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*J Immunol* 2015; 194:5472-5487; Prepublished online 27 April 2015; doi: 10.4049/jimmunol.1401343

http://www.jimmunol.org/content/194/11/5472

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**Supplementary Material**  [http://www.jimmunol.org/content/suppl/2015/04/25/jimmunol.1401343.DC1Supplemental](http://www.jimmunol.org/content/suppl/2015/04/25/jimmunol.1401343.DC1Supplemental)

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Prostaglandin E₂ Inhibits NLRP3 Inflammasome Activation through EP4 Receptor and Intracellular Cyclic AMP in Human Macrophages

Milena Sokolowska,* Li-Yuan Chen,* Yueqin Liu, Asuncion Martinez-Anton, Hai-Yan Qi,* Carolea Logun,* Sara Alsaaty,* Yong Hwan Park, Daniel L. Kastner, Jae Jin Chae, and James H. Shelhamer*

PGE₂ is a potent lipid mediator involved in maintaining homeostasis but also promotion of acute inflammation or immune suppression in chronic inflammation and cancer. Nucleotide-binding domain, leucine-rich repeat-containing protein (NLR)P3 inflammasome plays an important role in host defense. Uncontrolled activation of the NLRP3 inflammasome, owing to mutations in the NLRP3 gene, causes cryopyrin-associated periodic syndromes. In this study, we showed that NLRP3 inflammasome activation is inhibited by PGE₂ in human primary monocyte-derived macrophages. This effect was mediated through PGE₂ receptor subtype 4 (EP4) and an increase in intracellular cAMP, independently of protein kinase A or exchange protein directly activated by cAMP. A specific agonist of EP4 mimicked, whereas 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 small interfering RNA or inhibiting cyclooxygenase 2, resulting in inhibition of endogenous PGE₂ production, caused an increase in NLRP3 inhibition. PGE₂ caused an increase in intracellular cAMP. Blockade of adenylate cyclase by its inhibitor or small interfering RNA 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. The Journal of Immunology, 2015, 194: 5472–5487.
PGE2 belongs to a family of bioactive lipid mediators that 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 being 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). However, 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, depend on the species, cell, and tissue types or context of action (28). PGE2 synthesis is initiated by phospholipase A₂, which catalyzes the hydrolysis of membrane phospholipids and liberates 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 converts AA to prostaglandins. The key enzyme of this pathway is the cyclooxygenase (COX) enzymes, which catalyze the oxidative decarboxylation of AA to generate the unstable prostaglandin H₂ (PGH₂) (29). PGH₂ is then converted to downstream active prostaglandin 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 accounted for, at least in part, by the existence of four PGE₂ receptor subtypes (EPs), belonging to the family of G protein–coupled receptors (GPCRs), differentially expressed in cells and conferring distinct intracellular effects by a panel of cAMP-binding effector proteins (31). EP₃ signaling pathway inhibits AC activity by coupling to the Gα₃ subunit and decreases 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 inflammasomes further stimulate PGE₂ production, although probably in an NLRP3- and caspase-1–independent manner (39). Furthermore, PGE₂ and other prostaglandins 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, the 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. Owing to the growing evidence of the coexistence of potent lipid mediator signaling and inflammasome 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 monococyte-derived macrophages (MDMs) and in PBMCs from healthy donors as well as in PBMCs from CAPS patients. In this study, we found that PGE₂ decreased NLRP3 inflammasome activation, triggered by aluminum crystals, ATP, or nigericin. This effect was derived through EP4 and through an increase in intracellular cAMP, independently of protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac). In patients with CAPS, with mutations in the NBD 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, and it might serve as a significant autocrine and paracrine regulator.

