NLRP3 inflammasome inhibition is disrupted in a group of auto-inflammatory disease CAPS mutations

Leanne Mortimer¹, France Moreau¹, Justin A MacDonald² & Kris Chadee¹

Inflammasomes are positioned to rapidly escalate the intensity of inflammation by activating interleukin (IL)-1β, IL-18 and cell death by pyroptosis. However, negative regulation of inflammasomes remains poorly understood, as is the signaling cascade that dampens inflammasome activity. We found that rapid NLRP3 inflammasome activation was directly inhibited by protein kinase A (PKA), which was induced by prostaglandin E₂ (PGE₂) signaling via the PGE₂ receptor E-prostanoid 4 (EP4). PKA directly phosphorylated the cytoplasmic receptor NLRP3 and attenuated its ATPase function. We found that Ser295 in human NLRP3 was critical for rapid inhibition and PKA phosphorylation. Mutations in NLRP3-encoding residues adjacent to Ser295 have been linked to the inflammatory disease CAPS (cryopyrin-associated periodic syndromes). NLRP3-S295A phenocopied the human CAPS mutants. These data suggest that negative regulation at Ser295 is critical for restraining the NLRP3 inflammasome and identify a molecular basis for CAPS-associated NLRP3 mutations.

A controlled inflammatory response that resolves infection and tissue damage is fundamental to health. Inflammasomes, particularly in myeloid cells, are highly pro-inflammatory signaling platforms activated at sites of inflammation that are critical for microbial clearance and alerting the immune system to infection and tissue injury. Inflammasomes activate a proteolytic cascade driven by caspase-1, which cleaves the precursors of IL-1β and IL-18 into their active forms, followed by secretion via a poorly defined mechanism. Caspase-1 activation also initiates pyroptotic death of the cell and, in doing so, releases potent pro-inflammatory intracellular molecules into the tissue. The robust and often rapid inflammation evoked by inflammasomes necessitates that their activity be tightly regulated and tuned to prevent inappropriate and excessive inflammation. In humans, the effects of aberrant inflammasome activation are well recognized. Gain-of-function mutations in NLRP3 result in CAPS, which are a spectrum of diseases described as familial cold auto-inflammatory syndrome (FCAS), Muckle Wells syndrome (MW) and neonatal onset multisystem inflammatory disorder (NOMID), and are characterized by systemic inflammation affecting skin, joints, eyes, bone, muscles and central nervous system as a result of increased IL-1β secretion. Recently, gain-of-function mutations in NLRC4 have been associated with similar IL-1β-driven auto-inflammatory syndromes. Furthermore, NLRP3 is involved in the pathogenesis of numerous wide-spread chronic diseases, such as vascular disease, non-alcoholic fatty liver disease, obesity and type II diabetes, where dysregulated inflammation arising from malfunctioning tissues is a major disease component.

Presently, how active inflammasomes are attenuated is not well understood. We hypothesized that, to function properly at sites of inflammation, activated inflammasomes are wired to receive rapid inhibitory commands and components in the inflammatory milieu provide critical feedback to instantly adjust their activity. A number of constitutive and inducible pathways that regulate inflammasomes have been identified (reviewed in ref. 1). Regulatory pathways that immediately switch-off inflammasomes have not yet been defined.

PKA is a serine/threonine kinase that regulates a diverse number of pathways that are important for controlling the immune system by modulating gene transcription and post-translational signaling. PKA is promptly activated by increasing intracellular concentrations of cyclic adenylyl monophosphate (cAMP) synthesized by adenylyl cyclases. Recently, several groups reported that cAMP inhibits the NLRP3 inflammasome. Direct binding of cAMP to the NLRP3 NOD-binding domain (NBD) has been proposed, although only endogenous cAMP has been shown to interact with NLRP3, indicating that binding may occur through NLRP3-interacting proteins. Mechanistically, cAMP has been found to signal autophagy-mediated NLRP3 degradation in response to dopamine D1 receptor activation, where macrophages cultured with AMP and adenylyl cyclase agonists induce NLRP3 ubiquitination, leading to NLRP3 degradation several hours later.

PGE₂ is a lipid mediator that regulates the progression of inflammation. It signals through four different receptors of the E-prostanoid (EP) family: EP1, EP2, EP3 and EP4 (ref. 11). Both EP2 and EP4 activate adenylyl cyclases and turn on PKA via cAMP. PGE₂, signaling via the EP4 receptor was recently reported to reduce alum-induced activation of the NLRP3 inflammasome while having little suppressive effect on other NLRP3 stimuli. Although a role for PKA in inflammasome inhibition has not been described, PKA is the
principle cAMP-effector protein in mammalian cells. Here we investigated a mechanism involving rapid PGE₂-induced suppression of the NLRP3 inflammasome.

RESULTS

PGE₂ instantly suppresses the NLRP3 inflammasome

We treated lipopolysaccharide (LPS)-primed bone-marrow-derived macrophages (BMDMs) with inflammasome activators and PGE₂. PGE₂ can inhibit transcription of pro-inflammatory genes, including IL1B. In previous studies, however, IL-1β was assessed when macrophages were treated with PGE₂ during LPS priming. To assess whether PGE₂ could post-translationally suppress inflammasome activation, we first treated BMDMs with LPS for 3.5 h to upregulate inflammasome components and substrates, including NLRP3 and IL-1β (Fig. 1a,b and Supplementary Fig. 1a). We then applied PGE₂ 5 min before inflammasome stimulation. We initially selected the NLRP3 agonists nigericin and ATP because inflammasome activation can be assessed rapidly post-stimulation (30–45 min), and thus potential effects on secondary protein translation are minimized. We found that PGE₂ did not reduce expression of inflammasome components (Fig. 1a,b and Supplementary Fig. 1a). PGE₂ dose-dependently inhibited proteolytic activation and release of caspase-1 and IL-1β into the culture supernatants in response to nigericin and ATP and potently suppressed pyroptosis, a type of inflammatory cell death driven by caspase-1 following NLRP3 inflammasome activation (Fig. 1a-c and Supplementary Fig. 1b). PGE₂ also abruptly terminated inflammasome activation when it was added 30 min after nigericin stimulation and measured at 50 min post-activation, revealing that active inflammasomes were also rapidly shut off by PGE₂ (Fig. 1d and Supplementary Fig. 1c). The suppressive effect of PGE₂ was independent of PGE₂ metabolites produced by the enzyme 15-hydroxy PGDH, as 16,16-dimethyl PGE₂, an active PGE₂ analog that is not oxidized by 15-hydroxy PGDH, had the same suppressive effect (Fig. 1e,f and Supplementary Fig. 1d). Furthermore, the inactive PGE₂ metabolite 15-keto PGE₂, which does not stimulate EP receptors, had no inhibitory activity on inflammasome activation (Fig. 1e,f and Supplementary Fig. 1d). The rapid response to PGE₂ was mirrored in human THP-1 macrophages (Supplementary Fig. 1e-g). Assembly of the NLRP3 inflammasome is nucleated by the adaptor protein ASC, which itself undergoes oligomerization following NLRP3 activation and can be detected by immunoblot after chemical cross-linking cell lysates. ASC oligomerization and caspase-1 cleavage in cells treated for 5 min as indicated and stimulated with nigericin for 30 min. (h,i) Cells treated with indicated concentrations of PGE₂ and then stimulated with silica (250 μg/ml for 5 h) or MSU (150 μg/ml for 4 h). Immunoblot analyses of culture supernatants (SN) and lysates (LYS) are shown. *P < 0.005 (unpaired two-tailed t test). Data are from one experiment that is representative of three separate experiments with similar results. BMDMs were derived from two mice per experiment, mixed together. In c, four replicates per condition are shown. Each well of 500,000 ± s.e.m. BMDMs is a replicate.
show that PGE₂ broadly inhibits NLRP3 inflammasome activation. However, the strength of inhibition differed for rapid-acting extracellular agonists versus slow-acting particulate agonists that trigger the phagocytic pathway, indicating that the mechanism of suppression is different.

