP3 expressed in HEK293T cells seems to have a distinct conformation (Supplementary Fig. 1a,b), suggesting that the amino terminus and carboxyl terminus of the mutant NLRP3 are separated, compared to the wild-type NLRP3, and therefore pathological mutant NLRP3 could be in an ‘open’ conformation. This difference in BRET signal was not due to variations in protein expression (Supplementary Fig. 1c,d) and was also observed in other pathological mutants of NLRP3 (Supplementary Fig. 1c). NLRP3 BRET signal was intramolecular, as a stable signal was found when increased concentrations of the sensor was expressed (Supplementary Fig. 1b). The incubation of cells expressing the NLRP3 p.D305N BRET sensor with MCC950 at different doses or over increasing times, resulted in an increase of the BRET signal (Fig. 1a,b). These results suggest that MCC950 induces closure of the active NLRP3 p.D305N conformation without changing TFP signals (Supplementary Fig. 1e). MCC950 increased BRET signal from different NLRP3 pathological mutants, and slightly modified the wild-type NLRP3 structure (Supplementary Fig. 1f), suggesting that MCC950 can bind the resting NLRP3. The effect of MCC950 on the structure of NLRP3 was reversible (Supplementary Fig. 1g). The MCC950-driven increase in BRET signal from mutant NLRP3 is not necessarily indicative of a structural conformation identical to inactive wild-type NLRP3. To demonstrate that the increase in BRET and the ‘closure’ of the structure are indicative of NLRP3 inhibition, we expressed NLRP3 mutants in immortalized Nlrp3−/− macrophages, and recorded BRET while evaluating NLRP3 function. We found that the BRET signal of ectopic mutant NLRP3 in macrophages was similar to that of HEK293T cells (Supplementary Fig. 1h). MCC950 was able to increase the NLRP3 BRET signal and block, in parallel, the processing of the caspase-1 substrates IL-1β and GSDMD in macrophages (Fig. 1c). We then expressed N-terminal yellow fluorescent protein (YFP)-tagged NLRP3 in HEK293T cells, and found that gain-of-function NLRP3 mutations resulted in oligomerization (Fig. 1d and Supplementary Fig. 2a). NLRP3 oligomers were able to recruit ASC (Supplementary Fig. 2b), which suggests that they present a functional structure. The number of oligomers per cell varied for the three different gain-of-function mutations, with p.D305N and p.T350M mutations showing higher numbers of oligomers per cell when compared to p.R262W (Fig. 1d and Supplementary Fig. 2a). The p.D305N mutation was the strongest inducer of oligomers, consistent with clinical observations that the p.D305N mutation is associated with the most severe form of NLRP3-associated autoinflammatory syndromes1. Although MCC950 almost completely disaggregated all p.D305N oligomers, more than 50% of the cells retained at least one p.T350M oligomer (Fig. 1d). Monocytes from individuals carrying the p.D305N NLRP3 mutation released a higher concentration of IL-1β and contained a higher percentage of ASC specks compared to those from healthy donors in response to lipopolysaccharide (LPS) (Fig. 1e). LPS treatment resulted in an increase of NLRP3 expression in human monocytes (Supplementary Fig. 2c).
Fig. 1 | MCC950 closes the conformation of autoinflammatory-associated gain-of-function NLRP3. **a,b.** BRET signal in HEK293T cells expressing wild-type or p.D305N YFP-NLRP3-Luc, incubated for 24 h with different concentrations of MCC950 (a) or for different times with MCC950 (10 μM) (b). Vehicle control data are concentration zero (a) and time zero (b). c, BRET signal (bottom) and immunoblots for NLRP3, IL-1β, β-actin, BRET signal in HEK293T cells expressing Nlrp3β-actin BRET signal (bottom) and immunoblots for NLRP3, IL-1β actin.

Table: Percentage of ASC-specking monocytes

| Concentration (M) | Percentage of ASC-specking monocytes |
|-------------------|--------------------------------------|
| 0                 | 0%                                   |
| 0.1               | 10%                                  |
| 1                 | 20%                                  |
| 10                | 30%                                  |
| 100               | 40%                                  |

**d** Cells with:
- 0 NLRP3 oligomers
- 1–2 NLRP3 oligomers
- > 3 NLRP3 oligomers

**e** Inhibition of the percentage of ASC-specking monocytes (NLRP3 p.D305N) by MCC950 (10 μM, 30 min) alone or in combination with MCC950 (10 μM) and LPS (100 ng ml–1) alone or in combination with MCC950 (10 μM); GSDMD-Nt, GSDMD N-terminal. Blots are representative of n = 3 independent experiments with similar results (full blots are shown in Supplementary Fig. 10a). Mann–Whitney two-tailed test, ***P <0.0001 (U = 0).

**f** Quantification of the number of mutant NLRP3-YFP oligomers per HEK293T cell incubated (or not) for 24 h with MCC950 (10 μM); GSDMD-Nt, GSDMD N-terminal. Blots are representative of n = 3 independent experiments, and center values represent mean and error bars the s.e.m. from 1,000–1,500 cells. Percentage of ASC-specking monocytes and IL-1β secretion were determined by flow cytometry and ELISA, respectively. NLRP3 p.D305N oligomers.

