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Prostaglandin E₂ inhibits NLRP3 inflammasome activation through EP4 receptor and intracellular cAMP in human macrophages

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Abstract

Prostaglandin E₂ (PGE₂) is a potent lipid mediator involved in maintaining homeostasis but also promotion of acute inflammation or immune suppression in chronic inflammation and cancer. NLRP3 inflammasome plays an important role in host defense. Uncontrolled activation of NLRP3 inflammasome, due to mutations in the *NLRP3* gene causes cryopyrin-associated periodic syndromes (CAPS). Here, we showed that NLRP3 inflammasome activation is inhibited by PGE₂ in human primary monocyte-derived macrophages. This effect was mediated through prostaglandin E receptor 4 (EP4) and an increase in intracellular cAMP, independently of protein kinase A (PKA) or exchange protein directly activated by cAMP (Epac). A specific agonist of EP4 mimicked, while its antagonist or EP4 knockdown reversed PGE₂-mediated NLRP3 inhibition. PGE₂ caused an increase in intracellular cAMP. Blockade of adenylate cyclase by its inhibitor reversed PGE₂-mediated NLRP3 inhibition. Increase of intracellular cAMP by an activator of adenylate cyclase or an analog of cAMP, or a blockade of cAMP degradation by phosphodiesterase inhibitor decreased NLRP3 activation. PKA or Epac agonists did not mimic and their antagonists did not reverse PGE₂-mediated NLRP3 inhibition. In addition, constitutive IL-1 β secretion from LPS-primed PBMCs of CAPS patients was substantially reduced by high doses of PGE₂. Moreover, blocking cytosolic phospholipase A₂ α by its inhibitor or siRNA or inhibiting cyclooxygenase 2, resulting in inhibition of endogenous PGE₂ production, caused an increase in NLRP3 inflammasome activation. Our results suggest that PGE₂ might play a role in maintaining homeostasis during the resolution phase of inflammation and might serve as an autocrine and paracrine regulator.

Keywords

inflammasome; prostaglandin; PGE₂; phospholipase; cAMP; macrophage; COX2; cPLA₂

Introduction

The inflammasomes are a group of multimeric cytosolic protein complexes that consist of an inflammasome sensor molecule, adaptor protein ASC and caspase-1. Activation of caspase-1 by this complex leads to the processing of pro-IL-1 β and pro-IL-18 to their mature active forms (1). Inflammasome forming sensor molecules encompass the family of the nucleotide binding domain, leucine rich repeat-containing proteins (NLRs) including NLRP1, NLRP3, NLRP6, NLRP7, NLRP12 and NLRC4 as well as two structurally different proteins, AIM2 and IFI16 (2). They have been shown to form inflammasomes upon activation by a variety of stimuli, through several different activation pathways, mainly in dendritic cells and macrophages (3). Among them, NLRP3 is best characterized and is connected to the pathogenesis of a variety of diseases (4) including inflammatory bowel disease and colon cancer (5), diabetes type II (6), gout (7), atherosclerosis (8) and Alzheimer disease (9). NLRP3 consists of an N-terminal pyrin domain, a central nucleotide binding domain (NBD), and a C-terminal leucine rich domain (LRR) (10). Gain-of-function mutations in the *NLRP3* gene, mainly clustered in the NBD domain, result in its activation or predisposition for activation, and are associated with the cryopyrin-associated periodic fever syndromes (CAPS) including familial cold-induced autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS) and neonatal onset multisystem inflammatory disorder (NOMID) (11). In all three phenotypes the most common symptoms include periodic fever, arthralgia, rash and conjunctivitis (12). Both genetic and non-genetic diseases in which the inflammasome axis is dysregulated point to the importance of fine-tuning and modulation of its activity to maintain homeostasis. Since so many exogenous and endogenous factors are able to activate different inflammasomes, potent regulatory mechanisms must exist to allow the immune system to remove any sources of danger without causing excessive harm to the host. Recently, several factors and mechanisms have been identified to negatively regulate inflammasomes at different levels of their activation including autophagy (13), interferons type I (14), microRNAs (15), docosahexaenoic acid (16), nitric oxide (17) and cAMP (18). However the full spectrum, as well as downstream events involved in the regulation of inflammasome has not been elucidated.

Prostaglandin E₂ (PGE₂) belongs to a family of bioactive lipid mediators which have a broad range of effects (19). During the acute, initial stage of the inflammatory response PGE₂ acts as a vasodilator and facilitates tissue influx of neutrophils (20), macrophages (21) and mast cells (22) as well as a regulator of nociception (23). However, PGE₂ also has many potent immunosuppressive properties that contribute to the resolution phase of acute inflammation (24), facilitation of tissue regeneration (25) and the return to homeostasis (26). Yet in the context of many immunopathologies, those PGE₂-mediated effects can lead to aggravation of the disease phenotype such as chronic inflammation or cancer (27). PGE₂ regulates activities of both innate and adaptive immune cells. Its wide range of activities with often opposing effects depends on the species, cell and tissue types or context of action

(28). PGE₂ synthesis is initiated by phospholipases A₂, catalyzing the hydrolysis of membrane phospholipids, liberating free fatty acids. Cytosolic phospholipase A₂ group IVA (cPLA₂α) is selective for arachidonate in the sn-2 position of membrane phospholipids, thus generating arachidonic acid (AA), the substrate of cyclooxygenases (COX1 and COX2), that convert AA to PGH₂ (29). It is then converted to downstream active prostanoid by the terminal synthases. In many cells of innate immunity such as macrophages, cPLA₂α is the rate-limiting enzyme in PGE₂ production (30). The diverse effects of PGE₂ may be also accounted for, at least in part, by the existence of four EP receptors, belonging to the family of G protein coupled receptors (GPCRs), differentially expressed in cells and by coupling to more than one G protein, initiating various signal-transduction pathways (31). While, EP1 mediates cytosolic Ca²⁺ mobilization (32), EP2 and EP4 couple primarily to G_sα, which activates adenylate cyclase (AC) to convert ATP to cyclic AMP (cAMP) (33, 34). Changes in cAMP levels are further translated into pleiotropic intracellular effects by a panel of cAMP binding effector proteins (35). The EP3 signaling pathway inhibits AC activity by coupling to G_iα subunit and decreasing cAMP levels (36).

In macrophages, at the priming stage of NLRP3 inflammasome activation by TLR signaling, apart from induction of NLRP3 and pro-IL-1β expression, there is also an activation of cPLA₂α, release of AA and production of PGE₂ and other eicosanoids (37, 38). Moreover, aluminum salts and silica crystals (39), hyaluronan (40) as well as ATP (41) and other known activators of NLRP3 inflammasome further stimulate PGE₂ production, although probably in an NLRP3 and caspase-1 independent manner (39). Furthermore, PGE₂ and other prostanoids have been shown to be primarily responsible for several immediate reactions and sudden death in the “eicosanoid storm” after NLRC4 inflammasome activation by intracellular flagellin (42). In this case, this process appears to be NLRC4, casp-1 and COX1 dependent (42). However, the effects of exogenous or endogenous PGE₂ on the NLRP3 inflammasome activation have not been studied. Due to the growing evidence of the co-existence of potent lipid mediators signaling and inflammasomes formation in response to infection, tissue damage or other cellular stress, we sought to analyze the effect of exogenous PGE₂ on NLRP3 inflammasome activation. We also studied whether the endogenously produced lipid mediators might be involved in the modulation of NLRP3 inflammasome activation. We performed experiments in human primary monocyte-derived macrophages (MDM) and in peripheral blood mononuclear cells (PBMCs) from healthy donors as well as in PBMCs from CAPS patients. Here we found that PGE₂ decreased NLRP3 inflammasome activation, triggered by aluminum crystals, ATP or nigericin. This effect was derived through the EP4 receptor and through an increase in intracellular cAMP, independently of PKA and Epac. In patients with CAPS, with mutations in the NBD domain of *NLRP3*, resulting in the decrease of cAMP binding (18), the inhibitory effect of PGE₂ was observed only in high doses. Moreover, we found that blocking cPLA₂α, or COX2 resulting in inhibition of endogenous PGE₂ production, caused an increase in NLRP3 inflammasome activation. Our results suggest that PGE₂ might play an important role in maintaining homeostasis during the resolution phase of inflammation by preventing excessive inflammasome activation in infiltrating macrophages as well as it might serve as a significant autocrine and paracrine regulator.

Materials and Methods

Reagents and antibodies

Aluminum potassium sulfate (alum crystals), monosodium urate crystals (MSU), and ultrapure LPS were purchased from Invivogen (San Diego, CA). ATP, nigericin sodium salt, 8-bromo-cAMP and IBMX were obtained from Sigma-Aldrich (St. Louis, MO); KH7 and forskolin from Tocris (Bristol, UK). Prostaglandin E₂ (PGE₂), PF-04418948 (EP2 inhibitor), GW 627368X (EP4 inhibitor), butaprost, free acid (EP2 agonist), CAY10598 (EP4 agonist) and NS-398 (COX2 inhibitor) were purchased from Cayman Chemical (Ann Arbor, MI). cPLA₂α inhibitor (N-((2S,4R)-4-(Biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl-3-[4-(2,4-dioxothiazolidin-5-ylidene-methyl)-phenyl] acrylamide, HCl) was obtained from Calbiochem, EMD Millipore (Gibbstown, NJ). 8-(4-Chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic Monophosphate . sodium salt (Epac agonist; activates Epac 1 and Epac 2) and 8-Bromoadenosine 3',5'-cyclic Monophosphothioate, Rp-Isomer . sodium salt (PKA inhibitor) were purchased from Enzo Life Science (Farmingdale, NY). N6-Benzoyl-8-piperidinoadenosine-3',5'-cyclic monophosphate . sodium salt (PKA agonist) and 4-Cyclopentyl- 2- (2, 5- dimethylbenzylsulfanyl)- 6- oxo- 1, 6- dihydropyrimidine- 5- carbonitrile (Epac inhibitor) were purchased from Axxora (Lausen, Switzerland). Antibody against IL-1β (AF-201-NA) was purchased from R&D Systems (Minneapolis, MN). Antibodies against caspase-1 (2225S) and total cPLA₂α were purchased from Cell Signaling Technology (Danvers, MA); antibody against NLRP3 (AG-20B-0014-C100) from Adipogen (San Diego, CA); antibody against ASC (sc-22514-R) from Santa Cruz Biotechnology (Dallas, Texas). Monoclonal horseradish peroxidase (HRP)-conjugated beta actin antibody was obtained from GenScript (Piscataway, NJ) and Cell Signaling Technology. HRP-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

Primary cells and cell lines

Human elutriated monocytes from healthy donors were obtained by an Institutional Review Board-approved protocol from the National Institutes of Health Blood Bank (Bethesda, MD). Monocytes were resuspended in RPMI 1640 medium with 2 mM of L-glutamine and supplemented with 10% heat-inactivated FBS (Life Technologies, Thermo Scientific, Waltham, MA) for overnight. The next day, 18×10^6 monocytes were seeded into a T75 tissue culture flask in 15 ml of Iscove's modified Dulbecco's medium (IMDM) (Life Technologies) with 10% heat-inactivated FBS and 50 ng/ml of M-CSF (Life Technologies) for 7 days. Half of the medium was replaced after 3 days of culture. 24 hours prior to experiments, macrophages were treated with trypsin (Lonza, Walkersville, MD) for 1 minute, scraped and plated in the IMDM medium without M-CSF into 12-well plates or 6-well plates at a density of 0.35×10^6 or 0.85×10^6 , respectively.

Blood specimens from patients with CAPS were drawn after obtaining informed consent under a protocol approved by the NIAMS/NIDDK Institutional Review Board. Blood specimens from healthy volunteers were obtained under a protocol approved by the NHLBI Institutional Review Board. Blood samples were processed immediately using lymphocyte

separation medium (LSM) (Lonza), according to the manufacturer's protocol to obtain PBMCs. 2×10^6 PBMCs were seeded, allowed to attach to a 12-well plate containing RPMI medium without serum for 20 min, immediately followed by the inflammasome activation/inhibition experiments. THP-1 cells were obtained from ATCC (Manassas, VA) and cultured in RPMI medium containing 10% heat-inactivated FBS and 0.05 mM 2-mercaptoethanol. 0.5×10^6 THP-1 cells were plated into a 12-well plate in the presence of 50 nM PMA (Sigma-Aldrich) for 12 hours before the experiment was performed.