**Materials and Methods**

**Reagents and Abs**

Aluminum potassium sulfate (alum crystals), monosodium urate crystals, 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 were from Tocris Bioscience (Bristol, U.K.). 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 (6-[2-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]-piperidinoadenosine-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 Sciences (Farmingdale, NY). 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 IMDM (Life Technologies) with 10% heat-inactivated FBS and 0.05 mM 2-ME. THP-1 cells (0.5 × 10⁶) were seeded, allowed to attach to a culture flask in 15 ml IMDM (Life Technologies) with 10% heat-inactivated FBS and 2 mM L-glutamine and supplemented with 10% heat-inactivated FBS (Life Technologies/Thermo Scientific, Waltham, MA) overnight. The next day, 18 × 10⁶ monocytes were seeded into a 75 tissue culture flask in 15 ml IMDM (Life Technologies) with 10% heat-inactivated FBS and 50 ng/ml M-CSF (Life Technologies) for 7 d. Half of the medium was replaced every 3 d of culture. Twenty-four hours prior to experiments, macrophages were treated with trypsin (Lonza, Walkersville, MD) for 1 min, scraped, and plated in the IMDM medium without M-CSF into 12-well plates or 6-well plates at a density of 0.35 × 10⁶ or 0.85 × 10⁶, respectively.

Blood specimens from patients with CAPS were drawn after obtaining informed consent under a protocol approved by the National Institute of Arthritis and Musculoskeletal and Skin Diseases/National Institute of Diabetes and Digestive and Kidney Diseases Institutional Review Board. Blood specimens from healthy volunteers were obtained under a protocol approved by the National Heart, Lung, and Blood Institute Institutional Review Board. Blood samples were processed immediately using lymphocyte separation medium (Lonza) according to the manufacturer’s protocol to obtain PBMCs. PBMCs (2 × 10⁶) were seeded, allowed to attach to a 12-well plate containing RPMI 1640 medium without serum for 20 min, immediately followed by the inflammasome activation/inhibition experiments. THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium containing 10% heat-inactivated FBS and 0.05 mM 2-ME. THP-1 cells (0.5 × 10⁶) were plated into a 12-well plate in the presence of 50 nM PMA (Sigma-Aldrich) for 12 h before the experiment was performed.

**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 L-glutamine and supplemented with 10% heat-inactivated FBS (Life Technologies/Thermo Scientific, Waltham, MA) overnight. The next day, 18 × 10⁶ monocytes were seeded into a 75 tissue culture flask in 15 ml IMDM (Life Technologies) with 10% heat-inactivated FBS and 50 ng/ml M-CSF (Life Technologies) for 7 d. Half of the medium was replaced every 3 d of culture. Twenty-four hours prior to experiments, macrophages were treated with trypsin (Lonza, Walkersville, MD) for 1 min, scraped, and plated in the IMDM medium without M-CSF into 12-well plates or 6-well plates at a density of 0.35 × 10⁶ or 0.85 × 10⁶, respectively.

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**Inflammasome activation and inhibition**

Human primary MDMs 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...
FIGURE 1. PGE2 inhibits alum-induced NLRP3 inflammasome activation and IL-1β and IL-18 secretion. (A–E) Human primary MDMs 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–C) or indicated doses of PGE2 (D and E) and then stimulated with alum (400 μg/ml for 5 h) in serum-free medium. Supernatants, lysates, and cell pellets were collected for Western blot (WB) (A and D) or ELISA (B, C, and E). (A and D) The WBs are representative of three independent experiments from three healthy donors, each showing similar results. (B) IL-1β release data represent mean ± SEM from seven independent experiments from seven healthy donors, performed in duplicate. IL-1β data are presented as the percentage of the response after LPS/alum treatment, ranging from 61.6 to 831.6 pg/ml. (Figure legend continues)
(CAV10598; 0.1 μM), 8-bromo-cAMP (5–20 μM), PKA selective agonist (6-Brz-8-PIP-cAMP; 20 or 50 μM), or EPAC selective agonist (4-chlorophenylthio-2′,5′-O-methyladenosine 3′,5′-cyclic monophosphate sodium salt; 20 or 50 μM), followed by stimulation with alumn (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 (GW62736X; 2 μM), PKA inhibitor (8-bromo-adenosine 3′,5′-cyclic monophosphothioate, Rp-isomer sodium salt, 50 μM), EPAC inhibitor (4-cyclopropyl-2′,5′-dimethylbenzylsulfanylimidazo[6-oxo-1,6-dihydropyrimidine-5-carbonitrile; 10 μM), or vehicle (DMSO) were added 30 min prior to treatment with PGE2 or vehicle (EtOH). HK7 (25 μM), 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 MDMs, the inhibitor or DMSO was added before and after priming or only after priming, followed by treatment with alumn (400 μg/ml for 5 h) in Opti-MEM. In the experiments with COX2 inhibitor (NS398; 5 μM) in human primary MDMs, the inhibitor or DMSO was added before and after priming, followed by treatment with alumn (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 LPS and stimulated by monosodium urate (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-TARGETplus SMARTpool small interfering RNA (sRNA) (Thermo Scientific, Lafayette, CO) against human NLRP3 (L-017367-00-005 or sc-45469, Santa Cruz Biotechnology), EP2 (PTGER2)(L-005712-00-0005), EP4 (PTGER4) (L-005714-00-0005), and cPLA2α (PLA2G4A) (L-009886-00-005) together with ON-TARGETplus control nontargeting pool (D-00181970-05) were used to perform knockdown experiments in human primary MDMs or THP-1 cells. Human primary MDMs (0.5 × 10^6 cells per 100-μl cuvette) or THP-1 cells (5 × 10^5 per 100-μl cuvette) were transfected with siRNA pools (1 μm or 100 nM) using a P3 Primary Cell 4D-Nucleofector X Kit (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 h.