**PGE₂ selectively inhibits the NLRP3 inflammasome**

We next addressed whether PGE₂ is a selective NLRP3 inflammasome inhibitor or whether PGE₂ is a broad-spectrum inflammasome inhibitor and tested the other well-established inflammasomes. BMDMs were stimulated with anthrax lethal toxin, flagellin or poly(dA:dT) to activate the NLRP1, NLRC4 and AIM2 inflammasomes, respectively. In contrast with NLRP3, PGE₂ did not suppress the NLRP1, NLRC4 or AIM2 inflammasomes, indicating that PGE₂ is a potent and specific inhibitor of the NLRP3 inflammasome (Fig. 2a–c).

In addition to canonical activation, NLRP3 is triggered via a non-canonical pathway by the caspase-11 inflammasome. We tested whether PGE₂ inhibits the caspase-11 inflammasome and/or caspase-11-driven NLRP3 activation. To dissect this, we delivered *Escherichia coli* LPS (O111:B4) into the cytosol of PAM₃CSK₄-primed BMDMs. After 2 h, caspase-11 and IL-1β release were measured to assess the caspase-11-dependent, but NLRP3-inflammasome-independent, arm of the caspase-11 inflammasome. To assess the NLRP3-, ASC- and caspase-11-dependent arm, we measured IL-1β maturation (note that caspase-1 secretion was undetectable at 2 h post-LPS stimulation). We observed PGE₂ inhibited IL-1β in response to cytosolic LPS (Fig. 2d). Notably, however, caspase-11 activation and IL-1β release were increased (Fig. 2d). We concluded that, although PGE₂ enhanced caspase-11 inflammasome activation, it suppressed the NLRP3 inflammasome.

**EP4 mediates PGE₂ inhibition of NLRP3 inflammasomes**

LPS-primed BMDMs expressed all four EP receptors (Fig. 3a). We tested highly selective agonists of each EP receptor to determine which were involved in suppressing the NLRP3 inflammasome. EP agonists were applied at different concentrations 5 min before stimulation with nigericin or ATP. EP1 and EP3 agonists had no effect on inhibition of NLRP3 inflammasomes (Supplementary Fig. 3a–d). EP4 receptor agonists (ONO-AE1-329 and CAY10598) dose-dependently suppressed caspase-1 and IL-1β, and this was reversed in the presence of EP antagonists (L16198 and ONO-AE3-208) (Fig. 3b–d and Supplementary Fig. 3e–i). EP2 receptor activation (ONO-AE1-259) also inhibited inflammasome activation and was reversed by an antagonist (AH6809). However, EP2
inhibition was less robust than EP4, and the dose response occurred at lower agonist concentrations (Fig. 3e,f and Supplementary Fig. 4a–c). We noted EP4 agonists inhibition occurred at a similar concentration as PGE2. Correspondingly, ASC multimer formation and intracellular caspase-1 processing was abolished by EP4 agonists and was reversed in the presence of EP4 antagonists (Fig. 3g–i). EP4 agonists also abruptly terminated inflammasomes that were pre-activated and potently suppressed pyroptosis (Fig. 3h,i and Supplementary Fig. 4d). EP4 receptor stimulation also inhibited caspase-11-driven NLRP3 inflammasome activation while enhancing caspase-11 inflammasome activation (Fig. 3j). To determine which EP receptor was required for PGE2-mediated inhibition of the NLRP3 inflammasome, we added EP4 or EP2 antagonists to the culture media before addition of PGE2. Although PGE2-induced suppression was not reversed by EP2 receptor antagonism (AH6809), an EP4 receptor antagonist (ONO-AE3-208) almost completely restored activation and secretion of caspase-1 and IL-1β (Fig. 3k,l and Supplementary Fig. 4e–h). Similarly, the EP4 receptor antagonist restored ASC multimer formation and intracellular caspase-1 cleavage in the presence of PGE2 (Fig. 3l).

**CAMP-PKA mediates rapid PGE2 NLRP3 inflammasome inhibition**

EP4 and EP2 both couple through G12 to activate adenyl cyclase and increase intracellular CAMP11. Several studies have shown that CAMP inhibits NLRP3 activation and that autophagy-mediated degradation of NLRP3 is triggered by CAMP7–9. Similarly, we found that adenyl cyclase activation by forskolin and the CAMP analog dibutyryl cAMP (db-cAMP) suppressed nigericin- and ATP-elicited NLRP3 activation in both BMDMs and human THP-1 macrophages (Fig. 4a and Supplementary Fig. 5a–d). Similar to EP2 and EP4 receptor stimulation, CAMP inhibited NLRP3-driven pyroptosis and rapidly shut off the NLRP3 inflammasome post-activation (Fig. 4b,c and Supplementary Fig. 5e). CAMP also inhibited caspase-11-triggered NLRP3 activation while enhancing caspase-11 inflammasome activation (Fig. 4d). These data indicate