Inflammasome activation and inhibition

Human primary MDM were treated with/without LPS (100 ng/ml) for 4 h in IMDM with 10% FBS, followed by 30 min treatment with PGE₂ (0.1 μM or as indicated), EP2 agonist (butaprost, free acid; 0.5 μM), EP4 agonist (CAY10598; 0.1 μM), 8-bromo-cAMP (5-20 μM), PKA selective agonist (6-Bnz-8-PIP-cAMP; 20 or 50 μM) or EPAC selective agonist (8-(4-Chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic Monophosphate . sodium salt, 20 or 50 μM), followed by stimulation with alum (400 μg/ml for 5 h), ATP (2 mM for 1 h) or nigericin (4 μM for 1 h) in Opti-MEM medium (Life Technologies). EP2 inhibitor (PF-04418948, 0.5 μM), EP4 inhibitor (GW627368X, 2 μM), PKA inhibitor (8-Bromoadenosine 3',5'-cyclic Monophosphothioate, Rp-Isomer . sodium salt, 50 μM), EPAC inhibitor (4- Cyclopentyl- 2- (2, 5- dimethylbenzylsulfanyl)- 6- oxo- 1, 6- dihydropyrimidine- 5- carbonitrile, 10 μM) or vehicle (DMSO) were added 30 min prior treatment with PGE₂ or vehicle (EtOH). KH7 (25 μM) or IBMX (200 μM) or forskolin (50 μM) were added 1 h prior to treatment with PGE₂ or vehicle (EtOH). In the experiments with cPLA₂α inhibitor (2 μM) in human primary MDM, the inhibitor or DMSO were added before and after priming or only after priming, followed by treatment with alum (400 μg/ml for 5 h) in Opti-MEM. In the experiments with COX2 inhibitor (NS398, 5 μM) in human primary MDM, the inhibitor or DMSO were added before and after priming, followed by treatment with alum (400 μg/ml for 5 h) or ATP (2 mM for 1 h) in Opti-MEM. PBMCs from CAPS patients or healthy volunteers were primed for 3 h with LPS (1 μg/ml) in RPMI 1640 with 10% FBS and followed by indicated doses of PGE₂ (in CAPS patients) or PGE₂ with alum (400 μg/ml) or ATP (2 mM) (in healthy volunteers) for 30 min in RPMI 1640 without serum. PMA-treated THP-1 cells were treated with cPLA₂α inhibitor for 30 min, then primed with 100 ng/ml of LPS and stimulated by MSU (100 μg/ml for 5 h), alum (400 μg/ml for 5 h), ATP (2 mM for 1 h) or nigericin (4 μM for 45 min) in Opti-MEM medium. Supernatants, lysates and cell pellets were collected for the following analyses.

NLRP3, EP2, EP4 and PLA2G4A knockdown

ON-TARGET_{plus} SMART pool small interfering RNA (siRNA) (Dharmacon, Thermo Scientific, Lafayette, CO) against human NLRP3 (L-017367-00-0005), or sc-45469, Santa Cruz), EP2 (PTGER2) (L-005712-00-0005), EP4 (PTGER4) (L-005714-00-0005) and cPLA₂α (PLA2G4A) (L-009886-00-005) together with ON-TARGET_{plus} Control Non-targeting pool (D-00181970-05) were used to perform knockdown experiments in human primary MDM or THP-1 cells. Human primary MDM (0.5×10^6 cells per 100 μl cuvette) or THP-1 cells (5×10^6 per 100 μl cuvette) were transfected with siRNA pools (1 μM or 100 nM) using a P3 Primary Cell 4D-Nucleofector X Kit L (Amaxa, Cologne, Germany) according to the manufacturer's protocols for human macrophages and monocytes,

respectively. The silencing of gene expression was confirmed by RT-PCR. All experiments on transfected cells were performed after 48 hours.

Real-Time PCR

Total RNA was extracted from cells using QIAshredder columns and RNeasy mini kit and treated with DNase (Qiagen, Valencia, CA). Reverse transcription was performed using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Gene expression was assessed using RT-PCR performed on an ABI Prism ViiA7 sequence detection system (Applied Biosystems, Foster City, CA) using commercially available probe and primers sets (Applied Biosystems) as follows: GAPDH, Hs02758991_g1; IL-1 β , Hs01555410_m1; IL-6, Hs00174131_m1; TNF, Hs99999043_m1; cPLA₂ α (PLA2G4A), Hs00233352_m1; NLRP3, Hs00918082_m1; caspase-1, Hs00354836_m1; EP1 (PTGER1), Hs00168752_m1, EP2, Hs04183523_m1, EP3, Hs00168755_m1, EP4, Hs00168761_m1 and iTaq Universal Probes Supermix (Bio-Rad). Gene expression was normalized to GAPDH transcripts and represented as a relative quantification (RQ) compared with control.

ELISA

IL-1 β and IL-18 in cell culture supernatants were measured using Human IL-1 beta/IL-1F2 Quantikine Kit (SLB50) from R&D Systems or Human IL-18 ELISA kit from MBL (Nagoya, Japan) respectively, according to the manufacturer's directions. There were substantial differences in the IL-1 β and IL-18 release between the donors, as depicted in the Supplementary Figure 1 A and B. Therefore in order to combine similar experiments and calculate statistical significance, the values in all experiments are presented as the percentage of the response after LPS/alum treatment. The range of the raw values from combined experiments is included in each figure legend. PGE₂ in cell culture supernatants was measured using EIA kit from Cayman Chemical Co. TNF in cell culture supernatants was measured using Human TNF-alpha ELISA Kit (DTA00C) from R&D Systems. Intracellular cAMP quantification was performed using EIA kit from Enzo (Farmingdale, NY). Human primary MDM seeded on a 6-well plate were stimulated with PGE₂ (0.1 μ M), or forskolin (50 μ M) in the presence of IBMX (200 μ M) for 15 min, followed by 10 min cell lysis in 0.1 N HCl containing 0.1 % triton X-100 (Sigma-Aldrich). For the cAMP quantification in the experiments with NS398, primary MDM were treated with LPS (100 ng/ml) with/without NS398 (5 μ M), NS398 alone or DMSO for 4 h in complete medium and then stimulated with Alum for 15 min in serum-free medium followed by 10 min cell lysis in 0.1 N HCl containing 0.1 % triton X-100 and IBMX (200 μ M). Cell lysates were collected, centrifuged and stored at -80 °C before cAMP detection according to the manufacturer's protocol.

Western Blot

Supernatants were collected and cells were lysed with RIPA buffer (Thermo Scientific, Rockford, IL) containing protease inhibitor cocktails (Roche, Basel, Switzerland). Supernatants were precipitated with equal volume of methanol and ¼ volumes of chloroform (Sigma-Aldrich) and centrifuged at room temperature at 12,000 rpm for 5 min. The upper layer was aspirated, double volume of methanol added and centrifuged again. The liquid phase was discarded and the pellet was dried at 50°C for 5 min. The pellets were

dissolved in 1X NuPage Sample Buffer (Life Technologies) and heated at 70°C for 10 min and then subjected to electrophoresis using the 4-12% NuPAGE Bis-Tris gels using MES buffer (Life Technologies). The cell lysates were placed on ice for 15 min and then centrifuged at 13,000 rpm for 20 min. Protein concentration of cell lysates was determined by BCA kit (Pierce, Rockford, IL). Equal amounts of lysate proteins (20 µg) were resolved by 4–12% NuPAGE Bis-Tris gels using MOPS running buffer (Life Technologies) and then transblotted to the nitrocellulose membranes using the IBlot Dry Blotting System (Life Technologies). For ASC pyroptosome, cell pellets were cross-linked using 2 mM disuccinimidyl suberate (DSS) (Thermo Scientific) for 30 min in PBS in RT; 6x SDS sample buffer was added and samples were processed as cell lysates. The membranes were blocked with 5% nonfat skim milk in PBST (phosphate buffer saline containing 0.1% Tween 20) for 2 h and then incubated with primary antibodies overnight at 4°C. Subsequently, the membranes were washed and then incubated with specific secondary antibodies conjugated with horseradish peroxidase (HRP) for 1 h. The blots were developed using the SuperSignal West Femto Chemiluminescent Substrate kit (Thermo Scientific) and visualized on a Bio-Rad Image Station (Chemi-Doc-MP) (Hercules, CA).

Statistical analysis

Data were analyzed by one-way ANOVA with Holm-Sidak post hoc test, Kruskal-Wallis ANOVA on ranks with Dunn's post hoc test, RM (repeated measurements)-ANOVA, followed by Sidak post hoc test or unpaired Student t test, as appropriate. Differences were considered significant when $p < 0.05$. The data are presented as the mean \pm SEM from the indicated number of independent experiments, each performed in duplicate.

Results

PGE₂ inhibits NLRP3-triggered inflammasome activity in human macrophages

To assess the effect of PGE₂ on NLRP3 inflammasome activation, we first examined whether PGE₂ could inhibit caspase-1 cleavage and IL-1 β secretion. Human primary MDM were primed with LPS followed by treatment with PGE₂ and subsequent stimulation with alum, to activate IL-1 β maturation via NLRP3 inflammasome. We found that PGE₂ blocked alum-induced activation of caspase-1 and production of mature IL-1 β and IL-18 (Fig. 1A-C; Supplementary Fig. 1). We also observed that PGE₂, in these experimental conditions, slightly decreased the expression of pro-IL-1 β , without affecting NLRP3 or caspase-1 (Fig. 1A). The inhibitory effect of PGE₂ on caspase-1 cleavage, IL-1 β maturation, ASC oligomerization (Fig. 1D) and IL-1 β secretion (Fig. 1E) was dose-dependent and began with very low doses of PGE₂ (0.01 µM-0.1 µM) in human primary MDM. The effectiveness of these doses points to a physiologic and receptor-specific mode of PGE₂-mediated inhibition of NLRP3 activation (31). We also tested other activators of NLRP3 inflammasome, such as ATP and nigericin (43) to determine whether the suppression exerted by PGE₂ specifically affected alum-dependent NLRP3 inflammasome activity, a phagocytosis-dependent model of NLRP3 activation (44). We found that caspase-1 cleavage and IL-1 β secretion was also decreased by PGE₂ in an ATP- (Fig. 2A-C) or nigericin- (Fig. 2D, E) induced NLRP3 inflammasome activation model. To control for NLRP3 specificity of studied stimuli in human macrophages we performed NLRP3 siRNA experiments, with two different sources

of siRNAs, confirming that both alum and ATP specifically induced NLRP3-dependent caspase-1 and IL-1 β cleavage (Fig. 2F, G). Thus, PGE₂ specifically inhibited the activity of NLRP3 inflammasome.

PGE₂ differentially influences the gene expression of NLRP3 inflammasome complex components

To evaluate the role of PGE₂ on the expression of NLRP3 inflammasome components, we performed a time course analysis of PGE₂ on LPS-induced gene expression. Human primary MDM were treated with/without LPS, followed by treatment with PGE₂ for 1.5 h, 3 h and 5.5 h. We found that LPS-induced IL-1 β mRNA expression was not significantly altered by PGE₂ in either of the time points, though there was a downward trend (Fig. 3A). NLRP3 mRNA expression was significantly increased by PGE₂ at 1.5 h time point (Fig 3B), while caspase-1 mRNA expression was unchanged (Fig. 3C). TNF and IL-6 usually serve as controls for specific inflammasome and non-priming effecting mechanisms. However, both of these cytokines have been reported to be regulated by PGE₂ and cAMP in opposite directions in macrophages and through mechanisms not affecting intracellular canonical TLR signaling (45, 46). Interestingly, we found that LPS-induced IL-6 mRNA expression was not changed by the addition of PGE₂ after LPS stimulation (Fig. 3D), as opposed to TNF α , in which mRNA expression was significantly decreased at each time-point studied (Fig. 3E). Taken together, the lack of the effect of PGE₂ on the transcription of genes encoding proteins involved in the NLRP3 inflammasome complex formation, as well as its inhibitory effect on mature caspase-1, ASC oligomerization and constitutively expressed IL-18, as shown in the previous paragraph, suggests that PGE₂ acts on NLRP3 inflammasome assembly.

PGE₂ inhibits NLRP3 inflammasome through EP4 receptor

PGE₂ is known to exert various effects through at least four different receptors EP1, EP2, EP3 and EP4 (PTGER1-4), all belonging to the GPCR family (31). Therefore, we first analyzed which receptors are expressed on human primary MDM. In agreement with the literature on mouse macrophages, cell lines and human macrophages (34) we found that EP1 basal mRNA expression was at the edge of the detection level; EP3 receptor expression was very low, while EP2 and EP4 were abundantly expressed (Supplementary Fig. 2A). Furthermore, both EP2 and EP4 mRNA expression was significantly increased by LPS priming (Supplementary Fig. 2B, 2C). To determine which receptor is responsible for the PGE₂ effect on NLRP3 inflammasome, we used specific pharmacological agonists and antagonists of each receptor as well as a pool of siRNAs to knockdown each receptor. The EP4 agonist, CAY10598, but not the EP2 agonist, butaprost, blocked alum-induced secretion of mature IL-1 β and caspase-1 (Fig. 4A, 4B). Moreover, we found that the EP4 antagonist, GW627368X, blocked PGE₂-induced inhibition of NLRP3 inflammasome activation, while the EP2 antagonist, PF-04418948, had no effect (Fig. 4 C, 4D). Both inhibitors applied simultaneously had similar effect to EP4 antagonist alone (Fig. 4C, 4D). The function of EP2 and EP4 agonists and antagonists was controlled by measuring TNF (Supplementary Fig. 3 A, B), which inhibition was reported to be driven through both of these receptors in human macrophages (47). Knockdown of EP2 or EP4 receptors was performed by electroporation with the specific pools of siRNAs. The knockdown efficiency

for EP2 and EP4 was 97% and 65%, respectively. When both siRNA were applied together, then EP2 was reduced by 93% and EP4 by 50% as assessed by RT-PCR (Fig. 4 E). Similar to the antagonists results, EP4 knockdown, as opposed to EP2 knockdown reduced PGE₂-induced inhibition of NLRP3 inflammasome activation (Fig. 4F, 4G). Simultaneous knockdown did not have an effect on PGE₂-induced inhibition of NLRP3 inflammasome activation, probably because of the low efficiency of the knockdown of EP4 (Fig. 4 E, 4F, 4G). Altogether, these data suggest that PGE₂ decreases NLRP3-inflammasome activity through EP4 receptor.

cAMP, independent of PKA and Epac, is involved in PGE₂-mediated NLRP3 inflammasome inhibition