**g** Inhibition of IL-1β release from primary human monocytes treated with LPS (1 μg ml–1) and MCC950 (10 μM). Data are representative of n = 3 independent experiments, and center values represent mean and error bars the s.e.m. from 1,000–1,500 cells.

Fig. 3a), which is required to induce ASC specks and the release of IL-1β from cryopyrin-associated periodic syndrome (CAPS) monocytes11. Higher concentration of MCC950 was required to block mutant p.D305N NLRP3 in monocytes from CAPS than to block NLRP3 activation from healthy individuals (Fig. 1f,g). This effect was not due to interference with LPS priming11 (Supplementary Fig. 3b), or with high expression of NLRP3 or ASC specks in CAPS samples (Supplementary Fig. 3c,d). These suggest that the residue at position 305 could be important for the action of MCC950, which is indeed within the Walker B ATP-hydrolysis motif of NLRP3, an essential motif for NLRP3 activity19. We then performed NLRP3 NACHT homology modeling, followed by blind docking (BD) and molecular dynamics (MD) simulations. Our model systems disclosed that the residues within the Walker B motif in both clusters formed a hydrophobic pocket for MCC950 that was stabilized by hydrogen bonds with residues upstream and downstream of this
NLRP3 structure was predicted to be affected by MCC950 binding to both clusters found that the binding of MCC950 to both NLRP3 conformation. MD simulations of 100 ns starting from Fig. 4a). The consensus clusters could represent two potential BD calculations using ADP found the binding amino acids within (ns; U 10).

We then stimulated HEK293T cells expressing the wild-type NLRP3 BRET sensor with either nigericin or ATP (the latter in HEK293T cells stably expressing P2X7 receptor) and both stimuli decreased NLRP3 BRET signal (Fig. 3a). After stimulation, the NLRP3 BRET signal was still intramolecular (Supplementary Fig. 5a). Nigericin-induced NLRP3 oligomerization coincided with stabilization of the NLRP3 open structure, consistent with the idea that NLRP3-activating stimuli open NLRP3 to its active conformation by separation of the N and C termini (Supplementary Fig. 5b). This oligomerization was not due to non-specific aggregation induced by the YFP fluorescent tag, as GFP alone was unable to form oligomers in the cell after nigericin stimulation and NLRP3 without the YFP tag was also able to form oligomers after nigericin treatment (Supplementary Fig. 5c). Addition of MCC950 to the wild-type NLRP3 during an open stable conformation after nigericin stimulation resulted in a higher BRET signal (Fig. 3b). We next incubated HEK293T cells with MCC950 before and during nigericin or ATP (the latter in HEK293T cells expressing P2X7 receptor) and MCC950 was able to increase BRET signal after NLRP3 opening (Fig. 3c and Supplementary Fig. 5d).

MCC950 was also able to block the formation of NLRP3 oligomers (Fig. 2a). After 100 ns of MD simulations, cluster 2 residues within Walker B presented a more favorable binding energy to MCC950 (Supplementary Fig. 4d), with F304 establishing stacking interactions with F257 and the aromatic ring of MCC950 (Supplementary Fig. 4b,c). When key amino acids required for Walker B function (D302 and E306 in human, corresponding to D298 and E302 in mouse) were alanine-substituted in the NLRP3 p.D305N background, MCC950 lost its ability to increase the BRET signal (Fig. 2b).

Motif (Supplementary Fig. 4a,b), which is compatible with the reversible inhibitory effect of MCC950 (Supplementary Fig. 1g). BD calculations using ADP found the binding amino acids within the Walker A motif and also the distal H522 (Supplementary Fig. 4a). The consensus clusters could represent two potential MCC950 binding spots, which could be further stabilized and alter NLRP3 conformation. MD simulations of 100 ns starting from the two clusters found that the binding of MCC950 to both clusters remains stable (Supplementary Fig. 4a–c) and that NLRP3 structure was predicted to be affected by MCC950 binding (Fig. 2a). After 100 ns of MD simulations, cluster 2 residues within Walker B presented a more favorable binding energy to MCC950 (Supplementary Fig. 4d), with F304 establishing stacking interactions with F257 and the aromatic ring of MCC950 (Supplementary Fig. 4b,c). When key amino acids required for Walker B function (D302 and E306 in human, corresponding to D298 and E302 in mouse) were alanine-substituted in the NLRP3 p.D305N background, MCC950 lost its ability to increase the BRET signal (Fig. 2b).

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data suggest that when NEK7 expression is reduced, MCC950 is able to ‘close’ the NLRP3 active conformation more efficiently and that the change induced by MCC950 over the active ‘open’ structure of NLRP3 could disrupt the interaction with NEK7. The accompanying study by Coll et al. shows that MCC950 does not bind NEK7 but directly targets NLRP3 at a site within or close to the Walker B motif and blocks ATP hydrolysis, which blocks NLRP3 activation\textsuperscript{23}. Together, these complementary studies demonstrate that MCC950 prevents NLRP3 activation by interacting directly with NLRP3 and affecting Walker B function, leading to an inactive NLRP3 conformation.