**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-1α, Hs00174131_m1; IL-1β, Hs00168755_m1; IL-6, Hs00171431_m1; TNF, Hs99999043_m1; cPLA2α (PLA2G4A), Hs00233352_m1; NLRP3, Hs00918082_m1; caspase-1, Hs00354836_m1; EP2, Hs01168752_m1; EP4, Hs01438523_m1; PLA2G4A, Hs01438523_m1; and iTag universal probes supermix (Bio-Rad). Gene expression was normalized to GAPDH transcripts and represented as a relative quantification compared with control.

**ELISA**

IL-1β and IL-18 in cell culture supernatants were measured using a human IL-1β (D-00181970-05) or IL-18 Quantikine Kit (SLB50) from R&D Systems or a human IL-18 ELISA kit from Medical and Biological Laboratories (Nagoya, Japan), respectively, according to the manufacturers’ directions. There were substantial differences in the IL-1β and IL-18 release between the donors, as depicted in the Supplemental Fig. 1. Therefore, 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.

**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-1β secretion. Human primary MDMs were primed with LPS followed by treatment with PGE2 and subsequent stimulation with alum to activate IL-1β maturation via NLRP3 inflammasome. We found that PGE2 blocked alum-induced activation of caspase-1 and production of mature IL-1β and IL-18 (Fig. 1A–C, Supplemental Fig. 1). We also observed that PGE2 in these experimental conditions, slightly decreased the expression of pro–IL-1β without affecting NLRP3 or caspase-1 (Fig. 1A). The inhibitory effect of PGE2 on caspase-1 cleavage, IL-1β maturation,
ASC oligomerization (Fig. 1D), and IL-1β secretion (Fig. 1E) was dose-dependent and began with very low doses of PGE2 (0.01–0.1 μM) in human primary MDMs. 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-1β secretion were also decreased by PGE2 in an ATP-induced (Fig. 2A–C) or nigericin-induced (Fig. 2D, 2E) 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-1β cleavage (Fig. 2F, 2G). 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 MDMs were treated with/without LPS, followed by treatment with PGE₂ for 1.5, 3, and 5.5 h. We found that LPS-induced IL-1β mRNA expression was not significantly altered by PGE₂ in either of the time points, although there was a downward trend (Fig. 3A). NLRP3 mRNA expression was significantly increased by PGE₂ at the 1.5 h time point (Fig. 3B), whereas caspase-1 mRNA expression was unchanged (Fig. 3C). TNF and IL-6 usually serve as controls for specific inflammasome and nonpriming 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 significantly altered by 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.