The most widely described signaling pathway through EP4 in macrophages is coupling with G_sα subunit and activation of adenylyl cyclase (34). Adenylyl cyclase converts ATP to cAMP, which is an important second messenger. Intracellular cAMP levels are further controlled by the actions of phosphodiesterase, which converts cAMP to inactive 5'-AMP, by degrading the phosphodiester bond (48). Signaling through EP4 increases intracellular cAMP levels (34). It was previously demonstrated that cAMP inhibits NLRP3 inflammasome through direct interaction with NLRP3 protein in mouse BMDM and in human PBMCs (18). To determine, if this is the major mechanism of the observed effect of PGE₂-induced NLRP3 inhibition in human primary MDM, we applied several approaches that involved the key molecules in the regulation of intracellular cAMP levels. We used KH7, an adenylyl cyclase inhibitor which decreases basal or activated cAMP levels (49); forskolin, a direct adenylyl cyclase activator, that increases intracellular cAMP levels (50) and IBMX, a phosphodiesterase inhibitor, that increases cAMP levels by blocking its degradation (51). First, we confirmed that in human primary MDM, 15 min treatment with PGE₂ stimulated cAMP production (Fig. 5C). Next, we found that blocking adenylyl cyclase with KH7 in the presence or absence of alum significantly reduced PGE₂-mediated NLRP3-inflammasome inhibition (Fig. 5A, 5B) and IL-1β secretion (Fig. 5B). Forskolin treatment significantly decreased NLRP3 activation (Fig. 5A) and IL-1β secretion (Fig. 5B), which was further decreased by adding PGE₂, suggesting additive PGE₂-mediated mechanisms of adenylyl cyclase activation. Not surprisingly, prolonged IBMX treatment completely abrogated NLRP3 activation (Fig. 5A) and IL-1β secretion (Fig. 5B). To further confirm cAMP-mediated NLRP3 inflammasome inhibition, we used 8-Bromo-cAMP, a cell permeable analog of cAMP. We found that 8-Bromo-cAMP in a dose-dependent manner suppressed activation of caspase-1 and production of mature IL-1β (Fig. 5D) and in the dose of 20 μM significantly decreased IL-1β secretion (Fig. 5E). In macrophages, cAMP might act through ubiquitously expressed intracellular cAMP receptors, the classic protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac) or might directly bind to the NLRP3 protein (18). To study whether PGE₂-dependent cAMP-mediated effect on NLRP3 is derived through PKA or Epac, we used their specific agonists and antagonists. We found that PKA selective agonist (6-Bnz-8-PIP-cAMP) did not mimic PGE₂-dependent NLRP3 inflammasome inhibition (Fig. 6A, B). Also, we observed that PKA specific inhibitor (8-Bromo-adenosine 3',5'-cyclic Monophosphothioate) did not block PGE₂-mediated NLRP3 inflammasome inhibition (Fig. 6C, D). Similarly, Epac selective agonist (8-(4-Chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic Monophosphate) did not inhibit

before alum treatment (Fig. 8F). Under the same experimental conditions we found that cPLA₂α inhibitor added before or after priming increased NLRP3 inflammasome activation in human primary MDM (Fig. 8D, 8E, 8G, 8H). To more selectively block only PGE₂ production, we also inhibited COX2, by using its selective inhibitor NS398. First, we confirmed that NS398 blocked LPS/ATP- (Fig. 9A) as well as LPS/alum-induced PGE₂ (Fig. 9B) production. Similar to cPLA₂ inhibition, inhibition of COX2 led to an increase of NLRP3 inflammasome activation (Fig. 9C, D). Additionally, we also noted that treatment with NS398 decreased intracellular cAMP (Fig. 9E). Altogether, these data suggest that cPLA₂α and COX2, through PGE₂ production, are involved in the regulation of NLRP3 inflammasome activation.

Discussion

Monocytes and macrophages are among the first cells involved in the acute phase of inflammation. They infiltrate the tissue in response to chemotactic agents and respond to the invading pathogen or tissue trauma by several mechanisms including inflammasome activation. The inflammatory response should subside once it has carried out its function to maintain homeostasis. Activated NLRP3 inflammasome needs to be counterbalanced by the existence of local mediators or mechanisms that quickly quiet the response and lead to the resolution of acute inflammation. Here we have found that PGE₂ inhibited NLRP3 inflammasome activation in human primary MDM. We have demonstrated that this effect depends on the EP4 receptor and an increase in intracellular cAMP, but not on PKA or Epac-mediated pathways. We also found that PGE₂ decreased IL-1β secretion in patients with CAPS, but in higher doses than in healthy subjects. Moreover, we have demonstrated that this mechanism is involved in an autocrine or paracrine loop, possibly controlling the extent of NLRP3 inflammasome activation.

Our results are consistent with the recent findings that cAMP is directly involved in the endogenous control of NLRP3 inflammasome activation (18). Calcium sensing receptor (CASR), belonging to the GPCRs family in response to the extracellular Ca²⁺ increase activates phospholipase C (PLC) and inhibits adenylate cyclase (AC). As a result there is an increase in intracellular Ca²⁺ and decrease in cAMP, both of which can independently activate NLRP3 inflammasome (18). Here we also provided three lines of evidence showing that PGE₂-mediated inhibition of NLRP3 inflammasome occurred by an increase in intracellular cAMP. First, KH7, an AC inhibitor, reversed PGE₂-mediated NLRP3 inflammasome inhibition. Second, forskolin itself as an AC activator decreased NLRP3 activation and this effect was enhanced by cotreatment with PGE₂. Moreover, IMBX, a phosphodiesterase inhibitor totally blocked alum-induced NLRP3 activation. Finally, 8-Bromo-cAMP, a cell permeable analog of cAMP in a dose-dependent manner mimicked PGE₂-mediated NLRP3 inflammasome inhibition. Another recent study in which the authors used relatively low concentrations of forskolin (up to 20 μM) reports extracellular Ca²⁺ sensing by both CASR and GPRC6A in NLRP3 activation; however, cAMP involvement was not demonstrated (56). We have shown that even when forskolin was used in a concentration of 50 μM, there was still an additive effect of PGE₂, suggesting that AC might require stronger activation to elicit its full cAMP-producing potential. Interestingly, it has been demonstrated that this cAMP effect is not dependent on protein kinase A (PKA), a

major cAMP binding protein, but is driven by direct binding of cAMP to NBD domain of NLRP3 protein (18). We have confirmed these findings and extended them, showing that cAMP-mediated inhibition of NLRP3 is also not dependent on Epac, the second major intracellular cAMP binding protein. Similarly, we have also found that in patients with CAPS, carrying mutations in NBD domain of *NLRP3* gene that result in the impairment of cAMP binding (18), the PGE₂-mediated NLRP3 inflammasome inhibition was less efficient and required 10-100 times higher doses of PGE₂ to elicit similar effect as in healthy subjects. This suggests that patients with CAPS might be more resistant to signals leading to resolution of inflammation resulting in prolonged inflammation. They might also generate more PGE₂ as a derivative of an excessive need to extinguish NLRP3 inflammasome and increased IL-1 β signaling giving the positive feedback for activation of cPLA₂ α and expression of COX2 and mPGES1 (57). The pleiotropic and detrimental effects of prolonged, excessive PGE₂ signaling in various tissues might lead to secondary pathologies (27). Indeed, this has been demonstrated in patients with NOMID, the most severe phenotype among CAPS variants, who suffer from severe arthropathy and develop bulky masses in their long bones (58). There is an increase in PGE₂, cAMP and PKA activity in NOMID tumor cells, leading to abnormal *Wnt* signaling and proliferation, while inhibition of both PGE₂ and caspase-1 leads to a decrease in NOMID cell proliferation (58).

PGE₂ acts through four different receptors designated EP1, EP2, EP3 and EP4, with expression variations occurring in different cells and tissue types. Using multiple pharmacological and molecular approaches, we have determined that PGE₂ acted specifically through EP4 to decrease NLRP3 inflammasome activation in human primary MDM. Recently, two other GPCRs have been shown to be involved in the negative regulation of NLRP1 and NLRP3 inflammasomes. Yan et al have determined that ω -3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) acting through GPR120 and GPR40 and at least partially by their downstream scaffolding protein β -arrestin-2 decrease NLRP1 and NLRP3 activation (16). β -arrestin-2 was shown to bind to full-length NLRP3 and its LRR and NBD (NACHT) domains as well as to NLRP1 in a HEK293T overexpression system, but in *Arrb2*^{-/-} knockout mice the reversal of DHA or EPA-mediated inhibition was incomplete. However, DHA via this axis is able to suppress high fat diet-induced NLRP3 inflammasome activation and the prevention of NLRP3 inflammasome-dependent insulin resistance in vivo (16). We have recently determined that DHA also through GRP120 activates cPLA₂ α and induces PGE₂ production in RAW 264.7 cells and human primary macrophages (59). This suggests that the proposed PGE₂-mediated inhibition of NLRP3 by increase in cAMP might be an additional mechanism of ω -3 derived effects.

The role of PGE₂ on IL-1 β production in monocytes and macrophages has been previously suggested; however, several contradictory results have been reported and the mechanisms involved have not been described. Early studies suggested that PGE₂ inhibits IL-1 β secretion in macrophages (60-62) and monocytes (63, 64), not at the level of gene transcription but by a posttranscriptional mechanism which was not well defined (63). Others reported that in monocytes, PGE₂ enhances IL-1 β mRNA expression and protein production via cAMP (65-67) and PKA-CREB-dependent transcriptional events (68). With these differences in

mind, we studied the effect of PGE₂ on inflammasome activation. We found that PGE₂ added after LPS priming decreased NLRP3 inflammasome activation via a cAMP-dependent mechanism as assessed by the decrease in mature IL-1 β and mature caspase-1 release, and IL-18 release, as well as a decrease in ASC oligomerization. This might account for previous observations that were noted before the development of the inflammasome concept. Nonetheless, having controlled for IL-1 β mRNA and protein expression, we also noted a non-significant decrease at the mRNA level, though reflected by the slight decreases in total pro-IL-1 β levels in the cells, pointing out the complexity of PGE₂ in the control of IL-1 β release.

Our findings that cPLA₂ α or COX2 inhibition resulting in the decrease of PGE₂ led to an increase in NLRP3 inflammasome activation and IL-1 β secretion in macrophages are consistent with the several early works studying effects of various NSAIDs on IL-1 β production. Indomethacin (61-64, 69) and piroxicam (69) were reported to increase IL-1 β production. On the other hand, it was reported that inhibition of cPLA₂ or COX2 leads to a decrease in mature IL-1 β secretion (55, 70, 71). The differences with our findings might result from the dose and type of inhibitors used in those studies, as well as the type of cells, their redox state and EP receptors expression. Our results of pharmacological inhibition with a low dose of a highly specific COX2 inhibitor, cPLA₂ α inhibitor and cPLA₂ α knockdown by siRNA, in conjunction with the observations of the exogenous PGE₂ effects on NLRP3 inflammasome activation suggest that this axis might play a regulatory role in human macrophages.

In summary, our results suggest that PGE₂ might serve as a local inhibitor of NLRP3 inflammasome in monocytes and macrophages at the site of acute inflammation. In general this mechanism might serve as a switch to resolution of inflammation and return to homeostasis, but once dysregulated, it might lead to detrimental effects and pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

AA	Arachidonic acid
AC	Adenylate cyclase
AIM2	Absent in melanoma 2
CAPS	Cryopyrin-associated periodic fever syndromes
COX	Cyclooxygenase

cPLA₂α	Cytosolic phospholipase A ₂ group IVA
DHA	Docosahexaenoic acid
dsDNA	double-stranded DNA
Epac	Exchange protein directly activated by cAMP
FCAS	Familial cold-induced autoinflammatory syndrome
GPCR	G protein-coupled receptors
IFI16	Interferon, gamma-inducible protein 16
NBD	Nucleotide binding domain
LRR	C-terminal leucine rich domain
MDM	Monocyte-derived macrophages
MWS	Muckle-Wells syndrome
NLR	Nucleotide-binding domain, leucine rich repeat-containing proteins
NOMID	Neonatal onset multisystem inflammatory disorder
PG	Prostaglandin
PKA	Protein kinase A
PLC	Phospholipase C
siRNA	Small interfering RNA

References

1. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Molecular Cell*. 2002; 10:417–426. [PubMed: 12191486]
2. Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. *Nature Reviews Immunology*. 2013; 13:397–411.
3. De Nardo D, De Nardo CM, Latz E. New Insights into Mechanisms Controlling the NLRP3 Inflammasome and Its Role in Lung Disease. *The American Journal of Pathology*. 2014; 184:42–54. [PubMed: 24183846]
4. Davis BK, Wen H, Ting JP. The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annual review of immunology*. 2011; 29:707–735.
5. Allen IC, TeKippe EM, Woodford RM, Uronis JM, Holl EK, Rogers AB, Herfarth HH, Jobin C, Ting JP. The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer. *The Journal of Experimental Medicine*. 2010; 207:1045–1056. [PubMed: 20385749]
6. Wen H, Gris D, Lei Y, Jha S, Zhang L, Huang MT, Brickey WJ, Ting JP. Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. *Nature Immunology*. 2011; 12:408–415. [PubMed: 21478880]
7. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature*. 2006; 440:237–241. [PubMed: 16407889]
8. Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, Abela GS, Franchi L, Nunez G, Schnurr M, Espevik T, Lien E, Fitzgerald KA, Rock KL, Moore KJ, Wright SD, Hornung