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**Figure 3** PGE2 receptor EP4 and EP2 inhibit the NLRP3 inflammasome. (a–i,k,l) BMDMs were primed for 3.5 h with LPS. (b,c) 5-min treatment with the indicated concentration of EP4 agonists CAY10598 (b) and ONO-AE1-329 (c) or treatment first with EP4 antagonist ONO-AE3-208 (2 μM) followed by ONO-AE1-329 (500 nM) for 5 min (d) and then nigericin (5 μM for 30 min). (e,f) Treatment with the indicated concentration of EP2 agonist ONO-AE1-259-01 (e) or first with EP2 antagonist AH6809 (2 μM) (f) followed by nigericin. (g) Cells were treated as in d and cross-linked insoluble cell lysates were immunoblotted for ASC. (h) CAY10598 (500 nM) was added 30 min after nigericin stimulation and analyzed 50 min post nigericin. (i) LDH release after 5-min treatment with CAY10598 (500 nM) followed by nigericin for 60 min. (j) Cleaved caspase-11 and IL-1β (left) and total IL-1α and IL-1β (right) in cell supernatants (SN) and lysates (LYS) were analyzed by ELISA as indicated. * P < 0.005 (unpaired two-tailed t test). Data are from one experiment that is representative of three separate experiments with similar results. BMDMs were derived from two mice per experiment, mixed together. Four replicates (i) and three replicates (j) per condition are shown. Each well of 500,000 + s.e.m. BMDMs is a replicate.
that cAMP inhibition occurs too rapidly for autophagy-mediated NLRP3 degradation, which has been reported to take several hours\(^7\). NLRP3 expression was unchanged by forskolin or db-cAMP during a 30-min nigericin stimulation and was unchanged when cAMP was used to abruptly turn off pre-activated NLRP3 inflammasomes (Fig. 4c and Supplementary Fig. 5f). Assembly and auto-activation of the NLRP3 inflammasome occurs in HEK cells transfected with plasmids encoding the NLRP3 inflammasome\(^9\). Using this model, we tested whether rapid cAMP signaling directly triggers disassembly of active NLRP3 inflammasomes. When HEK cells were cultured with forskolin for 15 min, ASC oligomerization and NLRP3 secretion into the culture supernatants were abolished, whereas cellular expression of NLRP3 remained unchanged (Fig. 4e). We conclude that cAMP rapidly inhibits NLRP3

![Image](https://example.com/image.png)

**Figure 4** cAMP-PKA signaling mediates NLRP3 inflammasome inhibition. (a–f–j) BMDMs were primed for 3.5 h with LPS. (a) Indicated concentrations of forskolin were added 5 min before treatment with nigericin. (b) LDH release in cells treated for 5 min with forskolin (50 \(\mu\)M) and stimulated with nigericin for 60 min. (c) Forskolin (50 \(\mu\)M) was added 30 min after stimulation with nigericin and analyzed 50 min post initial nigericin stimulation. (d) Cleaved caspase-11 and IL-1\(\beta\) (top) and total IL-1\(\alpha\) and IL-1\(\beta\) were analyzed by ELISA (bottom) in cell supernatants of PAM\(_3\)CSK\(_4\)-primed BMDMs treated for 5 min with forskolin (50 \(\mu\)M) followed by LPS (2 \(\mu\)g/ml and 2.5 \(\mu\)l/ml FuGENE for 2 h). (e) HEK293A cells transfected with NLRP3 inflammasome components and stimulated with forskolin (50 \(\mu\)M) for 15 min. UT, untreated. (f–h) Indicated concentrations of PKA agonist 6-Bnz-cAMP (f). Epac1/2 agonist 8-CPT-2-O-Me-cAMP (h) or 50 \(\mu\)M of each (g) were added before nigericin for 30 min. (i–j) Rp-cAMP (2 \(\mu\)M) (i) or PKI (1 \(\mu\)M) (j) were added before db-cAMP (1 \(\mu\)M) or forskolin and treated with nigericin for 30 min. Culture supernatants (SN) and lysates (LYS) were analyzed by immunoblot as indicated. Cross-linked insoluble cell lysates (top) are shown in e and g. *\(P<0.005\) (unpaired two-tailed t-test). Data are from one experiment that is representative of three separate experiments with similar results. BMDM were derived from two mice per experiment mixed together Four replicates (b) and three replicates (d) per condition are shown. Each well of 500,000 + s.e.m. BMDMs is a replicate.
independent of autophagy-mediated degradation and promptly induces disassembly of active NLRP3 complexes.

Given that cAMP signaling is primarily mediated by PKA and the GTPase exchange proteins directly activated by cAMP 1 and 2 (Epac1/2), we first tested whether agonists that selectively activate PKA or Epac1/2 suppress the NLRP3 inflammasome. We found the PKA-selective small molecule cAMP analog 6-Bnz-cAMP dose-dependently inhibited caspase-1 and IL-1β activation in response to nigericin, whereas the Epac1/2-selective activator 8-pCPT-2′-O-Me-cAMP had no effect (Fig. 4f and Supplementary Fig. 6a,b). 6-Bnz-cAMP also abolished ASC oligomerization and intracellular caspase-1 processing (Fig. 4g). 6-Bnz-cAMP did not reduce NLRP3 expression during nigericin treatment (Supplementary Fig. 6c). Silica- and MSU-induced NLRP3 activation were not inhibited by 6-Bnz-cAMP, suggesting that PKA does not inhibit this pathway of NLRP3 activation (Supplementary Fig. 6d). To further confirm these results, we used two different antagonists of PKA and Epac1/2: Rp-cAMP and PKI-(Myr-24-22)-amide (PKI). Rp-cAMP acts as an inhibitor of both PKA and Epac1/2, whereas PKI, a cell-permeable version of the endogenously expressed PKI peptide, selectively inhibits PKA, but not Epac1/2 (refs. 21,22). Both Rp-cAMP and PKI restored activation and secretion of caspase-1 and IL-1β, assembly of ASC multimers, and intracellular caspase-1 cleavage in the presence of db-cAMP and forskolin (Fig. 4i and Supplementary Fig. 6e,f). Thus, we conclude that PKA mediates suppression of rapid NLRP3 inflammasome activation in response to elevated cAMP.