In conclusion, our study demonstrates that MCC950 closes the NLRP3 active conformation in pathological gain-of-function NLRP3 mutants or during stimulation of wild-type NLRP3. MCC950 showed different inhibitory effects compared to distinct pathological NLRP3 mutations, raising the possibility of developing personalized therapy for individuals with monogenic autoinflammatory syndromes carrying specific mutations for which the compound is more effective.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41589-019-0277-7.

Received: 9 July 2018; Accepted: 22 March 2019; Published online: 13 May 2019

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Acknowledgements
We thank M. C. Baños and A. I. Gómez for technical assistance with molecular biology and cell culture. We also wish to thank L. Martínez-Alarcon for help with healthy volunteer blood collection, I. Hafner-Bratkovič (National Institute of Chemistry, Ljubljana, Slovenia) for stable immortalized macrophages lines, and C. Vargas (Hospital Virgen de la Macarena, Sevilla, Spain), E. Ramos and S. Jimenez-Treviño (Hospital Central de Asturias, Oviedo, Spain), and M. Basaño Torreño (Hospital Universitario Germans Trias i Pujol, Badalona, Spain) for samples from individuals with autoinflammatory syndromes. We also thank the patients and healthy volunteers enrolled in this study, and the Biobanco en Red de la Región de Murcia (PT13/0010/0018), which is integrated into the Spanish National Biobanks Network (B.000859), for its collaboration. This research was partially supported by the e-infrastructure program of the Research Council of Norway, and the supercomputer centre of UiT at the Arctic University of Norway. The authors are also grateful for the computer resources at CTE-POWER and the technical support provided by Barcelona Supercomputing Center (RES-BCV-2018-3-0008). H.M.-B. was supported by a Rio Hortega fellowship from the Instituto Salud Carlos III (CM14/00008). D.A.-B. was supported by a Juan de la Cierva Grant. D.A.-B. was supported by a Juan de la Cierva Grant (RES-BCV-2018-3-0008). H.M.-B. was supported by a Rio Hortega fellowship from the Instituto Salud Carlos III (CM14/00008). D.A.-B. was supported by a Juan de la Cierva Grant.
postdoctoral fellowship from the Ministerio de Economía y Competitividad (FJCI-2014-22041). This work was supported by grants from the Instituto Salud Carlos III-Fondo Europeo de Desarrollo Regional (PI13/00174 to P.P.), the Ministerio de Economía, Industria y Competitividad-Fondo Europeo de Desarrollo Regional (project nos. SAF2017-88276-R to P.P. and CTQ2017-87974-R to H.P.-S.), Fundación Séneca (20859/PI/18 to P.P.), and the European Research Council (ERC-2013-CoG 614578 to P.P.).

Author contributions
A.T.-A., D.A.-B., H.M.-B. and C.dT.-M. conducted the experiments and interpreted data. J.P.C.-C. and H.P.-S. conducted BD and MD simulations. D.A.-B. performed structural modeling. J.I.A. coordinated human samples from autoinflammatory individuals. P.P. conceived, designed, and supervised this study, wrote the paper with feedback from all coauthors, and sourced funding.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41589-019-0278-6.

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Methods

Reagents. Key reagents and their sources were as follows: Escherichia coli LPS serotype O55:B5, nigericin sodium salt, MCC950 (CP-456773), ATP, bovine serum albumin, DAPI and siRNA NEK7 (Mission siRNA 1, SHH1-1048, and 2, SHH1-1049) and scrambled siRNA negative control (Mission siRNA negative control, SHH1-1030) were from Sigma-Aldrich. Uppercase E. coli LPS serotype O111:B4 was from InvivoGen. Coelenterazine-H and Lipofectamine 2000 were Life Technologies. The composition of the physiological buffer used in all experiments to stimulate cells was as follows: 147 mM NaCl, 10 mM HEPES, 13 mM d-glucose, 2 mM KCl, 2 mM CaCl₂, and 1 mM MgCl₂; pH 7.4.

Plasmid constructs. The different mutations of human NLRP3 were generated by overlapping PCR to introduce point mutations (p.R262W, p.D305N, or p.T350M) or triple mutant (p.D302A/D305N/E306A) (Uniprot Q96P20 annotation for human NLRP3) and cloned into pcDNA3.1/V5-His TOPO (Life Technologies). See Methods for all constructs. Stably expressed full-length NLRP3 C terminus was also produced to use as a control in all BRET assays. They were also cloned into pcDNA3.1/V5-His TOPO and sequenced to confirm correct alignment between tags and the NLRP3 sequence.