- V, Latz E. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature*. 2010; 464:1357–1361. [PubMed: 20428172]
9. Heneka MT, Kummer MP, Stutz A, Delekate A, Schwartz S, Vieira-Saecker A, Griep A, Axt D, Remus A, Tzeng TC, Gelpi E, Halle A, Korte M, Latz E, Golenbock DT. NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. *Nature*. 2013; 493:674–678. [PubMed: 23254930]
10. Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nature Genetics*. 2001; 29:301–305. [PubMed: 11687797]
11. Masters SL, Simon A, Aksentijevich I, Kastner DL. Horror autinflammaticus: the molecular pathophysiology of autoinflammatory disease (*). *Annual Review of Immunology*. 2009; 27:621–668.
12. Goldbach-Mansky R, Kastner DL. Autoinflammation: the prominent role of IL-1 in monogenic autoinflammatory diseases and implications for common illnesses. *The Journal of Allergy and Clinical Immunology*. 2009; 124:1141–1149. quiz 1150-1141. [PubMed: 20004775]
13. Shi CS, Shenderov K, Huang NN, Kabat J, Abu-Asab M, Fitzgerald KA, Sher A, Kehrl JH. Activation of autophagy by inflammatory signals limits IL-1 β production by targeting ubiquitinated inflammasomes for destruction. *Nature Immunology*. 2012; 13:255–263. [PubMed: 22286270]
14. Guarda G, Braun M, Staehli F, Tardivel A, Mattmann C, Forster I, Farlik M, Decker T, Du Pasquier RA, Romero P, Tschopp J. Type I interferon inhibits interleukin-1 production and inflammasome activation. *Immunity*. 2011; 34:213–223. [PubMed: 21349431]
15. Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri T, Wu J, Monks BG, Fitzgerald KA, Hornung V, Latz E. Cutting edge: NF- κ B activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol*. 2009; 183:787–791. [PubMed: 19570822]
16. Yan Y, Jiang W, Spinetti T, Tardivel A, Castillo R, Bourquin C, Guarda G, Tian Z, Tschopp J, Zhou R. Omega-3 fatty acids prevent inflammation and metabolic disorder through inhibition of NLRP3 inflammasome activation. *Immunity*. 2013; 38:1154–1163. [PubMed: 23809162]
17. Hernandez-Cuellar E, Tsuchiya K, Hara H, Fang R, Sakai S, Kawamura I, Akira S, Mitsuyama M. Cutting edge: nitric oxide inhibits the NLRP3 inflammasome. *J Immunol*. 2012; 189:5113–5117. [PubMed: 23100513]
18. Lee GS, Subramanian N, Kim AI, Aksentijevich I, Goldbach-Mansky R, Sacks DB, Germain RN, Kastner DL, Chae JJ. The calcium-sensing receptor regulates the NLRP3 inflammasome through Ca $^{2+}$ and cAMP. *Nature*. 2012; 492:123–127. [PubMed: 23143333]
19. Kalinski P. Regulation of immune responses by prostaglandin E $_2$. *J Immunol*. 2012; 188:21–28. [PubMed: 22187483]
20. Desouza IA, Franco-Penteado CF, Camargo EA, Lima CS, Teixeira SA, Muscara MN, De Nucci G, Antunes E. Inflammatory mechanisms underlying the rat pulmonary neutrophil influx induced by airway exposure to staphylococcal enterotoxin type A. *British Journal of Pharmacology*. 2005; 146:781–791. [PubMed: 16170330]
21. Tajima T, Murata T, Aritake K, Urade Y, Hirai H, Nakamura M, Ozaki H, Hori M. Lipopolysaccharide induces macrophage migration via prostaglandin D $_2$ and prostaglandin E $_2$. *The Journal of Pharmacology and Experimental Therapeutics*. 2008; 326:493–501. [PubMed: 18492946]
22. Weller CL, Collington SJ, Hartnell A, Conroy DM, Kaise T, Barker JE, Wilson MS, Taylor GW, Jose PJ, Williams TJ. Chemotactic action of prostaglandin E $_2$ on mouse mast cells acting via the PGE $_2$ receptor 3. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104:11712–11717. [PubMed: 17606905]
23. Natura G, Bar KJ, Eitner A, Boettger MK, Richter F, Hensellek S, Ebersberger A, Leuchtweis J, Maruyama T, Hofmann GO, Halbhuber KJ, Schaible HG. Neuronal prostaglandin E $_2$ receptor subtype EP3 mediates antinociception during inflammation. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110:13648–13653. [PubMed: 23904482]

24. Hellmann J, Zhang MJ, Tang Y, Rane M, Bhatnagar A, Spite M. Increased saturated fatty acids in obesity alter resolution of inflammation in part by stimulating prostaglandin production. *J Immunol.* 2013; 191:1383–1392. [PubMed: 23785121]
25. Kvirkvelia N, McMenamin M, Chaudhary K, Bartoli M, Madaio MP. Prostaglandin E2 promotes cellular recovery from established nephrotoxic serum nephritis in mice, pro-survival, and regenerative effects on glomerular cells. *American Journal of Physiology. Renal Physiology.* 2013; 304:F463–470. [PubMed: 23283994]
26. MacKenzie KF, Clark K, Naqvi S, McGuire VA, Noehren G, Kristariyanto Y, van den Bosch M, Mudaliar M, McCarthy PC, Pattison MJ, Pedrioli PG, Barton GJ, Toth R, Prescott A, Arthur JS. PGE₂ induces macrophage IL-10 production and a regulatory-like phenotype via a protein kinase A-SIK-CRTC3 pathway. *J Immunol.* 2013; 190:565–577. [PubMed: 23241891]
27. Nakanishi M, Rosenberg DW. Multifaceted roles of PGE₂ in inflammation and cancer. *Seminars in Immunopathology.* 2013; 35:123–137. [PubMed: 22996682]
28. Hirata T, Narumiya S. Prostanoids as regulators of innate and adaptive immunity. *Advances in Immunology.* 2012; 116:143–174. [PubMed: 23063076]
29. Dennis EA, Cao J, Hsu YH, Magrioti V, Kokotos G. Phospholipase A2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. *Chemical Reviews.* 2011; 111:6130–6185. [PubMed: 21910409]
30. Murakami M, Taketomi Y, Miki Y, Sato H, Hirabayashi T, Yamamoto K. Recent progress in phospholipase A(2) research: from cells to animals to humans. *Progress in Lipid Research.* 2011; 50:152–192. [PubMed: 21185866]
31. Hirata T, Narumiya S. Prostanoid receptors. *Chemical reviews.* 2011; 111:6209–6230. [PubMed: 21819041]
32. Katoh H, Watabe A, Sugimoto Y, Ichikawa A, Negishi M. Characterization of the signal transduction of prostaglandin E receptor EP1 subtype in cDNA-transfected Chinese hamster ovary cells. *Biochimica et Biophysica Acta.* 1995; 1244:41–48. [PubMed: 7766667]
33. Fujino H, Salvi S, Regan JW. Differential regulation of phosphorylation of the cAMP response element-binding protein after activation of EP2 and EP4 prostanoid receptors by prostaglandin E2. *Molecular Pharmacology.* 2005; 68:251–259. [PubMed: 15855407]
34. Yokoyama U, Iwatsubo K, Umemura M, Fujita T, Ishikawa Y. The prostanoid EP4 receptor and its signaling pathway. *Pharmacological Reviews.* 2013; 65:1010–1052. [PubMed: 23776144]
35. Lefkimmiatis K, Zaccolo M. cAMP signaling in subcellular compartments. *Pharmacology & Therapeutics.* 2014
36. Sugimoto Y, Namba T, Honda A, Hayashi Y, Negishi M, Ichikawa A, Narumiya S. Cloning and expression of a cDNA for mouse prostaglandin E receptor EP3 subtype. *The Journal of Biological Chemistry.* 1992; 267:6463–6466. [PubMed: 1372606]
37. Qi HY, Shelhamer JH. Toll-like receptor 4 signaling regulates cytosolic phospholipase A2 activation and lipid generation in lipopolysaccharide-stimulated macrophages. *The Journal of Biological Chemistry.* 2005; 280:38969–38975. [PubMed: 16176925]
38. Buczynski MW, Stephens DL, Bowers-Gentry RC, Grkovich A, Deems RA, Dennis EA. TLR-4 and sustained calcium agonists synergistically produce eicosanoids independent of protein synthesis in RAW264.7 cells. *The Journal of Biological Chemistry.* 2007; 282:22834–22847. [PubMed: 17535806]
39. Kuroda E, Ishii KJ, Uematsu S, Ohata K, Coban C, Akira S, Aritake K, Urade Y, Morimoto Y. Silica crystals and aluminum salts regulate the production of prostaglandin in macrophages via NALP3 inflammasome-independent mechanisms. *Immunity.* 2011; 34:514–526. [PubMed: 21497116]
40. Sokolowska M, Chen LY, Eberlein M, Martinez-Anton A, Liu Y, Alsaaty S, Qi HY, Logun C, Horton M, Shelhamer JH. Low molecular weight hyaluronan activates cytosolic phospholipase A2 α and eicosanoid production in monocytes and macrophages. *The Journal of Biological Chemistry.* 2014; 289:4470–4488. [PubMed: 24366870]
41. Barbera-Cremades M, Baroja-Mazo A, Gomez AI, Machado F, Di Virgilio F, Pelegrin P. P2X7 receptor-stimulation causes fever via PGE₂ and IL-1 β release. *FASEB Journal.* 2012; 26:2951–2962. [PubMed: 22490780]

42. von Moltke J, Trinidad NJ, Moayeri M, Kintzer AF, Wang SB, van Rooijen N, Brown CR, Krantz BA, Leppla SH, Gronert K, Vance RE. Rapid induction of inflammatory lipid mediators by the inflammasome in vivo. *Nature*. 2012; 490:107–111. [PubMed: 22902502]
43. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, Lee WP, Weinrauch Y, Monack DM, Dixit VM. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature*. 2006; 440:228–232. [PubMed: 16407890]
44. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, Fitzgerald KA, Latz E. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nature Immunology*. 2008; 9:847–856. [PubMed: 18604214]
45. Koga K, Takaesu G, Yoshida R, Nakaya M, Kobayashi T, Kinjo I, Yoshimura A. Cyclic adenosine monophosphate suppresses the transcription of proinflammatory cytokines via the phosphorylated c-Fos protein. *Immunity*. 2009; 30:372–383. [PubMed: 19285436]
46. Ma W, Quirion R. Up-regulation of interleukin-6 induced by prostaglandin E from invading macrophages following nerve injury: an in vivo and in vitro study. *Journal of Neurochemistry*. 2005; 93:664–673. [PubMed: 15836625]
47. Ratcliffe MJ, Walding A, Shelton PA, Flaherty A, Dougall IG. Activation of E-prostanoid4 and E-prostanoid2 receptors inhibits TNF-alpha release from human alveolar macrophages. *The European Respiratory Journal*. 2007; 29:986–994. [PubMed: 17331962]
48. Bender AT, Beavo JA. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacological Reviews*. 2006; 58:488–520. [PubMed: 16968949]
49. Kumar S, Kostin S, Flacke JP, Reusch HP, Ladilov Y. Soluble adenylyl cyclase controls mitochondria-dependent apoptosis in coronary endothelial cells. *The Journal of Biological Chemistry*. 2009; 284:14760–14768. [PubMed: 19336406]
50. Iwatsubo K, Tsunematsu T, Ishikawa Y. Isoform-specific regulation of adenylyl cyclase: a potential target in future pharmacotherapy. *Expert Opinion on Therapeutic Targets*. 2003; 7:441–451. [PubMed: 12783579]
51. Fawcett L, Baxendale R, Stacey P, McGrouther C, Harrow I, Soderling S, Hetman J, Beavo JA, Phillips SC. Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97:3702–3707. [PubMed: 10725373]
52. Frei R, Ferstl R, Konieczna P, Ziegler M, Simon T, Rugeles TM, Mailand S, Watanabe T, Lauener R, Akdis CA, O'Mahony L. Histamine receptor 2 modifies dendritic cell responses to microbial ligands. *The Journal of Allergy and Clinical Immunology*. 2013; 132:194–204. [PubMed: 23465664]
53. Aronoff DM, Canetti C, Serezani CH, Luo M, Peters-Golden M. Cutting edge: macrophage inhibition by cyclic AMP (cAMP): differential roles of protein kinase A and exchange protein directly activated by cAMP-1. *J Immunol*. 2005; 174:595–599. [PubMed: 15634874]
54. Gattorno M, Tassi S, Carta S, Delfino L, Ferlito F, Pelagatti MA, D'Osualdo A, Buoncompagni A, Alpigiani MG, Alessio M, Martini A, Rubartelli A. Pattern of interleukin-1beta secretion in response to lipopolysaccharide and ATP before and after interleukin-1 blockade in patients with CIAS1 mutations. *Arthritis and Rheumatism*. 2007; 56:3138–3148. [PubMed: 17763411]
55. Piccini A, Carta S, Tassi S, Lasiglie D, Fossati G, Rubartelli A. ATP is released by monocytes stimulated with pathogen-sensing receptor ligands and induces IL-1beta and IL-18 secretion in an autocrine way. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105:8067–8072. [PubMed: 18523012]
56. Rossol M, Pierer M, Raulien N, Quandt D, Meusch U, Rothe K, Schubert K, Schoneberg T, Schaefer M, Krugel U, Smajilovic S, Brauner-Osborne H, Baerwald C, Wagner U. Extracellular Ca²⁺ is a danger signal activating the NLRP3 inflammasome through G protein-coupled calcium sensing receptors. *Nature Communications*. 2012; 3:1329.
57. Park JY, Pillinger MH, Abramson SB. Prostaglandin E2 synthesis and secretion: the role of PGE2 synthases. *Clin Immunol*. 2006; 119:229–240. [PubMed: 16540375]
58. Almeida MQ, Tsang KM, Cheadle C, Watkins T, Grivel JC, Nesterova M, Goldbach-Mansky R, Stratakis CA. Protein kinase A regulates caspase-1 via Ets-1 in bone stromal cell-derived lesions: a