**FIGURE 3.** PGE₂ does not decrease gene expression of proteins involved in NLRP3 inflammasome complex formation. (A–E) Primary MDMs 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 mean ± SEM from four independent experiments from four healthy donors, each performed in duplicate. *p < 0.05 as assessed by Kruskal–Wallis ANOVA on ranks, followed by a Dunn post hoc test.
FIGURE 4. PGE2 inhibits NLRP3 inflammasome through EP4. (A and B) Primary MDMs 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) and then stimulated with alum (400 μg/ml for 5 h) in serum-free medium. Supernatants and lysates were collected for Western blot (WB) (A) or IL-1β ELISA (B). IL-1β release data represent mean ± SEM from three independent experiments from three 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. (Figure legend continues)
effect on PGE2-induced inhibition of NLRP3 inflammasome activation, probably because of the low efficiency of the knockdown of EP4 (Fig. 4E–G). Collectively, these data suggest that PGE2 decreases NLRP3 inflammasome activity through EP4.

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 Gαs subunit and activation of AC (34). AC 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 bone marrow–derived macrophages and in human PBMCs (18). To determine whether this is the major mechanism of the observed effect of PGE2–induced NLRP3 inhibition in human primary MDMs, we applied several approaches that involved the key molecules in the regulation of intracellular cAMP levels. We used KH7, an AC inhibitor that decreases basal or activated cAMP levels (49); forskolin, a direct AC 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 MDMs, 15 min treatment with PGE2 stimulated cAMP production (Fig. 5C). Next, we found that blocking AC 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 AC 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 it might directly bind to the NLRP3 protein (18). To study whether the 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 (6-8-P-P-8-IP-cAMP) did not mimic PGE2–dependent NLRP3 inflammasome inhibition (Fig. 6A, 6B). Also, we observed that PKA–specific inhibitor (8-bromoadenosine 3',5'-cyclic monophosphothioate) did not block PGE2–mediated NLRP3 inflammasome inhibition (Fig. 6C, 6D). Similarly, Epac selective agonist (8-(4-chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate) did not inhibit caspase-1 cleavage and production of mature IL-1β (Fig. 6E, 6F), and Epac selective inhibitor (4-cyclopentyl-2-(2,5-dimethylbenzylsulfanyl)-6-oxo-1,6-dihydropyrindine-5-carbonitrile) did not reverse PGE2–mediated NLRP3 inflammasome inhibition (Fig. 6G, 6H). At the same time, these agonists and antagonists had an effect on TNF production (Supplemental Fig. 3C–F), as previously reported (52, 53), proving that they were effective and, more importantly, suggesting separate pathways of cAMP–dependent NLRP3 inflammasome and TNF inhibition. Collectively, these data suggest that PGE2–mediated NLRP3 inflammasome inhibition is exerted through cAMP, but not through PKA or Epac.

PGE2-mediated NLRP3 inflammasome inhibition in CAPS patients requires higher doses

As mentioned above, it has been previously reported that CAMP binds directly to the NBD (NACHT) of NLRP3 protein, and this binding is impaired in cases of the most common mutations of NLRP3 gene, present in patients with CAPS (18). Therefore, to further analyze whether PGE2–mediated NLRP3 inhibition is CAMP–NBD specific, we studied the effect of PGE2 on IL-1β secretion in PBMCs from mutation-positive CAPS patients. PBMCs of these patients are especially prone to NLRP3 inflammasome activation and abundant secretion of mature IL-1β in response to LPS priming only, without other NLRP3 stimuli (54). Monocytes from healthy subjects are also reported to respond to LPS signal only, probably by intrinsic release of ATP, but the second signal makes their response much more robust (55). We found here that in monocytes from healthy subjects PGE2 decreased alun or ATP–dependent mature IL-1β secretion in similar doses as in monocyte–derived macrophages (0.1–10 μM) (Fig. 7A). However, in patients with familial cold autoinflammatory syndrome or Muckle–Wells syndrome with known mutations in NLRP3 (Fig. 7B), these doses were less effective and the PGE2 inhibitory effect was visible in doses 10– to 100-fold higher than in healthy subjects (Fig. 7B). These results suggest that PGE2–mediated NLRP3 inhibition is diminished in patients carrying an NLRP3 mutation in the NBD.