To determine whether PKA mediates rapid PGE2-induced inhibition of NLRP3 inflammasome, we tested Rp-cAMP and PKI

**Figure 5** Rapid PGE2 NLRP3 inflammasome inhibition by PKA. (a-f) BMDMs were primed for 3.5 h with LPS. (a,b) Rp-cAMP (2 μM) (a) or PKI (1 μM) (b) were added before PGE2 (500 nM) for 5 min followed by nigericin (5 μM for 30 min). (c) Non-targeting siRNA (1 μM) or PKA C-α and PKA C-β siRNA (0.5 μM each) were added for 96 h. Cells were treated for 5 min with PGE2 (500 nM) before nigericin (5 μM for 30 min). (d-f) PKI (d) or Rp-cAMP (f) were added before EP2 agonist ONO-AE1-259-01 (50 nM) (d) or EP4 agonist ONO-AE1-329 (500 nM) (e,f) followed by nigericin for 30 min. Culture supernatants (SN) and lysates (LYS) were analyzed by immunoblot as indicated. (a,b,e,f) Cross-linked insoluble cell lysates (top) are shown in a, b, e and f. *P < 0.005 (unpaired two-tailed t test). Data are from one experiment that is representative of three separate experiments with similar results. BMDMs were derived from two mice per experiment, mixed together. Three replicates (c) per condition are shown. Each well of 500,000 + s.e.m. BMDMs is a replicate.
with PGE₂. PGE₂-induced inhibition of nigericin-induced NLRP3 inflammasome was reversed by both Rp-cAMP and PKI (Fig. 5a,b and Supplementary Fig. 6g,h). To further verify these results, we treated BMDM with or without PGE₂ in the presence of siRNA knockdown of the PKA catalytic α and β (PKA C-α and C-β) subunits. When compared with cells transfected with non-targeting siRNA, cells transfected with PKA-targeting siRNA had substantially less inhibition of NLRP3 activation in response to PGE₂ stimulation (Fig. 5c). Similarly, blockade of nigericin-elicited NLRP3 inflammasome activation via EP2 and EP4 receptor pathways was prevented if PKA antagonists were added before cells were stimulated with either an EP4 or EP2 agonist (Fig. 5d–f and Supplementary Fig. 7a–d). PGE₂-induced inhibition of MSU and silica was not reversed by PKI or Rp-cAMP, indicating that PGE₂-induced suppression of MSU and silica was not PKA mediated (Supplementary Fig. 7e,f). Collectively, our data support the idea that rapid PGE₂-mediated suppression of the NLRP3 inflammasome via the EP4 receptor occurs via cAMP-PKA signaling. Similarly, the EP2 represses NLRP3 activation via the same pathway.

**PKA directly phosphorylates NLRP3 and turns off the ATPase**

We next sought to understand how PKA inhibits the NLRP3 inflammasome. We scanned human and mouse NLRP3 for consensus patterns of cAMP- and cGMP-dependent protein kinase phosphorylation sites. Using Prosite, we identified two sites in mouse NLRP3 and four sites in human NLRP3 that matched the [RK](2)–X–[ST] consensus sequence (Fig. 6a). Both sites present in mouse NLRP3 are conserved in human NLRP3: an RKPS site at residues 292–295

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**Figure 6** cAMP-PKA induces NLRP3 phosphorylation. (a) Diagram of NLRP3 human and locations of consensus patterns for cAMP and cGMP-dependent protein kinase phosphorylation determined using Prosite and the [RK](2)–X–[ST] consensus sequence indicated in blue and underlined. Asterisks indicate the predicted PKA phosphorylation residue. Amino acids in red are disease-causing CAPS mutations. Dots indicate mutations tested in Figures 7 and 8. (b–d) Human GST-NLRP3 fusion protein was used as a substrate in in vitro kinase reactions using purified PKA catalytic subunit. (b) Coomassie stain (left) and γ-[32P]-ATP autoradiograph (right) at increasing times. (c) Stoichiometry of NLRP3 phosphorylation by PKA for the indicated times. (d) Reactions were carried out in the presence of PKI (1 μM). Phosphorylated NLRP3 was detected using a phospho-PKA substrate antibody. (e) ATP-P32 autoradiograph of GST-NLRP3 + PKA (min). Phosphorylated NLRP3 was detected using a phospho-PKA substrate antibody. (f) P04 release from ATP in the presence of human GST-NLRP3 that was first incubated in PKA kinase reaction followed by separation on a 100-kDa cut-off filter. (g–i) Immunoprecipitation of indicated protein in PMA-treated THP-1 cells treated with forskolin (50 μM) for 5 min in the presence or absence of PKI (1 μM) or PMA-differentiated THP-1 cells (i) treated with non-targeting siRNA (1 μM) or PKA C-α and PKA C-β siRNA (0.5 μM each) for 96 h. *P < 0.005 (unpaired two-tailed t test). Data in d–i are from one experiment that is representative of three separate experiments with similar results. Data in b and c are from one experiment performed once. Data in e represent four reactions with two technical replicates ± s.d.
(human numbering) located in the NBD and a KKLS site at 594–597 positioned just outside the NBD (Fig. 6a). Notably, among the four consensus patterns in human, the RKPS and KKLS are uniquely adjacent to NLRP3 disease-causing CAPS mutations (Fig. 6a)23–25. Ser597 in the KKLS site is a reported NOMID mutation, the most severe form of CAPS (Fig. 6a)1.3. This analysis prompted us to investigate whether PKA phosphorylates NLRP3 and whether the consensus sites function in rapid cAMP-induced suppression of the NLRP3 inflammasome.

To determine whether NLRP3 is a target for PKA phosphorylation, we performed in vitro PKA kinase assays using a commercial full-length NLRP3 construct, GST-NLRP3, as a substrate and the PKA catalytic subunit in the presence of [γ-32P]ATP (Fig. 6b). Autoradiography of the reaction products revealed that PKA phosphorylated the 145-kDa GST-NLRP3 in a time-dependent manner (Fig. 6b). In a stoichiometry assay, PKA phosphorylated GST-NLRP3 to about 0.6 mol of 32P per mol of GST-NLRP3, whereas 32P was not incorporated into GST incubated with PKA, NLRP3 incubated without PKA or PKA incubated alone (Fig. 6c). We also determined that a phospho-PKA substrate antibody that recognizes the [RK](2)-X-[ST] consensus sequence when arginine is in the –3 position detected GST-NLRP3 in a PKA kinase reaction and was inhibited by the PKA inhibitor PKI (Fig. 6d). Active PKA is recognized by the phospho-PKA substrate antibody30 and we used it as a positive control (Fig. 6d). Together, these data indicate that NLRP3 is a target of PKA phosphorylation.