Cells and transfections. HEK293T cells (CRL-11288, American Type Culture Collection) immortalized in DMEM/F12 (Lonza) (Lonza) supplemented with 10% fetal calf serum (FCS) (Life Technologies), 2 mM Glutamax (Life Technologies), and 1% penicillin-streptomycin (Life Technologies). HEK293T cells stably expressing the rat P2X7 receptor have been described previously and were cultured in F-12 media (Lonza) supplemented with 10% FCS. Lipofectamine 2000 was used for the transfection of HEK293T cells according to the manufacturer’s instructions. After 2 days of transfection, stable selection of HEK293T cells expressing the different NLRP3 constructs was initiated by supplementing culture media with 418 (2 mg/ml), Acros Organic. After 4 weeks of culture in G418, cell cloning was performed by serial dilution in 96-well plates in the presence of G418 for a further 4–8 weeks. Positive clones were expanded and tested for correct expression by western blot and fluorescence microscopy. HEK293T cells stably expressing NLRP3 constructs were maintained in DMEM/F12 (1:1) supplemented with 10% FCS, 2 mM Glutamax, and 1% penicillin–streptomycin.

All cells were tested routinely for Mycoplasma contamination with a Mycoplasma Detection Kit (Roche). siRNA were diluted to a final concentration of 2.5 pmol µl⁻¹ and HEK293T cells stably expressing the wild-type NLRP3 BRET sensor were transfected using Lipofectamine 2000 with 10 µM siRNA 1 and 10 pmol of siRNA 2, or 20 pmol of scramble RNA, both for 48 h.

Construct preparation and transduction of immortalized mouse macrophages Nrps⁻⁻. For doxycycline-inducible expression of NLRP3 BRET sensors in immortalized mouse macrophages, we used the Tet-ON retroviral system (Clontech 631188). NLRP3 BRET sensors were subcloned into pRETRO Tre3G plasmid (Clontech) using BamHI/XhoI sites and transfected using Lipofectamine 2000 into the packaging cell line G418 Ampho Amphi cell line (Aallele Biotechnology, ABP-RVC-10001). Nrps⁻⁻ immortalized mouse macrophages stably expressing the Tet-3G transgene (gift from Dr. Marta Novak, National Institute of Chemistry, Ljubljana, Slovenia) were transfected with different NLRP3 BRET sensors or empty vector encoding retroviruses for 2 d. Positive macrophages were selected with puromycin (6 µg ml⁻¹) and G418 (1.5 mg ml⁻²).

Bone marrow-derived macrophages. Wild-type C57 BL/6 mice were purchased from Harlan. For all experiments, mice between 8–10 weeks of age and bred under specific-pathogen-free conditions were used in accordance with the Hospital Clínico Universitario Virgen Arrixaca animal experimentation guidelines, and the Spanish national (RD 1201/2005 and Law 32/2007) and EU guidelines, and the Spanish national (RD 1201/2005 and Law 32/2007) and EU guidelines. Samples were stored in the Biobanco en Red de la Región de Murcia (PT13/0010/0018) integrated in the Spanish National Biobanks Network (B.000859). Peripheral blood mononuclear cells (PBMCs) were collected using Histopaque-1077 (Sigma-Aldrich) and cultured in RPMI 1640 medium (Lonza) with 10% FCS, 2 mM Glutamax and 100 U ml⁻¹ penicillin–streptomycin. PBMCs from patients were left unstimulated or were stimulated with 20 ng ml⁻¹ of LPS for 4 h at 37 °C. In the presence of 350 mM MCC950 (from 0.1 µM to 10 µM). MCC950 was added 30 min before and during LPS stimulation. To calculate dose inhibition curves for MCC950, PBMCs from healthy donors were similarly treated with LPS and MCC950, but were then stimulated with nigericin (5 µM, 30 min at 37 °C).

BRET assays. HEK293T cells or immortalized mouse macrophages expressing the different NLRP3 BRET sensors (wild-type and mutants) were plated on a poly-L-lysine–coated white opaque 96-well plate; after adhesion, cells were incubated with different doses of MCC950 or MCCNP (as stated in the figure legends) or vehicle, washed with PBS with calcium and magnesium, and BRET readings were performed 5 min after the addition of 5 µM coelenterazine-H substrate. BRET signals were detected with two filter settings (Renilla Luc filter (485 ± 20 nm) and YFP filter (530 ± 25 nm)) at 37 °C using the Synergy MX plate reader (BioTek) as described before. In some experiments, BRET signal was recorded every 35 s before and after nigericin or ATP automatic injection for a total of 15 min. For experiments measuring basal BRET signal, a stable signal for 5 min kinetic was recorded and averaged. BRET signal was similar when comparing HEK293T cells stably expressing the different NLRP3 BRET sensor with transient transfections for the same construct. Titration was performed by transfection of different amounts of plasmids encoding YFP-NLRP3-Luc or YFP-NLRP3-Luc (pcDNA empty plasmid was used to have equal amounts of total DNA in all the transfections). Each cell was transfected with a different amount of plasmid. BRET signal was measured using a plate reader by analyzing the fluorescence of individual cells by fluorescence microscopy. Titration of the sensor determines whether the recorded BRET is intra- or intermolecular. For intramolecular BRET the increase in the BRET sensor expression results in a stable BRET signal while for intermolecular BRET the increase in the BRET sensor expression results in a proportional increase of the BRET rate. The BRET ratio was defined as the difference of the emission ratio 530 nm/485 nm of the BRET sensor minus this ratio of NLRP3 tagged with Luc only. Results were expressed in milliBRET units (mBU).