cPLA2α and COX2 are involved in endogenous NLRP3 inflammasome regulation

Having found that exogenous PGE2 inhibits NLRP3 inflammasome activation in human primary MDMs, we wondered whether endogenous PGE2 production might serve as a regulatory autocrine or paracrine mechanism against excessive NLRP3 activation. Along with others, we have previously shown that LPS induces

*p < 0.05 as assessed by Kruskal–Wallis ANOVA on ranks, followed by a Dunn post hoc test (B). (C and D) Primary MDMs 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 μM), EP4 inhibitor (GW627368X, 2 μM), both inhibitors, or vehicle (DMSO), followed by 30 min treatment with PGE2 (0.1 μM) or vehicle (EtOH) and stimulation with alun (400 μg/ml for 5 h) in serum-free medium. Supernatants and lysates were collected for WB (C) or IL-1β ELISA (D). IL-1β release data represent mean ± SEM from five independent experiments from five 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–G) MDMs were transfected with EP2 siRNA (1 μM), EP4 siRNA (1 μM), 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 μM) or vehicle (EtOH), and then stimulated with alun (400 μg/ml for 5 h) in serum-free medium. Supernatants and lysates were collected for RT-PCR (E), WB (F), or IL-1β 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 mean ± SEM from three independent experiments from three healthy donors, each performed in duplicate. *p < 0.05 as assessed by Kruskal–Wallis ANOVA on ranks, followed by a Dunn post hoc test (E). IL-1β release data represent mean ± SEM from three independent experiments from three 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 < 0.05 as assessed by Kruskal–Wallis ANOVA on ranks, followed by a Dunn post hoc test (D and G). The WBs are representative of three independent experiments from three healthy donors, each showing similar results (A, C, and F).
FIGURE 5. cAMP is involved in PGE2-mediated NLRP3 inhibition. (A and B) Primary MDMs were treated with/without LPS (100 ng/ml) for 4 h in complete medium, followed by treatment for 1 h with Kh7 (25 μM), 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 Western blot (WB) (A) or IL-1β ELISA (B). IL-1β release data represent mean ± SEM from three independent experiments from three 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 < 0.05 as compared with LPS/alum treatment, as assessed by one-way ANOVA, followed by a Holm–Sidak post hoc test (B). (C) Primary MDMs 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 mean ± SEM from three independent experiments from three healthy donors, each performed in triplicate. *p < 0.05 as assessed by Kruskal–Wallis ANOVA on ranks, followed by a Dunn post hoc test. (D and E) MDMs 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 mean ± SEM from three independent experiments from three 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 < 0.05 as compared with LPS/alum treatment, as assessed by Kruskal–Wallis ANOVA on ranks, followed by a Dunn post hoc test (E). The WBs are representative of three independent experiments from three healthy donors, each showing similar results (A and D).
cPLA2 activation, AA release, and downstream PGE2 production (37, 38). Moreover, ATP or crystals can lead to cPLA2-dependent PGE2 production (39, 41). Therefore, we studied whether blockade of AA release and PGE2 production would influence NLRP3 activation. PMA-induced THP-1 macrophages, human primary MDMs, and cPLA2α siRNA-transfected THP-1 cells were used. We found that inhibition of cPLA2α enhanced NLRP3 inflammasome activation in THP-1 cells (Fig. 8A). Similarly, knockdown of cPLA2α in THP-1 cells resulted in an increase of NLRP3 inflammasome activation (Fig. 8B). We also studied pri-
FIGURE 7. PGE₂-induced NLRP3 inflammasome inhibition requires higher doses in CAPS patients. (A) PBMCs from four healthy subjects were treated with LPS (1 µg/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 µg/ml) for 30 min in serum-free medium. Supernatants and lysates were collected for Western blot (WB). (B) PBMCs from five CAPS patients with designated mutations in NLRP3 were treated with LPS (1 µg/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.
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) and 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 5 h) or alum (200 μg/ml, for 5 h). Supernatants and lysates were collected for Western blot (WB). The WBs are representative of two independent experiments, each showing (Figure legend continues)
primary human MDMs, which were treated with cPLA2 inhibitor before the priming step or after priming to block cPLA2α-dependent signals during inflammasome activation. We first confirmed that PGE2 production was induced by LPS treatment and further increased by alum (Fig. 8C, 8F). PGE2 production induced by LPS alone was blocked in the presence of cPLA2 inhibitor added before priming (Fig. 8C) and was significantly decreased when added 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 MDMs (Fig. 8D, 8E, 8G, 8H). To more selectively block only PGE2 production, we also inhibited COX2 by using its selective inhibitor NS398 (5 μM), or DMSO for 4 h in complete medium, followed by subsequent treatment with NS398 (5 μM) 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 (A and B) or Western blot (WB) (C and D). The WBs are representative of three independent experiments from three healthy donors, each performed in duplicate. *p < 0.05 as compared to LPS. #p < 0.05 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 MDMs were treated with LPS (100 ng/ml) with/without NS398 (5 μM), 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 ± SEM from three independent experiments from three healthy donors, each performed in triplicate. *p < 0.05 as assessed by Kruskal–Wallis ANOVA on ranks, followed by a Dunn post hoc test.