We next determined whether PKA phosphorylation directly inhibits NLRP3 ATPase function. ATP hydrolysis by the NBD regulates NLRP3 self-oligomerization and assembly of the inflammasome complex required for caspase-1 and IL-1β activation31. We performed an ATPase assay to assess NLRP3 activity after PKA phosphorylation. (Fig. 6e and Supplementary Fig. 8a). PKA incubated in kinase buffer

![Figure 7](https://www.nature.com/natureimmunology/journal/v13/n11/fig/supplementary/natureimmunology201611fig7f07-i1-9974.jpg)

**Figure 7** NLRP3 Ser295 mediates rapid inhibition by cAMP. (a–c) HEK293A cells transfected with plasmids encoding WT NLRP3, T169V NLRP3, S295A NLRP3, S975A NLRP3 or D305G NLRP3, along with plasmids encoding pro-caspase-1, ASC and IL-1β. 20 h after transfection, cells were changed into OptiMEM (serum-free medium) and stimulated with forskolin (50 µM) for 60 min (a,b) or 15 min (c). (d) In vitro PKA kinase assay of His-NLRP3 proteins (WT, S295A, D305G) produced in HEK293T cells. P32-labeled proteins were detected by autoradiography (top left). NLRP3 immunoblot in which equivalent volumes His-NLRP3 proteins were loaded is shown at the bottom left. Densitometry of P32-labeled proteins normalized to NLRP3 loading control are shown at right. (e) PO4 release from ATP in the presence of indicated human His-NLRP3 proteins that were first incubated in PKA kinase reactions and separated on a 100-kDa cut-off filter (left). NLRP3 immunoblot in which equivalent volumes of purified His-NLRP3 proteins were loaded is shown at right. Culture supernatants (SN) and lysates (LYS) were analyzed by immunoblot as indicated. Cross-linked insoluble cell lysates (top) are shown in c. *P < 0.005 (unpaired two-tailed t test). Data are from one experiment that is representative of three separate experiments with similar results. Data in e represent two separate reactions with two technical replicates ± s.d.
and passed through the 100-kDa cut-off filter served as a negative control and retained no ATPase activity in the fraction above 100 kDa (Fig. 6e). When NLRP3 was incubated without PKA, the high-molecular-weight fraction hydrolyzed ATP, indicating that NLRP3 was functional (Fig. 6e). When NLRP3 and PKA were incubated in kinase buffer together, the high-molecular-weight fraction containing GST-NLRP3 had undetectable levels of ATPase activity following separation, similar to the negative control (Fig. 6e). Thus, PKA phosphorylation of NLRP3 inhibited the NLRP3 ATPase activity, which is required for assembly of NLRP3-ASC complexes.

We next sought to determine whether NLRP3 is phosphorylated by PKA endogenously and whether PKA and NLRP3 are in a complex together. We immunoprecipitated the catalytic isoforms of PKA expressed in macrophages, PKA C-α and PKA C-β, as well as NLRP3 from PMA-primed THP-1 cells and LPS-primed BMDMs (Fig. 6f–j and Supplementary Fig. 8b,c). We observed NLRP3 in PKA C-α immunoprecipitates from unstimulated macrophages (Fig. 6f and Supplementary Fig. 8b). However, the amount of NLRP3 substantially increased following forskolin stimulation and was reduced by PKA inhibition with PKI. NLRP3 was present in PKA C-β immunoprecipitates in THP-1 cells similar to PKA C-α, but not in BMDMs, suggesting that both isoforms may regulate NLRP3 in human macrophages (Fig. 6g). When THP-1 cells and BMDMs were treated with forskolin we detected active PKA C-α in NLRP3 immunoprecipitates (Fig. 6h and Supplementary Fig. 8c).

Furthermore, the phospho-PKA substrate antibody detected immunoprecipitated NLRP3 following forskolin treatment and was abrogated by PKI (Fig. 6h). The phospho-PKA substrate antibody detected NLRP3 in forskolin-stimulated BMDMs, but much less was detected than in THP-1 cells (Supplementary Fig. 8c). To further confirm the role of PKA in forskolin-induced NLRP3 phosphorylation, we treated THP-1 cells with or without forskolin in the presence of siRNA knockdown of PKA C-α and C-β (Fig. 6j). Note that we achieved PKA C-α knockdown in THP-1 cells, whereas PKA C-β expression was unaltered. When compared with cells transfected with non-targeting siRNA, the phospho-PKA substrate antibody detected significantly less NLRP3 phosphorylation in response to forskolin in cells transfected with PKA-targeting siRNA (Fig. 6j). Furthermore, NLRP3 immunoprecipitates contained less PKA C-α in cells that received PKA-targeting siRNA (Fig. 6j). The level of reduced NLRP3 phosphorylation and PKA C-α in NLRP3 immunoprecipitates correlated with the degree of total PKA C-α knockdown. Taken together, these data suggest that endogenous NLRP3 is phosphorylated by PKA and they are present in the same complex.

Ser295 is essential for rapid cAMP-induced inhibition

Next we addressed the PKA consensus sites involved in rapid cAMP-induced NLRP3 inhibition. To study this, we transfected HEK cells,
which do not express NLRP3 inflammasome components, with plasmids for pro-caspase-1, ASC, pro-IL-1β and NLRP3 (ref. 19). The serine or threonine residues that would be PKA phosphorylated in each consensus site of human NLRP3 were mutated to alanine and valine, respectively, and assessed for whether they abolished cAMP-induced inhibition (Fig. 7a). HEK cells were stimulated with forskolin for 60 min, after which culture media was measured for secreted NLRP3, active caspase-1 and mature IL-1β (Fig. 7a). In this system, the NLRP3 inflammasome is auto-activated and does not require a stimulus10. In HEK cells expressing wild-type NLRP3, forskolin inhibited secretion of NLRP3, active caspase-1 and mature IL-1β without reducing the cellular expression of NLRP3, ASC or pro-caspase-1 (Fig. 7a). The mutants T169V (KRYT), S597A (KKLS) and S957A (RKLS) all suppressed NLRP3 inflammasome activation in response to forskolin similar to wild type (Fig. 7a). However, we found the S295A (RKPS) mutant nullified forskolin-induced inhibition. We observed increased secretion of active inflammasome components (most prominently NLRP3 and IL-1β) following forskolin treatment in cells expressing S295A, indicating that NLRP3 inflammasome activation was enhanced by cAMP (Fig. 7a). Cellular expression of NLRP3, ASC and pro-caspase-1 was similar between wild-type and the NLRP3 mutants and was not decreased by forskolin (Fig. 7a).