Fluorescence microscopy. Poly-L-lysine coated coverslips (Corning) were used to seed 10³ HEK293T cells stably expressing the different NLRP3 constructs tagged with YFP at the N terminus. For NLRP3–NEK7 co-localization experiments, stable cell lines were transfected with myc-human NEK7 expressing plasmid (GeneScript) on the coverslips. To study NLRP3 oligomerization without YFP tag, NLRP3-flag or GFP control plasmid were transfected into HEK293T. To analyze wild-type or p.D305N NLRP3 oligomerization with ASC, a stable HEK293T cell line expressing the desired NLRP3 construction was transfected with ASC. Cells were treated and stimulated as indicated in the figure legends, washed twice with PBS and fixed for 15 min at room temperature with 4% paraformaldehyde, and were then washed three times with PBS. For NEK7, NLRP3, or ASC immunofluorescence, cells were blocked with 2% bovine serum albumin and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 20 min at room temperature. Cells were stained for 1.5 h at room temperature with an Alexa Fluor 488 (1:1000; Invitrogen) and the primary monoclonal mouse antibody anti-ASC (N-15)-R (sc-22514-R, Santa Cruz, 1:1500) followed by staining with a donkey anti-rabbit alexa-488 antibody (1:1000, Life Technologies) as described previously. Monoclones were gated using the PE-conjugated human CD14 antibody (61D3, 50-0149-TO25, TONBO Biosciences, 1 µl 10⁶ cells) and stained cells were acquired on a FACs Canto cytometer (BD Biosciences). The gating strategy is shown in Supplementary Fig. 7.
Immunoprecipitation and western blot. BMDMs and immunolated mouse macrophages expressing BRET sensors were lysed in ice-cold lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 2% Triton X-100) supplemented with 100 μM of protease inhibitor mixture (Sigma), 10 mM NaN3, 5 mM EDTA, 1% Igepal CA-630) supplemented with 100 μM of protease inhibitor mixture (Sigma–Aldrich) for 30 min on ice and then were clarified by centrifugation (16,000 g for 15 min at 4°C). Cleared cell lysates (1 mg) were incubated at 4°C overnight with 5 μl (1:100) anti-NEK7 rabbit monoclonal (EPR4900 clone, ab133514, Abcam) with 25 μl of protein A/G magnetic beads (Pierce Protein A/G Magnetic Beads, Thermo Fisher Scientific 88802) in a final volume of 500 μl. Immunoprecipitated complexes were washed twice in lysis buffer and eluted in Laemmli buffer (Sigma–Aldrich) after 2 min of incubation. Immortalized mouse macrophages expressing wild-type NLRP3-BRET sensors were added above were treated with doxycycline (1 μg ml−1) and ultrapure LPS (100 ng ml−1) for 16 h and then stimulated with 10 μM of nigericin during 30 min in the presence or absence of 10 μM of MCC950. Cells were lysed in ice-cold lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Igepal CA-630) supplemented with 100 μM of protease inhibitor mixture (Sigma–Aldrich) for 30 min on ice and then were clarified by centrifugation (400 g for 10 min at 4°C). Cleared cell lysates (1 mg) were incubated at 4°C overnight with 5 μl (1:100) anti-NEK7 rabbit monoclonal (EPR4900 clone, ab133514, Abcam) with 25 μl of protein A/G magnetic beads (Pierce Protein A/G Magnetic Beads, Thermo Fisher Scientific 88802) in a final volume of 500 μl. Immunoprecipitated complexes were washed twice in lysis buffer and eluted in Laemmli buffer after 2 min of incubation. In the case of immortalized mouse macrophages expressing pathological mutants NLRP3-BRET sensors, MCC950 (10 μM) was added at the same time as the other stimuli. Western blots were performed using the appropriate antibodies and were visualized using the chemiluminescence system (Bio-Rad). Immunoprecipitated complexes were resolved on 15% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were probed with the anti-β-actin antibody (dilution 1:5000) to determine protein loading and were reprobed with the anti-β-actin antibody (dilution 1:15000) for actin normalization. Data shown are representative of three independent experiments. Protein Fold Recognition Server (http://www.sbg.bio.ic.ac.uk/~phyre2) using the 8 structural model for the human NACHT NLRP3 was constructed using the Phyre2 Human NLRP3 homology modeling and molecular dynamics. ELISA. Individual culture cell-free PBMCs supernatants were collected, clarified by centrifugation (16,000 g for 15 min at 4°C). Cleared cell lysates (1 mg) were incubated at 4°C overnight with 5 μl (1:100) anti-NEK7 rabbit monoclonal (EPR4900 clone, ab133514, Abcam) with 25 μl of protein A/G magnetic beads (Pierce Protein A/G Magnetic Beads, Thermo Fisher Scientific 88802) in a final volume of 500 μl. Immunoprecipitated complexes were washed twice in lysis buffer and eluted in Laemmli buffer after 2 min of incubation. In the case of immortalized mouse macrophages expressing pathological mutants NLRP3-BRET sensors, MCC950 (10 μM) was added at the same time as the other stimuli. Western blots were performed using the appropriate antibodies and were visualized using the chemiluminescence system (Bio-Rad). Immunoprecipitated complexes were resolved on 15% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were probed with the anti-β-actin antibody (dilution 1:5000) to determine protein loading and were reprobed with the anti-β-actin antibody (dilution 1:15000) for actin normalization. Data shown are representative of three independent experiments.
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Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
- Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Clearly defined error bars