- link between cyclic AMP and pro-inflammatory pathways in osteoblast progenitors. *Human Molecular Genetics*. 2011; 20:165–175. [PubMed: 20940146]
59. Liu Y, Chen LY, Sokolowska M, Eberlein M, Alsaaty S, Martinez-Anton A, Logun C, Qi HY, Shelhamer JH. The fish oil ingredient, docosahexaenoic acid, activates cytosolic phospholipase A via GPR120 receptor to produce prostaglandin E and plays an anti-inflammatory role in macrophages. *Immunology*. 2014; 143(1):81–95. [PubMed: 24673159]
60. Brandwein SR. Regulation of interleukin 1 production by mouse peritoneal macrophages. Effects of arachidonic acid metabolites, cyclic nucleotides, and interferons. *The Journal of Biological Chemistry*. 1986; 261:8624–8632. [PubMed: 2424900]
61. Shirahama M, Ishibashi H, Tsuchiya Y, Kurokawa S, Hayashida K, Okumura Y, Niho Y. Kupffer cells may autoregulate interleukin 1 production by producing interleukin 1 inhibitor and prostaglandin E2. *Scandinavian Journal of Immunology*. 1988; 28:719–725. [PubMed: 2852846]
62. Goss JA, Mangino MJ, Flye MW. Kupffer cell autoregulation of IL-1 production by PGE2 during hepatic regeneration. *The Journal of Surgical Research*. 1992; 52:422–428. [PubMed: 1619908]
63. Knudsen PJ, Dinarello CA, Strom TB. Prostaglandins posttranscriptionally inhibit monocyte expression of interleukin 1 activity by increasing intracellular cyclic adenosine monophosphate. *J Immunol*. 1986; 137:3189–3194. [PubMed: 3021848]
64. Hart PH, Whitty GA, Piccoli DS, Hamilton JA. Control by IFN-gamma and PGE2 of TNF alpha and IL-1 production by human monocytes. *Immunology*. 1989; 66:376–383. [PubMed: 2495247]
65. Sung SS, Walters JA. Increased cyclic AMP levels enhance IL-1 alpha and IL-1 beta mRNA expression and protein production in human myelomonocytic cell lines and monocytes. *The Journal of Clinical Investigation*. 1991; 88:1915–1923. [PubMed: 1661292]
66. Ohmori Y, Strassman G, Hamilton TA. cAMP differentially regulates expression of mRNA encoding IL-1 alpha and IL-1 beta in murine peritoneal macrophages. *J Immunol*. 1990; 145:3333–3339. [PubMed: 2172382]
67. Oshima H, Hioki K, Popivanova BK, Oguma K, Van Rooijen N, Ishikawa TO, Oshima M. Prostaglandin E(2) signaling and bacterial infection recruit tumor-promoting macrophages to mouse gastric tumors. *Gastroenterology*. 2011; 140:596–607. e597. [PubMed: 21070778]
68. Chandra G, Cogswell JP, Miller LR, Godlevski MM, Stinnett SW, Noel SL, Kadwell SH, Kost TA, Gray JG. Cyclic AMP signaling pathways are important in IL-1 beta transcriptional regulation. *J Immunol*. 1995; 155:4535–4543. [PubMed: 7594450]
69. Otterness IG, Bliven ML, Eskra JD, Reinke M, Hanson DC. The pharmacologic regulation of interleukin-1 production: the role of prostaglandins. *Cellular Immunology*. 1988; 114:385–397. [PubMed: 2968844]
70. Andrei C, Margiocco P, Poggi A, Lotti LV, Torrisi MR, Rubartelli A. Phospholipases C and A2 control lysosome-mediated IL-1 beta secretion: Implications for inflammatory processes. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:9745–9750. [PubMed: 15192144]
71. Hua KF, Chou JC, Ka SM, Tasi YL, Chen A, Wu SH, Chiu HW, Wong WT, Wang YF, Tsai CL, Ho CL, Lin CH. Cyclooxygenase-2 Regulates NLRP3 Inflammasome-Derived IL-1beta Production. *J Cell Physiol*. 2014; 230:863–874. [PubMed: 25294243]

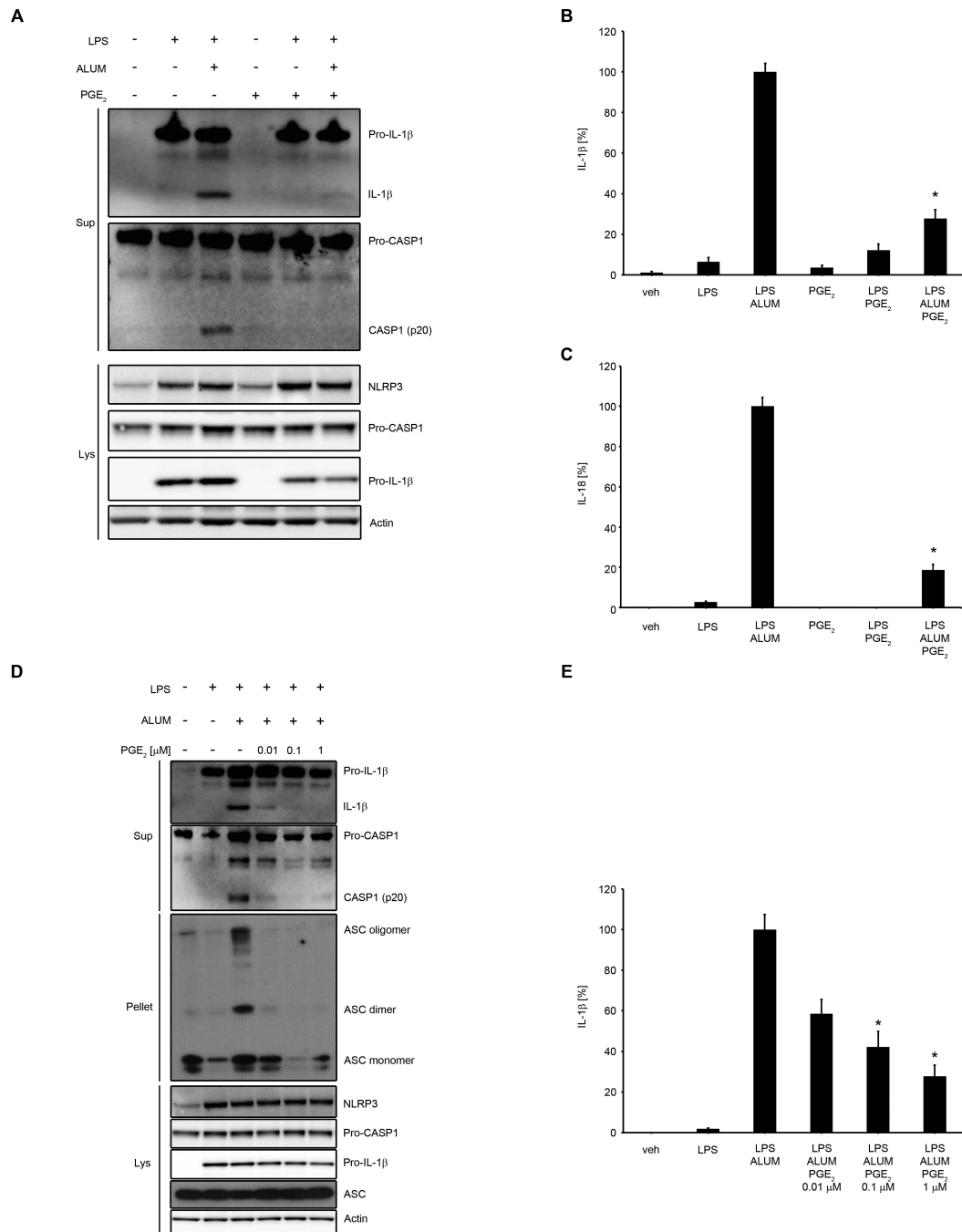


Figure 1. PGE₂ inhibits alum-induced NLRP3 inflammasome activation and IL-1β and IL-18 secretion

(A-E) human primary MDM were treated with/without LPS (100 ng/ml) for 4 h in complete medium, followed by 30 min treatment with PGE₂ (0.1 μM) or vehicle (EtOH) (A, B and C) or indicated doses of PGE₂ (D and E) then stimulated with alum (400 μg/ml for 5 h) in serum-free medium. Supernatants, lysates and cell pellets were collected for WB (A and D) or ELISA (B, C and E). (A and D) the WB are representative of 3 independent experiments from 3 healthy donors, each showing similar results. (B) IL-1β release data represent the

mean \pm SEM from 7 independent experiments from 7 healthy donors, performed in duplicate. IL-1 β data are presented as the percentage of the response after LPS/alum treatment, ranging from 61.6 to 831.6 pg/ml. (C) IL-18 release data represent the mean \pm SEM from 5 independent experiments from 5 healthy donors, performed in duplicate. IL-18 data are presented as the percentage of the response after LPS/alum treatment, ranging from 18.2 to 821.3 pg/ml. (E) IL-1 β release data represent the mean \pm SEM from 6 independent experiments from 6 healthy donors, performed in duplicate. IL-1 β data are presented as the percentage of the response after LPS/alum treatment, which ranged from 11.4 to 299 pg/ml.* $P < .05$ as compared to LPS/alum treatment, as assessed by Kruskal-Wallis ANOVA on ranks, followed by Dunn's post hoc test.

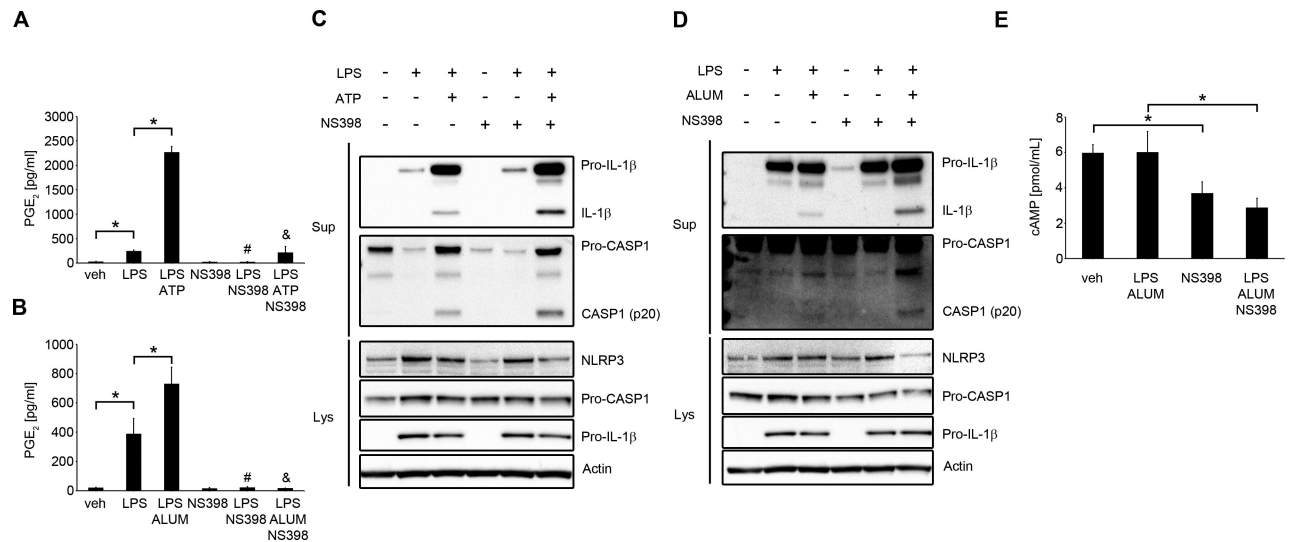


Figure 2. PGE₂-induced inhibition of IL-1 β production is driven through NLRP3 inflammasome (A-E) primary MDM were treated with/without LPS (100 ng/ml) for 4 h in complete medium, followed by 30 min treatment with PGE₂ (0.1 μ M) or vehicle (EtOH), then stimulated with ATP (2 mM for 1 h) (A, B and C) or nigericin (4 μ M for 1 h) (D and E) in serum-free medium. Supernatants and lysates were collected for WB or ELISA. (A and E) the WB are representative of 3 independent experiments from 3 healthy donors, each showing similar results. (B) IL-1 β release data represent the mean \pm SEM from 3 independent experiments from 3 healthy donors, performed in duplicate. Data are presented as the percentage of the response after LPS/ATP treatment, ranging from 226.6 to 5851.4 pg/ml. (C and D) mature caspase-1 is presented in optical density units [OD] after subtraction of background from 3 independent experiments from 3 healthy donors. (F and G) MDM were transfected with NLRP3 siRNA (1 μ M; L-017367-00-0005). After 48 h cells were treated with/without LPS (100 ng/ml) for 4 h in complete culture medium, followed by stimulation with alum (400 μ g/ml for 5 h) (F) or ATP (2 mM for 1 h) (G) in serum-free medium. The WB are representative of 3 independent experiments from 3 healthy donors, each showing similar results.