The RKPS site is embedded in a large cluster of CAPS mutations from residues 291–315 located in the NBD (Fig. 6a). The molecular bases for activating NLRP3 mutations that cause CAPS have been elusive. We tested the hypothesis that mutations near the RKPS disrupt rapid cAMP-induced NLRP3 inflammasome inhibition. Initially, we mutated residue Asp305, as it is reported to be mutated in NOMID, MW and FCAS. We selected a NOMID mutation, D305G, expecting that it would have the most exaggerated phenotype, as NOMID is the most severe manifestation of CAPS. Similar to the S295A mutant, cAMP-induced inhibition of the NLRP3 inflammasome was completely attenuated in cells expressing D305G (Fig. 7b). The D305G mutant phosphorylated S295A in that forskolin enhanced secretion of NLRP3, as well as mature IL-1β (Fig. 7b). We tested whether cAMP inhibited NLRP3-dependent ASC oligomerization in cells expressing the S295A and D305G mutations. In contrast with wild-type NLRP3, in cells expressing S295A or D305G NLRP3, ASC oligomerization remained intact in response to forskolin (Fig. 7c). Thus, we conclude that S295A and D305G NLRP3 are unresponsive to cAMP-induced disassembly of ASC complexes.

Next, we purified full-length NLRP3 constructs with N-terminal histidine tags from HEK293T cells and performed in vitro PKA kinase assays in the presence of [γ-32P]ATP. We compared phosphorylation between wild-type NLRP3 and the S295A and D305G mutants. To determine the relative phosphorylation of each protein, we used an NLRP3 immunoblot containing equivalent sample volumes to normalize the data. Autoradiography of the reaction products revealed that, compared with wild-type NLRP3, the S295A and D305G mutants were significantly less phosphorylated by PKA and that the reduction was similar between each mutant (Fig. 7d). This indicates that D305G does not undergo phosphorylation on Ser295. We next assessed the ATPase activity of the NLRP3 S295A and D305G mutants and whether PKA affects ATP hydrolysis. We performed ATPase assays using full-length NLRP3 constructs with N-terminal histidine tags from HEK293T cells. In contrast with wild-type NLRP3, the S295A and D305G mutants retained ATPase activity after PKA incubation (Fig. 7e). Thus, ATP hydrolysis, which regulates inflammasome assembly, was not inhibited by PKA in S295A and D305G NLRP3 mutants. We observed slightly elevated levels of ATP hydrolysis when S295A and D305G mutants were incubated with PKA (Fig. 7e). This may explain why increased secretion of active inflammasome components occurred following forskolin treatment when cells expressed the S295A and D305G mutants. An NLRP3 immunoblot loaded with equivalent sample volumes was used to determine relative amounts of each protein in the ATPase assay. The S295A and D305G purifications that we used contained slightly less NLRP3, accounting for the slightly lower basal ATPase activity (Fig. 7e). Together, these data suggest that Ser295 is the site of PKA phosphorylation that mediates NLRP3 inhibition, and concur with NLRP3 inflammasome activation in HEKs, where the S295A and D305G mutants did not suppress activation in response to forskolin.

**CAPS mutations linked to Ser295 are unresponsive to cAMP**

On the basis of our findings with D305G, we investigated whether other CAPS mutations surrounding Ser295 respond to rapid cAMP-induced inhibition. We tested an additional seven CAPS mutations (Fig. 8a–d). For six of the seven mutations NLRP3 inflammasome activation was not inhibited by forskolin, as determined by secretion of active inflammasome components and ASC oligomerization in the HEK model (Fig. 8a–d). Similar to S295A and D305G NLRP3, for some of these mutants we detected increased secretion of active inflammasome components (either the IL-1β p17 fragment, NLRP3 or both) and ASC oligomerization in response to forskolin. For the CAPS mutant M301V, forskolin suppressed activation, but it was significantly weaker than in wild type (Fig. 8a,c). We observed that NLRP3 secretion and ASC oligomerization was only slightly decreased for M301V, suggesting that inhibition was limited. These data reveal that CAPS mutations proximal to Ser295 render NLRP3 unresponsive to rapid cAMP-induced inhibition.

**DISCUSSION**

A fine balance between inflammasome activation and inhibition must be maintained during an effective inflammatory response. The underlying mechanisms that achieve this balance are just starting to be understood. Our data suggest that PGE2–EP4 induction of cAMP–PKA functions to tune active NLRP3 inflammasomes in real time. cAMP–PKA is among the most common and versatile signaling machinery employed in cells. Spatial and temporal specificity of cAMP–PKA signaling modules depend on the adenylyl cyclases, phosphodiesterases, PKA isozymes (comprised of different regulatory and catalytic subunits) and A kinase anchoring proteins (AKAPs) that are wired to a particular G-protein-coupled receptor. Thus, outstanding questions remain about how cAMP–PKA rapidly terminates NLRP3 and how this might differ between cell types. In particular, we noted differences between HEKs and macrophages in the duration of forskolin inhibition. The inhibitory response was relatively short-lived in HEKs and differences remain about how cAMP–PKA rapidly terminates NLRP3 and how this might differ between cell types. In particular, we noted differences between HEKs and macrophages in the duration of forskolin inhibition. The inhibitory response was relatively short-lived in HEK cells: by 30 min, reversal of cAMP-induced inhibition was evident on the basis of ASC oligomerization and disappeared by 60 min. Thus, for secreted NLRP3 inflammasome components, which lag slightly behind ASC oligomerization, we observed forskolin-inhibition for wild-type NLRP3 at 60 min. However, this was much less pronounced by 90 min. This indicates that PKA inhibition is reversible and modified by other signaling, and happens differently among cell types. This may explain why PKA activation did not inhibit slow-acting NLRP3 stimuli. It may also explain why PGE2-induced inhibition was not observed in peripheral blood mononuclear cells (PBMCs) with nigericin or ATP. Given that a longer PGE2 pre-treatment was used, differences in reversal rates of PGE2-induced cAMP–PKA activity in PBMCs versus macrophages might exist.
Together, the emerging picture reveals that PGE$_2$ and cAMP regulation of inflammasomes is multifaceted. PGE$_2$ appears to selectively inhibit NLRP3, but does this by PKA-dependent and PKA-independent mechanisms depending on the NLRP3 stimulus. It would be relevant to further investigate how PGE$_2$-EP4-cAMP pathway enhances caspase-11 inflammasome activation and its functional relevance in tuning inflammasome functions. Given the pathological role of the NLRP3 inflammasome in chronic inflammatory diseases, as well as the critical role of caspase-11 in driving sepsis, it would be interesting to determine whether dysregulated PGE$_2$ and/or cAMP regulation contributes to these conditions.

In human NLRP3, our data support the idea that Ser295 is phosphorylated and is the critical site for rapid cAMP-induced inhibition. Our methods, however, have not excluded the existence of additional PKA phosphorylation sites. In stoichiometry assays, γ-32P incorporation may have been upwards of 1 mol/mol, as contaminating proteins prevented a precise determination of the amount of NLRP3-GST in the reactions. We were surprised that the S957A (KKLS) mutant, which is the position of a NOMID mutation located in the other broadly conserved cAMP/GCAMP-dependent protein kinase consensus pattern, was responsive to forskolin-induced inhibition. Potentially, PKA or other kinases regulate Ser597 by a different mechanism.