Software and code

Policy information about availability of computer code

Data collection

- Gene5 & Gene 5 Secure (Biotek Instruments, USA), Image Lab 5.2.1 (Biorad, USA), IQS Optical System software v2.0 (Biorad, USA), FACS Canto (BD, Bioscience), Imaging Software NIS-Elements v4.3 (Nikon, Japan), Chromas v2.6.4 (Technleysium Pty Ltd, Australia), Gaussian 16 Revision A.03 (Gaussian Inc.), Maestro 2018-1 (Schrödinger LLC), GROMACS - gmx_mpi, 2016.3 (LINCS algorithm and Steepest Descent algorithm are directly implemented into GROMACS), AutoDock Vina v1.1.2 (Molecular Graphics Lab, The Scripps Research Institute), ACPYPE v0.1.0.

Data analysis

- Prism (GraphPad Inc., v6 and v7), FCS Express 4 Flow Research (De novo software), ImageJ (US National Institutes of Health, Bethesda, MD-USA), Phyre2 Protein Fold Recognition Server (Imperial College, London, UK), AutoDock Vina software (The Scripps Research Institute, USA), Gene5 & Gene 5 Secure (Biotek Instruments, USA), Clustal Omega (EMBL-EBI), APE-A Plasmid Editor v2.0.47, Chimera (Resource for Biocomputing, Visualization, and Informatics, University of California, USA), Protein-Ligand Interaction Profiler (PLIP) v1.3.2 (Nucl. Acids Res. 2015;43:W443-W447), Poseview 1.1.2 (Center for Bioinformatics, University of Hamburg).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Source data for main figures is presented in Supplementary Source Data files. Supplementary Fig. 8 and 9 present data for main figures 1a,f,g and 3a-c,e as dot-plots to show data distribution. Supplementary Fig. 10 present uncropped Western blots presented in main and supplementary figures. All other data supporting the findings of this study are available from the corresponding author upon reasonable request.

Field-specific reporting

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☑ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | The number of in vitro assays performed was defined by a precision inter assay using the coefficient of variation (c.v) lower than 10% |
|-------------|----------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data points were excluded from any results.                                                                                   |
| Replication | Experiments were repeated at least two times and/or with sufficient cells per group to demonstrate statistical significance. All replication experiments give a similar result as the presented representative in main figures. In the case of human samples, due to the limitation of the samples and the cell numbers, the experiment from each patient or donor was done once for each patient. |
| Randomization | not relevant for in vitro assays.                                                                                               |
| Blinding    | not relevant for in vitro assays.                                                                                               |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☐   | Unique biological materials |
| ☒   | Antibodies |
| ☒   | Eukaryotic cell lines |
| ☐   | Palaeontology |
| ☒   | Animals and other organisms |
| ☐   | Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | ChIP-seq |
| ☐   | Flow cytometry |
| ☒   | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

The different mutations of human NLRP3 were generated by overlapping PCR to introduce point mutations (p.R262W, p.D305N or p.T350M) or triple mutant (p.D302A/D305N/E306A) (Uniprot #O96P20 annotation was used) and cloned into pcDNA3.1/V5-His TOPO (Life Technologies). Sequencing of all constructs was performed to confirm correct modification and the absence of unwanted mutations. All constructs were fused using overlapping PCR in the N-terminus to YFP for microscopy assays or double tagged with YFP in the N-terminus and Renilla Luciferase (Luc) in the C-terminus to generate the various BRET sensors. NLRP3 single fused to Renilla Luciferase (Luc) in the C-terminus was also produced to use as a control in all BRET assays. They were also cloned into pcDNA3.1/V5-His TOPO and sequenced to confirm correct alignment between tags and the NLRP3 sequence. HEK293T cells expressing the different NLRP3 constructs. Stable selection of HEK293T clones was initiated by supplementing
culture media with G418 (2 mg/ml, Acros Organic). After 4 weeks of culture in G418, cell cloning was performed by serial dilution in 96 well plates in the presence of G418 for a further 4-8 weeks. Positive clones were expanded and tested for correct expression by Western blot and fluorescence microscopy.