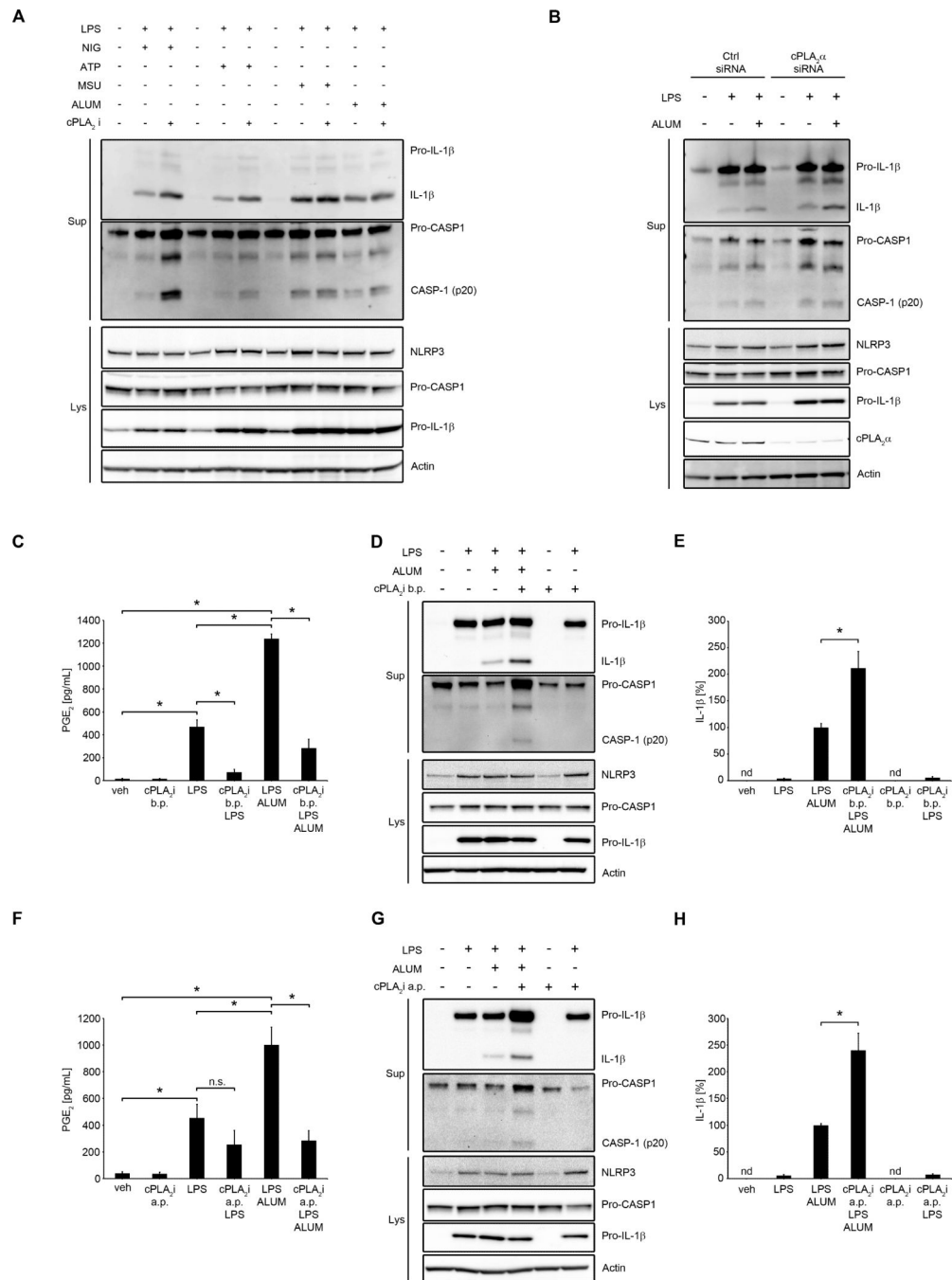


Figure 3. PGE₂ does not decrease gene expression of proteins involved in NLRP3 inflammasome complex formation

(A-E) primary MDM were treated with/without LPS (100 ng/ml) for 4 h in complete medium, followed by treatment with PGE₂ (0.1 μM) or vehicle (EtOH) for indicated time in serum-free medium. At each time point, cells were lysed and total RNA was extracted. Gene expression was assessed using RT PCR, normalized to GAPDH transcripts and represented as a relative quantification (RQ) compared with vehicle treated cells from the first time-point. Data represent the mean ± SEM from 4 independent experiments from 4 healthy

donors, each performed in duplicate. * $P < .05$ as indicated, as assessed by Kruskal-Wallis ANOVA on ranks, followed by Dunn's post hoc test.

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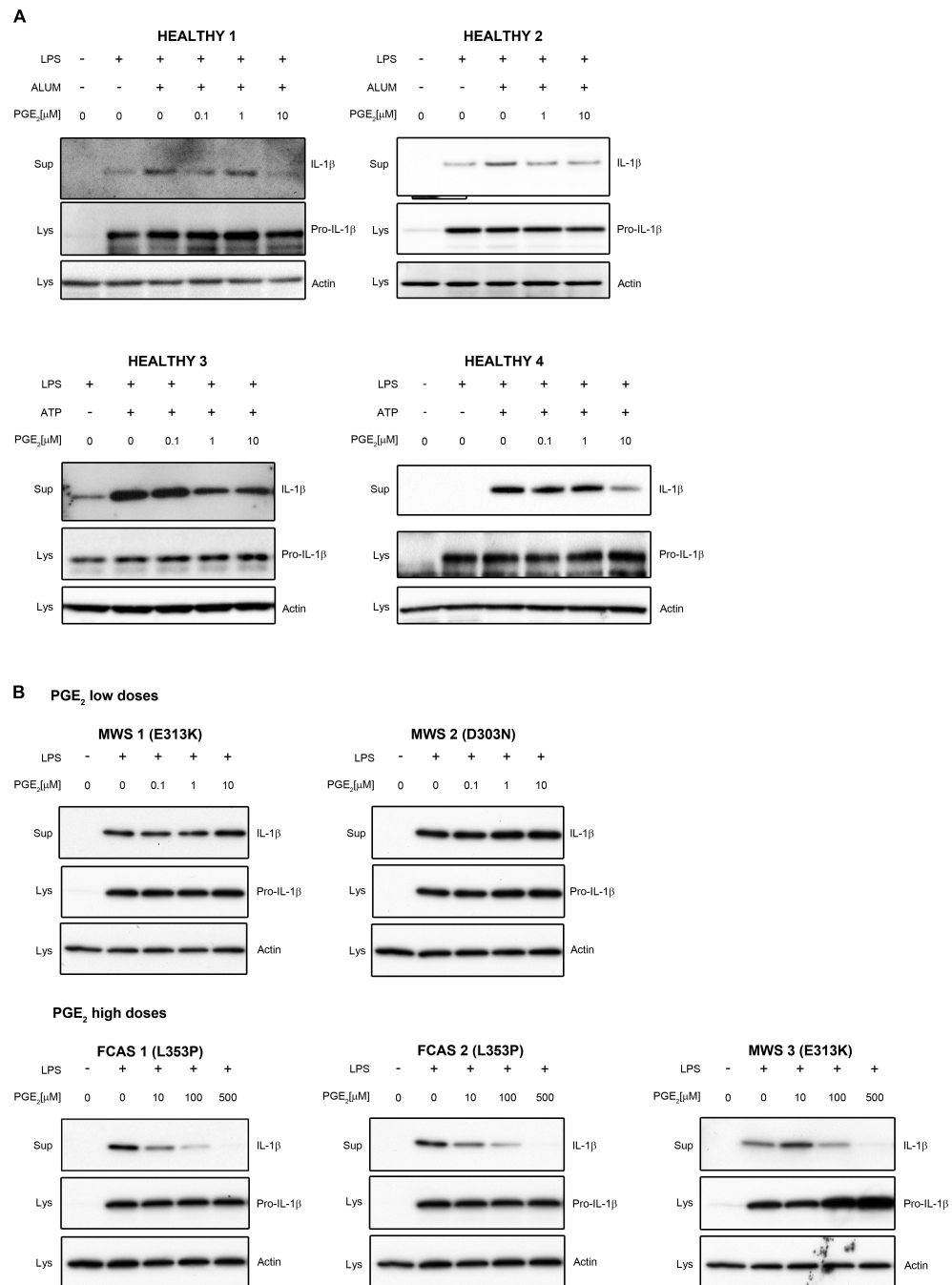


Figure 4. PGE₂ inhibits NLRP3 inflammasome through EP4 receptor (A and B) primary MDM were treated with/without LPS (100 ng/ml) for 4 h in complete medium, followed by 30 min treatment with EP2 agonist (butaprost, free acid, 0.5 μM), EP4 agonist (CAY10598, 0.1 μM), PGE₂ (0.1 μM) or vehicle (EtOH), then stimulated with alum (400 μg/ml for 5 h) in serum-free medium. Supernatants and lysates were collected for WB (A) or IL-1β ELISA (B). IL-1β release data represent the mean ± SEM from 3 independent experiments from 3 healthy donors, each performed in duplicate. Data are presented as the percentage of the response after LPS/alum treatment, which ranged from 111.5 to 139.9 pg/

ml. * $P < .05$ as indicated, as assessed by Kruskal-Wallis ANOVA on ranks, followed by Dunn's post hoc test (**B**). (**C** and **D**) primary MDM were treated with/without LPS (100 ng/ml) for 4 h in complete culture medium, then 30 min with EP2 inhibitor (PF-04418948, 0.5 μ M), EP4 inhibitor (GW627368X, 2 μ M), both inhibitors or vehicle (DMSO), followed by 30 min treatment with PGE₂ (0.1 μ M) or vehicle (EtOH), and stimulation with alum (400 μ g/ml for 5 h) in serum-free medium. Supernatants and lysates were collected for WB (**C**) or IL-1 β ELISA (**D**). IL-1 β release data represent the mean \pm SEM from 5 independent experiments from 5 healthy donors, each performed in duplicate. Data are presented as the percentage of the response after LPS/alum treatment, which ranged from 36.6 to 372.6 pg/ml (**D**). (**E**, **F** and **G**), MDM were transfected with EP2 siRNA (1 μ M), EP4 siRNA (1 μ M) or both. After 48 h cells were treated with/without LPS (100 ng/ml) for 4 h in complete culture medium, followed by 30 min treatment with PGE₂ (0.1 μ M) or vehicle (EtOH), and then stimulated with alum (400 μ g/ml for 5 h) in serum-free medium. Supernatants and lysates were collected for RT-PCR (**E**), WB (**F**) or IL-1 β ELISA (**G**). Gene expression was normalized to GAPDH transcripts and represented as a relative quantification (RQ) compared with vehicle-treated control siRNA transfected cells. Data represent the mean \pm SEM from 3 independent experiments from 3 healthy donors, each performed in duplicate. * $P < .05$ as indicated, as assessed Kruskal-Wallis ANOVA on ranks, followed by Dunn's post hoc test (**E**). IL-1 β release data represent the mean \pm SEM from 3 independent experiments from 3 healthy donors, performed in duplicate. Data are presented as the percentage of the response after LPS/alum treatment, which ranged from 34.6 to 491.5 pg/ml (**G**). * $P < .05$ as indicated, as assessed by Kruskal-Wallis ANOVA on ranks, followed by Dunn's post hoc test (**D** and **G**). The WB are representative of 3 independent experiments from 3 healthy donors, each showing similar results (**A**, **C** and **F**).