We found that the NOMID mutant D305G phenocopied the S295A mutation in function and loss of phosphorylation. Further study is warranted to determine whether all mutations linked to Ser295 directly disrupt NLRP3 phosphorylation or whether indirect mechanisms exist, such as disrupted binding of a PKA-containing regulatory complex. Notably, it was recently reported that CAPS PBMCs stimulated with LPS are unresponsive to PGE$_2$-induced suppression in what are considered to be physiologically relevant PGE$_2$ concentrations. The CAPS PBMCs that showed a null response to PGE$_2$ harbored D303N and E313K mutations, which are proximal to Ser295 (ref. 9). Dissecting the mechanisms of CAPS mutations in other regions of NLRP3 will provide understanding of the molecular regulation of the NLRP3 inflammasomes.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

L.M., J.A.M. and K.C. conceived of and designed the experiments and wrote the paper; L.M., F.M. and J.A.M. performed the experiments and analyzed the data; and L.M., F.M., J.A.M. and K.C. contributed reagents, materials and analysis tools.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Male C57BL/6 and BALB/c 8–10-week-old mice were from Charles River. In each experiment, two wild-type mice were used to derive BMDM and bone marrows were pooled. The researchers were not blinded to outcome assessments for experiments. The Health Sciences Animal Care Committee from the University of Calgary, have examined the animal care and treatment protocol (M08123) and approved the experimental procedures proposed and certifies with the applicant that the care and treatment of animals was in accordance with the principles outlined in the most recent policies on the Guide to the Care and Use of Experimental Animals by The Canadian Council on Animal Care.

Reagents. Ultra-pure PS (E. coli O127:B8), PMA, forskolin, nigricin and ATP were from Sigma. Silica and MSU were a gift from Y. Shi (University of Calgary). PAM3CSK4 and ultra-pure LPS (E. coli O111:B4 for caspase-11 activation) flagglin from Salmonella typhimurium and poly (da:dT) were from Invivogen. PGE2, 15-keto PGE2, 16-16 dimethyl PGE2, CAY10598, L161982, AH6809 were from Cayman. Myristilated PKI (14-22 amide) and 6-Bnz-cAMP was from Tocris. Dibutyryl-cAMP, 8-CPT-2-O-Me-cAMP and Rp-cAMP were from Enzo. EP4 agonist (ONO-AE-329), EP4 antagonist (ONO-AE-308), EP2 agonist (ONO-AE-259-01), EP1 agonist (ONO-DI-004) and EP3 agonist (ONO-AE-248) were kindly provided by Ono Pharmaceutical. Anthrax lethal toxin, consisting of protective antigen and lethal factor was from List Biological Laboratories. Human and murine IL-1β and IL-1α (1:1,000, 2021 and 12703, Cell Signaling)23, anti-IL-1β human (1:700, 7884, Santa Cruz)24, anti-caspase-1 human (1:1,000, 622, Santa Cruz)25, anti-IL-1β mouse (1:3,000, AF401, R&D Systems)26, anti-caspase-1 (p20) mouse (1:3,000, clone Casper-1, Adipogen)27, for some cell lyses anti-caspase-1 mouse (1:1,000, 514, Santa Cruz)28 was used, anti-NLRP3 (1:1,000, clone Cryo-2, Adipogen)29, anti-caspase-11 (1:1,000, clone 17D2, Novus Biologicals)23, anti-ASC (1:1,000, 30153, Santa Cruz)27, for some experiments anti-ASC (1:1,000 AL177, Adipogen)30 was used, anti-PKA C-α (1:1,000, 4782, Cell Signaling)39, anti-PKA C-β (1:1,000, 904, Santa Cruz)39, anti-phospho (Ser/Thr) PKA substrate antibody (1:1,000, 9621, Cell Signaling)30, anti-EpC-1 receptor (1:1,000, 101740, Cayman Chemical)40, anti-EpC-2 receptor (1:1,000, 101750, Cayman Chemical)41, anti-EP3 receptor (1:1,000, 101760, Cayman Chemical)41, anti-EP4 receptor (1:1,000, 101775, Cayman Chemical)41, anti-GAPDH (1:10,000, clone 6C5, Calbiochem)44.

Immunoblotting. THP-1 or BMDM supernatants from four wells were pooled and centrifuged at 4 °C for 5 min at 2,000g. Pelleted debris was discarded and supernatants were concentrated by TCA precipitation. Precipitated supernatants were resuspended in 50 µl (THP-1) or 100 µl (BMDM) Laemmli buffer, boiled for 5 min and equal volumes were resolved on 12.5% polyacrylamide gels and transferred to nitrocellulose for cell lysates, plates were washed in cold PBS before lysis buffer (NaCl, Tris (pH 8), SDS, Triton 100-X, EDTA, PMSE, F-64, leupeptin, aprotinin, supplemented with protease inhibitor cocktail (Sigma)) and centrifuged at 4 °C for 15 min at 14,000g. Equal amounts of proteins boiled for 5 min in Laemmli buffer were resolved on 7.5–12.5% polyacrylamide gels and transferred to nitrocellulose. Membranes were blocked in 5% skim milk or in 3% BSA for detection of phospho-proteins, incubated overnight at 4 °C in primary antibodies and visualized with secondary HRP-conjugated antibodies. Supernatants were detected with SuperSignal Chemiluminescence Reagents (Pierce) and lysates with ChemiDoc (Molecular Dynamics) detection (EMD Millipore). Primary antibodies were anti-IL-1β cleaved human (1:1,000, 2021 and 12703, Cell Signaling)23, anti-IL-1β human (1:700, 7884, Santa Cruz)24, anti-caspase-1 human (1:1,000, 622, Santa Cruz)25, anti-IL-1β mouse (1:3,000, AF401, R&D Systems)26, anti-caspase-1 (p20) mouse (1:3,000, clone Casper-1, Adipogen)27, for some cell lysates anti-caspase-1 mouse (1:1,000, 514, Santa Cruz)28 was used, anti-NLRP3 (1:1,000, clone Cryo-2, Adipogen)29, anti-caspase-11 (1:1,000, clone 17D2, Novus Biologicals)23, anti-ASC (1:1,000, 30153, Santa Cruz)27, for some experiments anti-ASC (1:1,000 AL177, Adipogen)30 was used, anti-PKA C-α (1:1,000, 4782, Cell Signaling)39, anti-PKA C-β (1:1,000, 904, Santa Cruz)39, anti-phospho (Ser/Thr) PKA substrate antibody (1:1,000, 9621, Cell Signaling)30, anti-EpC-1 receptor (1:1,000, 101740, Cayman Chemical)40, anti-EpC-2 receptor (1:1,000, 101750, Cayman Chemical)41, anti-EP3 receptor (1:1,000, 101760, Cayman Chemical)41, anti-EP4 receptor (1:1,000, 101775, Cayman Chemical)41, anti-GAPDH (1:10,000, clone 6C5, Calbiochem)44.