For doxycycline-inducible expression of NLRP3 BRET sensors in immortalized mouse macrophages we used Tet-ON retroviral system (Clontech 631188). NLRP3 BRET sensors were subcloned into pRETROX Tre3G plasmid (Clontech) using BamHI/EcoRI and transfected using Lipofectamine 2000 into the packaging cell line Gryphon Ampho cell line (Alelle Biotechnology, ABP-RVC-10001). Nlrp3-/- immortalized mouse macrophages stably expressing the Tet-On 3G transactivator (a kindly gift from Dr. Iva Hafner-Bratkovič, National Institute of Chemistry, Ljubljana, Slovenia) were transduced with different NLRP3 BRET sensors or empty vector encoding retroviruses for two days. Then cells were selected with puromycin (6 μg/mL) and G418 (1.5 mg/ml).

All unique materials are available from the corresponding author upon reasonable request.

### Antibodies

| Antibody name | Supplier name | Catalogue number | Clone name | Lot number | Dilution used |
|---------------|---------------|------------------|------------|------------|---------------|
| anti-GFP rabbit polyclonal | Abcam | ab6556 | - | GR3216572-1 | WB 1:2500 |
| anti-il-1β rabbit polyclonal | Santa Cruz Biotechnology | sc-7884 | - | #B1115 | WB 1:1000 |
| anti-GSDMD rabbit monoclonal | Abcam | ab209845 | EPR19828 | GR3187186-8 | WB 1:5000 |
| Horseradish peroxidase-anti-beta-actin mouse monoclonal | Santa Cruz Biotechnology | sc-47778 HRP | C4 | #J2915 | WB 1:10000 |
| anti-ASC (N-15)-R rabbit polyclonal | Santa Cruz Biotechnology | sc-22514-R | - | #E1613 | 1:1500 |
| PE-conjugated anti-human CD14 mouse monoclonal | TONBO Biosciences | 50-0149-T025 | 61D3 | C0149081517503 | 1ul /100000 cells |
| anti-myc mouse monoclonal | Thermo Scientific | MA1-980 | 9E10 | QD216104 | IF 1:1000 |
| anti-ASC mouse monoclonal | BioLegend | 653902 | HASC-71 | B197851 | IF 1:1000 |
| anti-NLRP3 mouse monoclonal | Adipogen | AG-20B-0014 | - | - | - |
Clone name: Cryo-2  
Lot number: A27381510  
Dilution used: Immunoprecipitation (IP) 5 μg, Western blot (WB) 1:1000, Immunofluorescence (IF) 1:1000

Antibody name: anti-NEK7 rabbit monoclonal  
Supplier name: Abcam  
Catalogue number: ab133514  
Clone name: EPR4900  
Lot number: GR96239-11  
Dilution used: WB 1:5000, IP 1:100

Antibody name: Alexa Fluor 647 donkey anti-mouse IgG (H+L)  
Supplier name: Life Technologies  
Catalogue number: A31571  
Lot number: 1900251  
Dilution used: 1:200

Antibody name: ECL Anti-mouse IgG, horseradish peroxidase linked whole antibody (from sheep)  
Supplier name: GE Healthcare  
Catalogue number: NA931V  
Lot number: 9557666  
Dilution used: WB 1:10000

Antibody name: Alexa fluor 488 donkey anti-rabbit IgG (H+L)  
Supplier name: Life Technologies  
Catalogue number: A21206  
Lot number: 1796375  
Dilution used: 1:1000

Antibody name: ECL Anti-rabbit IgG, horseradish peroxidase linked F(ab′)2 (from donkey)  
Supplier name: GE Healthcare  
Catalogue number: NA9340V  
Lot number: 9784574  
Dilution used: WB 1:10000

Antibody name: Native protein A conjugated to horseradish peroxidase  
Supplier name: Calbiochem  
Catalogue number: 539253  
Lot number: D00149040  
Dilution used: IP 1:8000

Validation

Antibody name: anti-GFP rabbit polyclonal  
Specificity: This antibody is reactive against all variants of Aequorea Victoria GFP such as: S65T-GFP, RS-GFP, YFP, CFP, RFP and EGFP  
Application: Suitable for: IHC-FoFr, Electron Microscopy, IHC-P, IHC-Fr, IP, WB, ICC/IF, Flow Cyt (as manufacturer specifications for the antibody)

Antibody name: anti-IL-1β rabbit polyclonal (H-153)  
Specificity: mouse, rat, human  
Application: Suitable for: WB, IP, IF, IHC(P) and ELISA (as manufacturer specifications for the antibody)

Antibody name: anti-GSDMD rabbit monoclonal  
Specificity: This product works well in western blot using mouse cell lines  
Application: Suitable for: IP, WB (see Front Immunol 9:1121, 2018)

Antibody name: Horseradish peroxidase-anti-beta-actin mouse monoclonal  
Specificity: mouse, rat, human, avian, bovine, canine, porcine, rabbit, Dictyostelium discoideum and Physarum polycephalum  
Application: Suitable for: WB, IHC(P) and ELISA (as manufacturer specifications for the antibody)

Antibody name: anti-ASC (N-15)-R rabbit polyclonal  
Specificity: mouse, rat and human  
Application: Flow Cyt (J. Immunol. 194, 455–462, 2014).