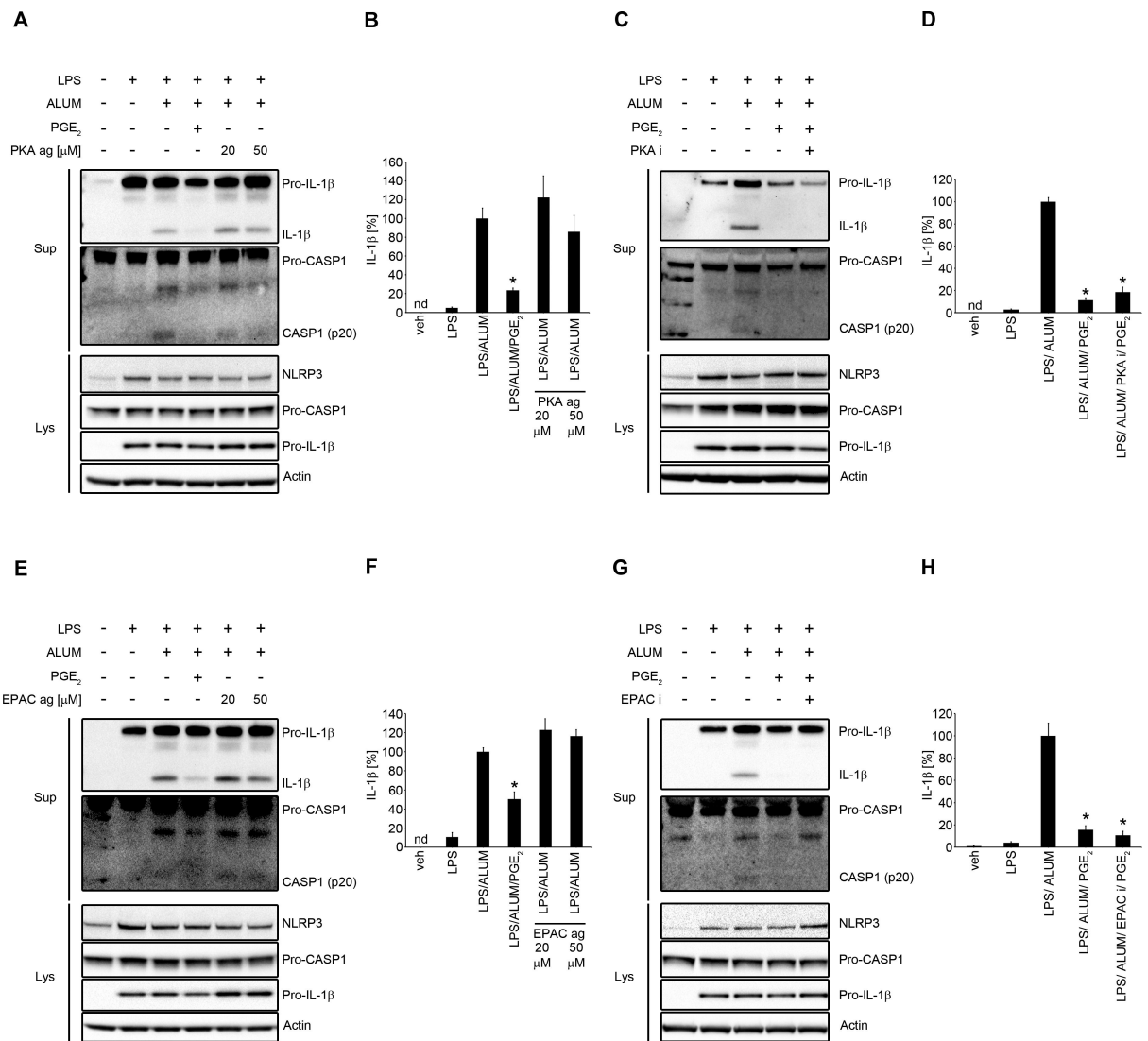


Figure 5. cAMP is involved in PGE₂-mediated NLRP3 inhibition

(A, B) primary MDM were treated with/without LPS (100 ng/ml) for 4 h in complete medium, followed by treatment for 1 h with KH7 (25 μM) or IBMX (200 μM) or forskolin (50 μM), and followed by PGE₂ (0.1 μM) or vehicle (EtOH) for 30 min and stimulated with alum for 5 h (400 μg/ml) in serum-free medium. Supernatants and lysates were collected for WB (A) or IL-1β ELISA (B). IL-1β release data represent the mean ± SEM from 3 independent experiments from 3 healthy donors, each performed in duplicate. Data are presented as the percentage of the response after LPS/alum treatment, which ranged from 76.7 to 562.8 pg/ml. # $P < .05$ as compared to LPS/alum treatment, * $P < .05$ as indicated, as assessed by one-way ANOVA, followed by Holm-Sidak post hoc test (B). (C) primary MDM were treated with IBMX (200 μM) with either vehicle (EtOH), PGE₂ (0.1 μM) or forskolin (50 μM) for 15 min. cAMP measured in cell lysates represents the mean ± SEM from 3 independent experiments from 3 healthy donors, each performed in triplicate. * $P < .05$ as indicated, as assessed by Kruskal-Wallis ANOVA on ranks, followed by Dunn's post hoc test. (D and E) MDM were treated with/without LPS (100 ng/ml) for 4 h in complete

medium, followed by 30 min treatment with indicated doses of 8-Bromo-cAMP or vehicle (Tris) and then stimulated with alum for 5 h (400 µg/ml) in serum-free medium. Supernatants and lysates were collected for WB (**D**) or IL-1β ELISA (**E**). IL-1β release data represent the mean ± SEM from 3 independent experiments from 3 healthy donors, each performed in duplicate. Data are presented as the percentage of the response after LPS/alum treatment, which ranged from 70.2 to 1100.9 pg/ml.* $P < .05$ as compared to LPS/alum treatment, as assessed by Kruskal-Wallis ANOVA on ranks, followed by Dunn's post hoc test (**E**). The WB are representative of 3 independent experiments from 3 healthy donors, each showing similar results (**A** and **D**).

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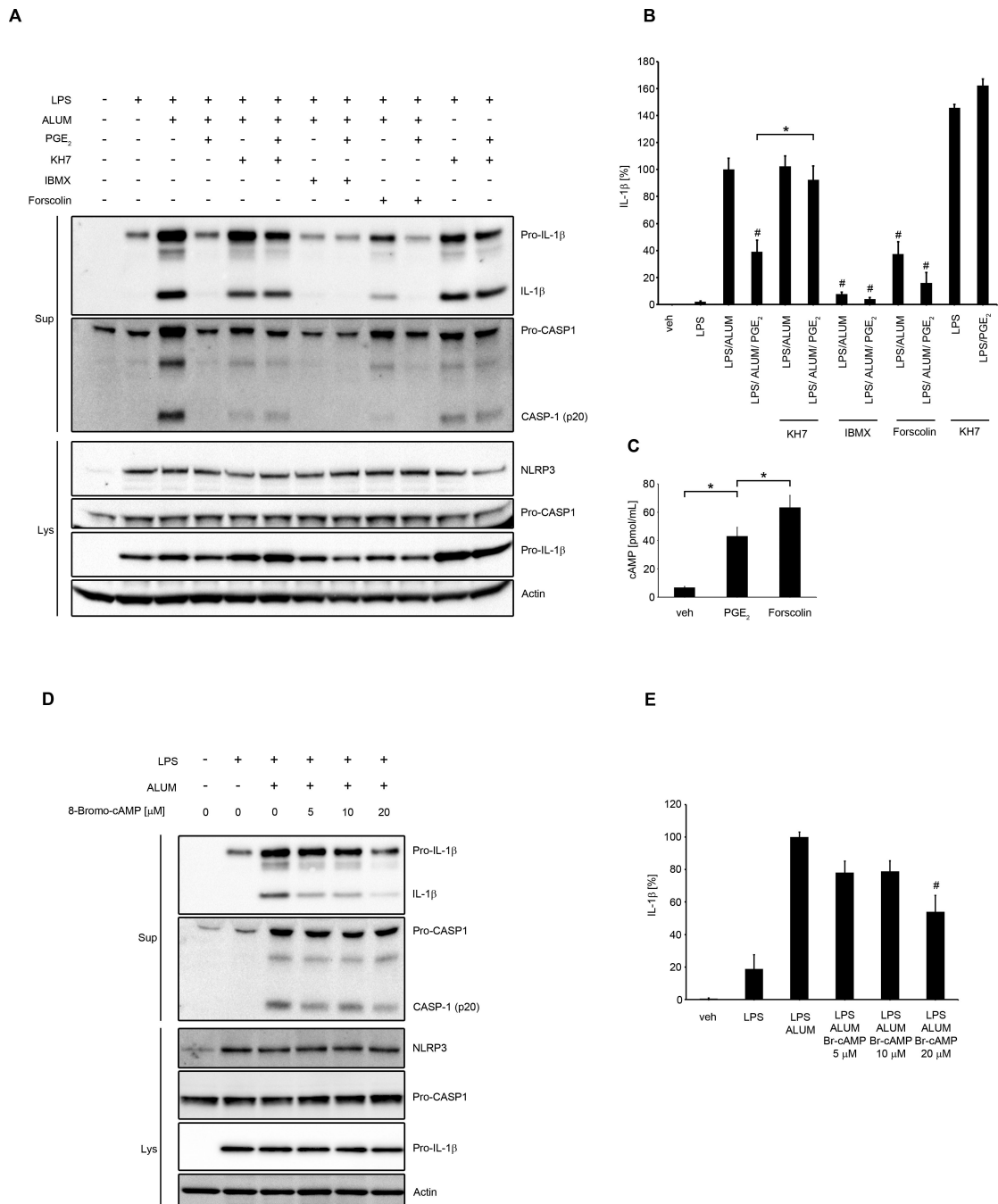


Figure 6. PGE₂-derived NLRP3 inflammasome inhibition does not require PKA or EPAC (A and B) primary MDM were treated with/without LPS (100 ng/ml) for 4 h in complete medium, followed by 30 min treatment with PKA selective agonist (6-Bnz-8-PIP-cAMP, 20 or 50 μM), PGE₂ (0.1 μM) or vehicle, then stimulated with alum (400 μg/ml for 5 h) in serum-free medium. Supernatants and lysates were collected for WB (A) or IL-1β ELISA (B). IL-1β release data represent the mean ± SEM from 3 independent experiments from 3 healthy donors, each performed in duplicate. Data are presented as the percentage of the response after LPS/alum treatment, which ranged from 44.7 to 103 pg/ml. * *P* < .05 as

compared to LPS/alum, as assessed by Kruskal-Wallis ANOVA on ranks, followed by Dunn's post hoc test (**B**). (**C** and **D**) primary MDM were treated with/without LPS (100 ng/ml) for 4 h in complete culture medium, then 30 min with PKA inhibitor (8-Bromoadenosine 3',5'-cyclic Monophosphothioate, Rp-Isomer . sodium salt, 50 μ M) or vehicle (DMSO), followed by 30 min treatment with PGE₂ (0.1 μ M) or vehicle (EtOH), and stimulation with alum (400 μ g/ml for 5 h) in serum-free medium. Supernatants and lysates were collected for WB (**C**) or IL-1 β ELISA (**D**). IL-1 β release data represent the mean \pm SEM from 3 independent experiments from 3 healthy donors, each performed in duplicate. Data are presented as the percentage of the response after LPS/alum treatment, which ranged from 43.9 to 167.7 pg/ml. * $P < .05$ as compared to LPS/alum, as assessed by Kruskal-Wallis ANOVA on ranks, followed by Dunn's post hoc test (**D**). (**E** and **F**) primary MDM were treated with/without LPS (100 ng/ml) for 4 h in complete medium, followed by 30 min treatment with EPAC selective agonist (8-(4-Chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic Monophosphate . sodium salt, 20 or 50 μ M), PGE₂ (0.1 μ M) or vehicle, then stimulated with alum (400 μ g/ml for 5 h) in serum-free medium. Supernatants and lysates were collected for WB (**E**) or IL-1 β ELISA (**F**). IL-1 β release data represent the mean \pm SEM from 3 independent experiments from 3 healthy donors, each performed in duplicate. Data are presented as the percentage of the response after LPS/alum treatment, which ranged from 82.9 to 245.6 pg/ml.* $P < .05$ as compared to LPS/alum, as assessed by Kruskal-Wallis ANOVA on ranks, followed by Dunn's post hoc test (**F**). (**G** and **H**) primary MDM were treated with/without LPS (100 ng/ml) for 4 h in complete culture medium, then 30 min with EPAC inhibitor (4- Cyclopentyl- 2- (2, 5- dimethylbenzylsulfanyl)- 6- oxo- 1, 6- dihydropyrimidine- 5- carbonitrile, 10 μ M) or vehicle (DMSO), followed by 30 min treatment with PGE₂ (0.1 μ M) or vehicle (EtOH), and stimulation with alum (400 μ g/ml for 5 h) in serum-free medium. Supernatants and lysates were collected for WB (**G**) or IL-1 β ELISA (**H**). IL-1 β release data represent the mean \pm SEM from 3 independent experiments from 3 healthy donors, each performed in duplicate. Data are presented as the percentage of the response after LPS/alum treatment, which ranged from 206.2 to 226.4 pg/ml * $P < .05$ as compared to LPS/Alum, as assessed by Kruskal-Wallis ANOVA on ranks, followed by Dunn's post hoc test (**H**).