Immunoprecipitation. For co-immunoprecipitation of NLRP3 and PKA C-α, 2 × 106 cells were lysed in NP-40 buffer (50 mM Tris HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM PMSE, protease inhibitors), centrifuged at 14,000g for 5 min at 4 °C, and supernatants were pre-cleared with a 2.5 µl volume of A/G beads (Santa Cruz) for 30 min at 4 °C. Antibodies against NLRP3 (1:50, AG-208B-0014, Adipogen), PKA C-α (1:50, 4782, Cell Signaling), PKA C-β (1:50, 904, Santa Cruz) were used to precipitate proteins overnight at 4 °C, followed by addition of a 10% volume of A/G beads for 3 h at 4 °C. Protein complexes were washed three times with NP-40 lysis buffer, boiled for 5 min in Laemmli buffer and resolved by immunoblot analysis.

ASC oligomerization assay. Cells were lysed in PBS containing 0.5% Triton X-100 and 150 mM NaCl, and the cell lysates were centrifuged at 8,000g for 15 min at 4 °C13. Triton X-100-insoluble pellets were washed three times with PBS, suspended in 200 µl PBS and then cross-linked at 22 °C for 30 min by adding DSS (2 mM) (Thermo Scientific). Reactions were terminated by adding Tris (20 mM) for 15 min at 22 °C. The cross-linked pellets were centrifuged at 8,000g for 15 min, boiled in Laemmli buffer for 5 min, and resolved by immunoblot analysis.

Generation and mutation of His-NLRP3. Plasmid pCDNA3.1-N-His encoding human NLRP3 (NLRP3-His) was purchased from GenScript (clone OHu268515). Substitution of Thr169 with Val, or Ser295, Ser597 and Ser975 with Ala, Ile290 with Met, Met301 with Val, Gly303 with Ser, Asp305 with Glu, Gly306 with lys, Gly308 with lys, Gly309 with Ser or Phe311 with Leu on the NLRP3-His plasmid was performed using the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer's directions. Recombinant proteins were expressed in HEK293T cells and purified on Ni-NTA Agarose following the manufacturer's instructions (Qiagen). Purified proteins were separated on an 8% SDS-polyacrylamide gel and stained with silver stain or immunoblotted for NLRP3 to verify the integrity and relative concentrations of expressed proteins. For NLRP3-His that was used for ATPase assays, following stimulation with nigericin (5 µM) and LPS (50 ng/ml) for 30 min or for THP-1 cells treated with forskolin (50 µM) for 5 min.

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Ni-NTA Agarose purification proteins were purified on a 100-kDa molecular weight spin column before addition to PKA phosphorylation reactions.

**NLRP3 inflammasome reconstitution in HEK293A cells.** HEK293A Cells were transfected with plasmids encoding NLRP3 inflammasome components (NLRP3, pro-caspase-1, ASC and IL-1β). After 20 h, media was changed to optimum and cells were stimulated with forskolin (50 µM) for 60 min or time stated. Cell lysates and secreted cell supernatants were analyzed for NLRP3, caspase-1 and IL-1β.

**In vitro PKA phosphorylation assay and measurement of NLRP3 ATPase activity.** To test in vitro PKA phosphorylation of NLRP3, NLRP3-GST fusion protein (0.4 µg) (Abnova) or 30 µl of purified NLRP3-His proteins were resuspended in a buffer containing 100 mM HEPES, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol, 10 mM β-glycerophosphate, 100 µM ATP, 10 units of the catalytic subunit of PKA (P2645, Sigma) and 10 µCi [γ-32P]ATP in a final reaction volume of 50 µl and incubated at 22 °C. Reactions were stopped by adding 5 x SDS-PAGE loading buffer. 32P-labeled proteins were resolved on an 8% SDS-polyacrylamide gel, dried and detected by autoradiography.

For stoichiometry assays phosphorylation of NLRP3-GST (0.4 µg) by PKA was carried out at 22 °C in buffer containing 100 mM HEPES, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol, 10 mM β-glycerophosphate, 100 µM ATP, 10 units of the catalytic subunit of PKA and 0.2 mM [γ-32P]ATP. The reactions were initiated by addition of [γ-32P]ATP and the stoichiometry of NLRP3-GST phosphorylation was measured by terminating the kinase reactions with 25% TCA, and the radioactivity incorporated into the precipitated proteins was assessed by Cerenkov counting.

For in vitro PKA phosphorylation of NLRP3 for ATPase assay, NLRP3-GST fusion protein (0.4 µg) or 10 µl of purified NLRP3-His proteins were suspended in buffer containing 50 mM Tris-HCL (pH 7.4), 10 mM MgCl2, 1 mM dithiothreitol, 10 mM β-glycerophosphate, 100 µM ATP, and 10 units of the catalytic subunit of PKA in a final 30 µl reaction volume. Phosphorylation reactions were incubated at 30 °C for 40 min and terminated by spinning the reaction on a 100-kDa molecular weight spin column at 4 °C 5 times for 10 min and once for 30 min in ATPase buffer without ATP to remove the PKA catalytic subunit (42 kDa) and PKA kinase buffer from phosphorylated GST-NLRP3 (145 kDa) or NLRP3-His proteins (120 kDa). Phosphorylated GST-NLRP3 or NLRP3-His proteins were then incubated for 1 h at 37 °C in reaction buffer (25 mM Tris-HCL, pH 7.5, 150 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 1 mM PMSE, and 1× protease inhibitor cocktail, 250 µM ATP). The hydrolysis of ATP by NLRP3 was measured by P, ColorLock Gold phosphate detection system (Innova Bioscience) according to manufacturer’s instructions.

**Statistical analysis.** All experiments shown are representative of three independent experiments unless otherwise indicated. Treatment groups were compared using the paired two-tailed Student’s t-test. GraphPad Prism4 was used for statistical analysis. A P value of ≤ 0.05 was considered statistically significant. The number of reproduced experimental repeats is described in the relevant figure legends. The investigators were not blinded to allocation during experiments and outcome assessment.