Antibody name: PE-conjugated anti-human CD14 mouse monoclonal  
Specificity: human  
Application: Flow Cyt (J. Immunol. 194, 455–462, 2014).

Antibody name: anti-myc mouse monoclonal  
Specificity: human, mouse  
Application: WB, IP, IF, IHC(P), ICC, ChIP and ELISA (as manufacturer specifications for the antibody)

Antibody name: anti-ASC mouse monoclonal  
Specificity: human  
Application: IF; this work: see Supplementary Figure 2b for specificity controls where we use cells do not expressing ASC vs cells
expressing ASC. Also after inflammasome activation, ASC localisation changes and form the well-described ASC speck.

Antibody name: anti-NLRP3 mouse monoclonal
Specificity: Recognizes mouse and human NLRP3/NALP3.
Application: IP in RIPA buffer (Mol. Cell 49, 331, 2012), WB see online protocol in the Adipogen webpage: 1μg/ml (as manufacturer specifications for the antibody), IF (Nat. Immunol. 13, 255, 2012)

Antibody name: anti-NEK7 rabbit monoclonal
Specificity: mouse, rat, human
Application: Suitable for: WB, IP (Nature 530:354-7, 2016)

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  HEK293T cells (CRL-11268; American Type Culture Collection), HEK293T cells stably expressing the rat P2X7 receptor (Virginio, C. et al. J. Physiol. 519 Pt 2, 335–346; 1999), Nlrp3-/- immortalized mouse macrophages stably expressing the Tet-On 3G transactivator (a kindly gift from Dr. Iva Hafner-Bratkovič, National Institute of Chemistry, Ljubljana, Slovenia), Gryphon Ampho cell line (Alelle Biotechnology, ABP-RVC-10001).

Authentication  None of the cell lines used were authenticated

Mycoplasma contamination  Cell lines were routinally tested for Mycoplasma contamination and were clean for Mycoplasma

Commonly misidentified lines  No commonly misidentified cell lines were used.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals  We worked with bone marrow-derived macrophages obtained from laboratory mice (Mus musculus) wild-type C57BL/6j background purchased from Harlan. Males and females were used at the age of 8-10 weeks. No procedure was performed to the animals, so no ethical committee approval was required under Spanish legislation RD53/2013 and Law 6/2013.

Wild animals  none

Field-collected samples  none

Human research participants

Policy information about studies involving human research participants

Population characteristics  Healthy donors: 5 females between 25-40 age
Patients: 2 males with cryopyrin-associated periodic syndrome carrying the NLRP3 p.D305N mutation ages 43 and 39

Recruitment  CAPS is a rare disease, and we aimed to recruit CAPS patients carrying a specific NLRP3 mutation p.D303N, since this was one of the mutations studied in this work, therefore two patients carrying that particular mutation were selected. The recruitment of other CAPS patients with other mutations could affect the IC50 found in this study for MCC950.

Healthy individuals were recruited to have at least two control healthy samples to process in parallel to the sample of CAPS.

Informed consent was obtained from all individuals enrolled in the study following the principles set out in the WMA Declaration of Helsinki and samples were stored in the Biobanco en Red de la Región de Murcia (P13/0010/0018) integrated in the Spanish National Biobanks Network (B.000859)

The Institutional Review Board of the Hospital Clínico Universitario Virgen de la Arrixaca approved the use of these blood samples.
Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

PBMCs from healthy blood donors or patients carrying NLRP3 p.D303N mutation were isolated from whole peripheral blood using Histopaque-1077 (Sigma-Aldrich) and cultured in RPMI 1640 medium (Lonza) with 10% of fetal calf serum, 2mM glutamax and 100U/ml penicillin-streptomycin. PBMCs from patients were treated with either nothing or with 1 μg/ml of LPS for 4h at 37°C in the presence of different concentrations of MCC950 (from 0.1 μM to 10 μM). MCC950 was added 30 min before and during LPS stimulation. PBMCs from healthy donors were similarly treated with LPS and MCC950 but then were stimulated with nigericin (5 μM, 30 min at 37°C). Stimulated PBMCs were fixed with 2% paraformaldehyde and stained for the detection of Apoptosis-associated Speck-like protein containing a Caspase recruitment domain (ASC) specks by Time of Flight Inflammasome Evaluation using the rabbit polyclonal antibody anti-ASC (N-15)-R (Santa Cruz Biotechnology, Dallas, Texas, United States).

Instrument

FACS Canto cytometer (BD Biosciences)

Software

FCS Express 4 Flow Research (De novo software)

Cell population abundance

Monocytes were the population of interest and accounted between 15-20% of PBMCs after Ficoll gradient isolation from whole blood.

Gating strategy

Monocytes were gated using SSC vs CD14-PE. ASC specking monocytes were gated using FITC-W vs FITC-A.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.