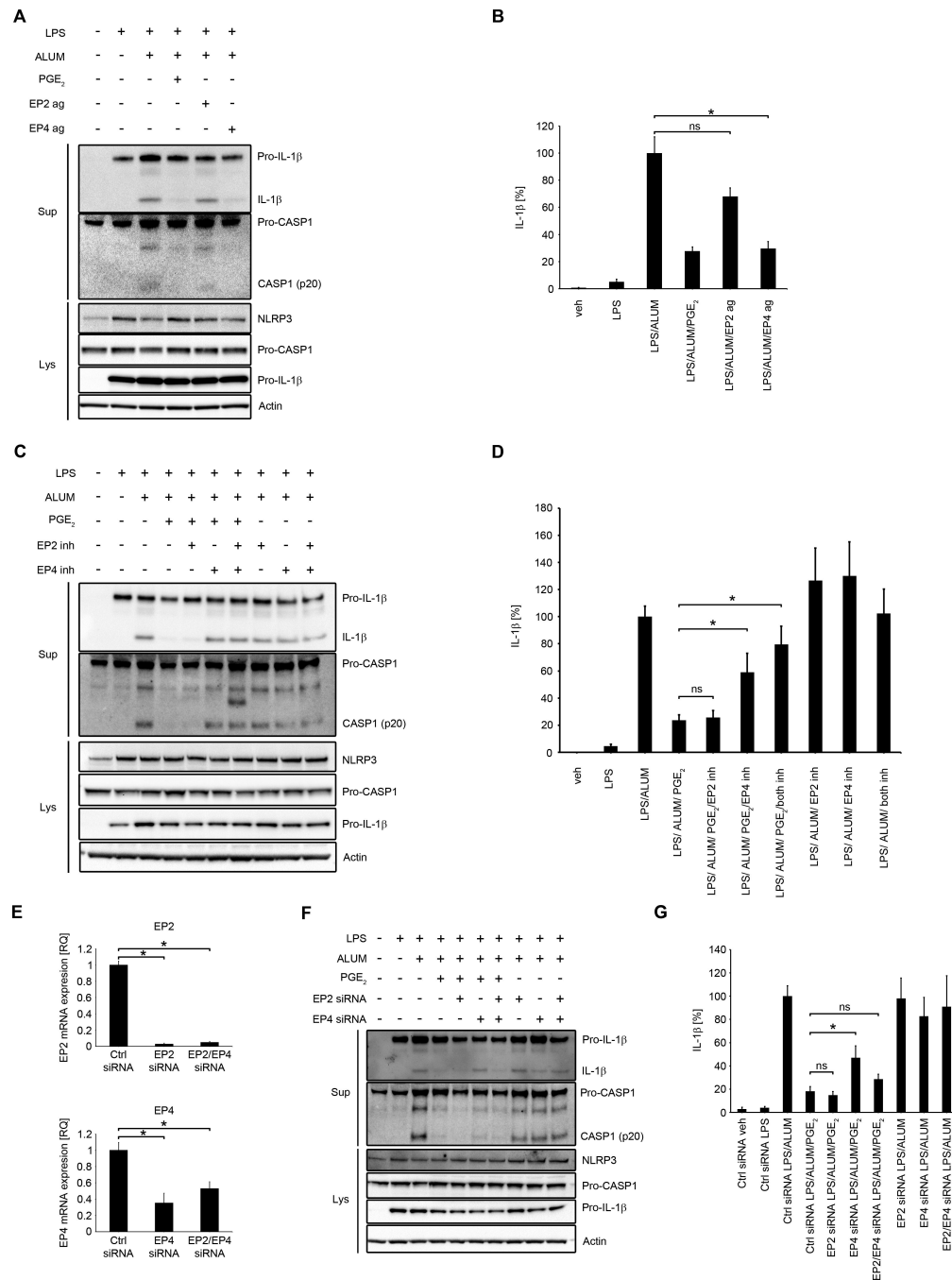


Figure 7. PGE₂-induced NLRP3 inflammasome inhibition requires higher doses in CAPS patients

(A) PBMCs from four healthy subjects were treated with LPS (1 μ g/ml) for 3 h in complete medium, followed by treatment with the indicated doses of PGE₂ or vehicle (EtOH) and ATP (2 mM) or alum (400 μ g/ml) for 30 min in serum-free medium. Supernatants and lysates were collected for WB. (B) PBMCs from five CAPS patients with designated mutations in *NLRP3* were treated with LPS (1 μ g/ml) for 3 h in complete medium, followed

by treatment with indicated doses of PGE₂ or vehicle (EtOH) for 30 min in serum-free medium. Supernatants and lysates were collected for WB.

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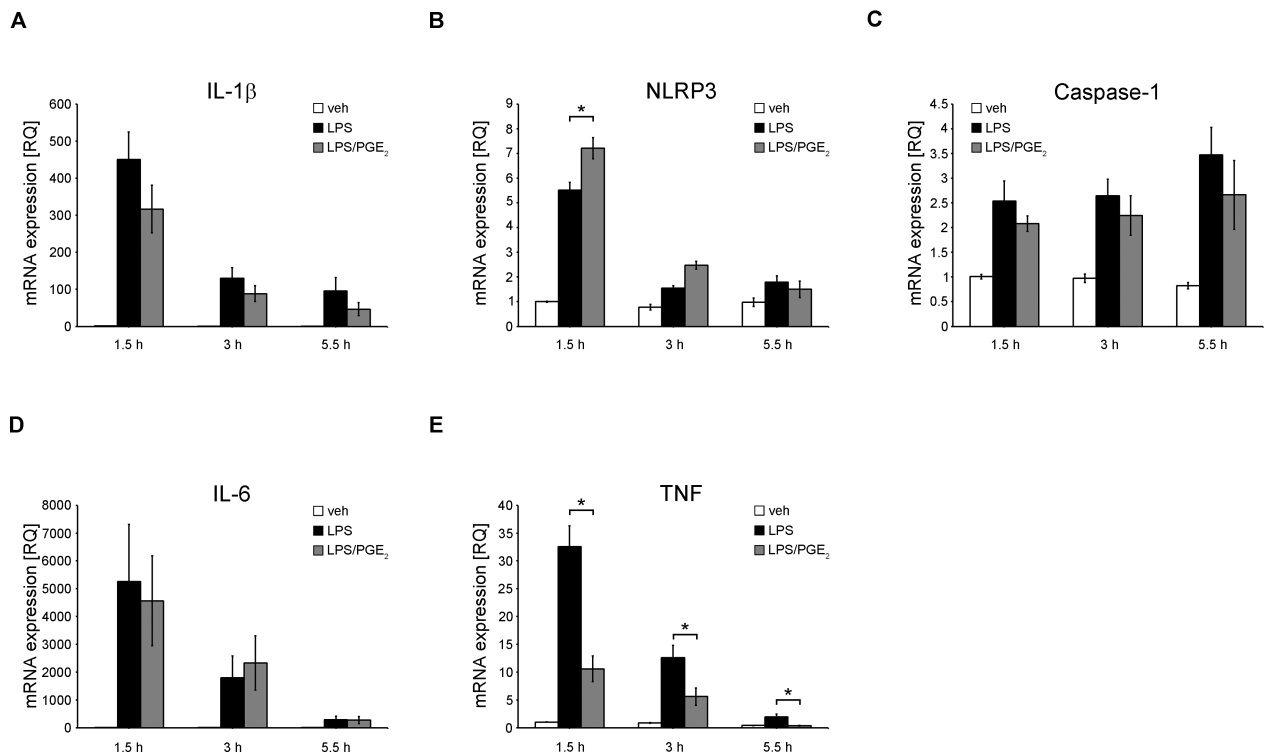


Figure 8. cPLA₂α is involved in endogenous NLRP3 inflammasome regulation

(A) THP-1 cells were treated with cPLA₂ inhibitor (pyrrolidone derivative, 2 μM, for 30 min) or vehicle (DMSO) then primed with LPS (100 ng/ml) for 4 h in serum-free medium, followed by treatment with nigericin (4 μM for 45 min), ATP (1 mM, for 1 h), monosodium urate crystals (MSU, 100 μg/ml, for 5h) or alum (200 μg/ml, for 5h). Supernatants and lysates were collected for WB. The WB are representative of 2 independent experiments, each showing similar results. (B) THP-1 cells were transfected with cPLA₂α siRNA or control siRNA (1 μM). After 48 h transfected cells were primed with LPS (100 ng/ml) for 4 h in serum-free medium, followed by stimulation with alum (200 μg/ml, for 5h). Supernatants and lysates were collected for WB. The WB are representative of 2 independent experiments, each showing similar results. (C, D and E) human primary MDM were treated with/without LPS (100 ng/ml) with cPLA₂ inhibitor (2 μM) or DMSO for 4 h in complete medium, followed by subsequent treatment with cPLA₂ inhibitor or DMSO for 30 min, and then stimulated with alum (400 μg/ml for 5 h) in serum-free medium. Supernatants and lysates were collected for PGE₂ ELISA (C), WB (D) or IL-1β ELISA (E). PGE₂ (C) and IL-1β (E) release data represent the mean ± SEM from 3 independent experiments from 3 healthy donors, each performed in duplicate. IL-1β data are presented as the percentage of the response after LPS/alum treatment, which ranged from 409.4 to 647.6 pg/ml (E). (F, G and H) human primary MDM were treated with/without LPS (100 ng/ml) for 4 h in complete medium, followed by treatment with cPLA₂ inhibitor or DMSO for 30 min, and then stimulated with alum (400 μg/ml for 5 h) in serum-free medium. Supernatants and lysates were collected for PGE₂ ELISA (F), WB (G) or IL-1β ELISA (H). PGE₂ (F) and IL-1β (H) release data represent the mean ± SEM from 3 independent experiments from 3 healthy donors, each performed in duplicate. IL-1β data are presented as the percentage of

the response after LPS/alum treatment, which ranged from 261.2 to 516.7 pg/ml (**E**). The WB are representative of 3 independent experiments from 3 healthy donors, each showing similar results (D, G). * $P < .05$ as indicated, as assessed by Kruskal-Wallis ANOVA on ranks, followed by Dunn's post hoc test. b.p. (before priming), a.p. (after priming)

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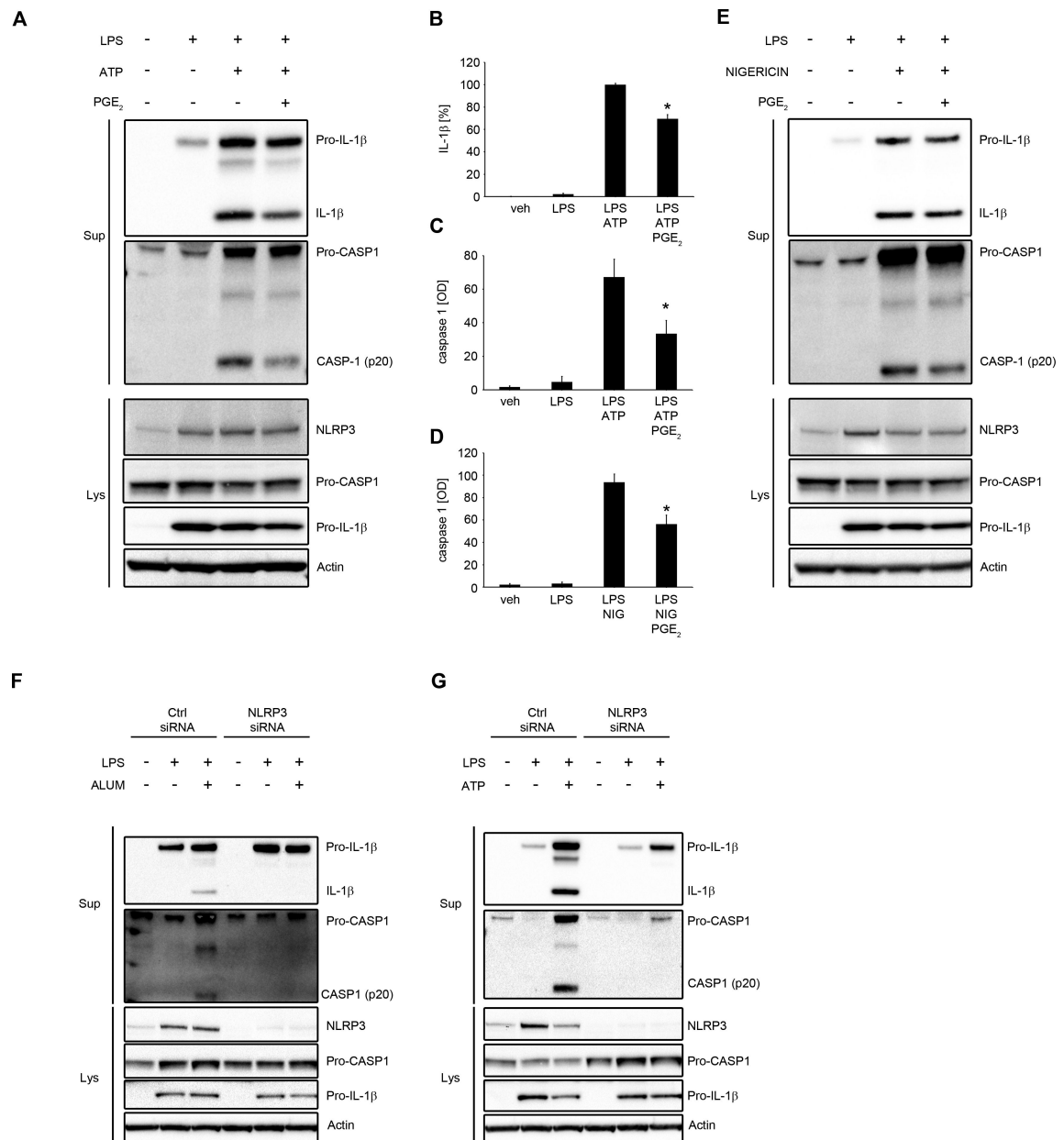


Figure 9. COX2 is involved in endogenous NLRP3 inflammasome regulation

(A-D) primary MDM were treated with/without LPS (100 ng/ml) with NS398, a specific COX2 inhibitor (5 μ M) or DMSO for 4 h in complete medium, followed by subsequent treatment with NS398 (5 μ M) or DMSO for 30 min, and then stimulated with ATP (2 mM for 1 h) (A and C) or alum (400 μ g/ml for 5 h) (B and D) in serum-free medium. Supernatants and lysates were collected for PGE₂ ELISA (A and B) or WB (C and D). The WB are representative of 3 independent experiments from 3 healthy donors, each showing similar results. PGE₂ release data represent the mean \pm SEM from 3 independent experiments from 3 healthy donors, each performed in duplicate. * $P < .05$ as indicated, # $P < .05$ as compared to LPS, & $P < .05$ as compared to LPS/ATP or LPS/alum, as assessed by Kruskal-Wallis ANOVA on ranks, followed by Dunn's post hoc test. (E) Primary MDM

were treated with LPS (100 ng/ml) with/without NS398 (5 μ M), NS398 alone or DMSO for 4 h in complete medium and then stimulated with alum for 15 min in serum-free medium. cAMP measured in cell lysates represents the mean \pm SEM from 3 independent experiments from 3 healthy donors, each performed in triplicate. * $P < .05$ as indicated, as assessed by Kruskal-Wallis ANOVA on ranks, followed by Dunn's post hoc test.

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