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. In the present study, we have found that PGE2 inhibited NLRP3 inflammasome activation in human primary MDMs. We have demonstrated that this effect depends on EP4 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 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 5 h). Supernatants and lysates were collected for WB. The WBs are representative of two independent experiments, each showing similar results. (C-E) Human primary MDMs 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 mean ± SEM from three independent experiments from three 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-H) Human primary MDMs 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 cPLA2 inhibitor (2 μM) 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 mean ± SEM from three independent experiments from three 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 WBs are representative of three independent experiments from three healthy donors, each showing similar results (D and G). *p < 0.05 as assessed by Kruskal–Wallis ANOVA on ranks, followed by a Dunn post hoc test. a.p., after priming; b.p., before priming.
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, belonging to the GPCR family, in response to the extracellular Ca\(^{2+}\) increase activates phospholipase C and inhibits AC. As a result there is an increase in intracellular Ca\(^{2+}\) and decrease in cAMP, both of which can independently activate NLRP3 inflammasome (18). In this study, we also provided three lines of evidence showing that PGE\(_2\) mediated inhibition of NLRP3 inflammasome occurred by an increase in intracellular cAMP. First, KH7, an AC inhibitor, reversed PGE\(_2\) mediated NLRP3 inflammasome inhibition. Second, forskolin itself as an AC activator decreased NLRP3 activation, and this effect was enhanced by cotreatment with PGE\(_2\). Moreover, IMX, 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 PGE\(_2\)-mediated NLRP3 inflammasome inhibition. Another recent study in which the authors used relatively low concentrations of forskolin (up to 20 \(\mu\)M) reports extracellular Ca\(^{2+}\) sensing by both calcium sensing receptor 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 \(\mu\)M, there was still an additive effect of PGE\(_2\), 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 PKA, a major cAMP binding protein, but is driven by direct binding of cAMP to the NBD 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 the NBD of NLRP3 gene that result in the impairment of cAMP binding (18), the PGE\(_2\)-mediated NLRP3 inflammasome inhibition was less efficient and required 10- to 100-fold higher doses of PGE\(_2\) 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 PGE\(_2\) as a derivative of an ex- cessive need to extinguish NLRP3 inflammasome and increased IL-1\(\beta\) secretion, while inhibition of both PGE\(_2\) and caspase-1 leads to a decrease in NLRP3 cell proliferation (58). There is an increase in PGE\(_2\), cAMP, and PKA activity in NOMID tumor cells, leading to abnormal Wnt signaling and proliferation, whereas inhibition of both PGE\(_2\) and caspase-1 leads to a decrease in NOMID cell proliferation (58).

PGE\(_2\) 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 PGE\(_2\) acted specifically through EP4 to decrease NLRP3 inflammasome activation in human primary MDMs. Recently, two other GPCRs have been shown to be involved in the negative regulation of NLRP1 and NLRP3 inflammasomes. Yan et al. (16) have determined that omega-3 fatty acids such as eicosapentaenoic acid and docosahexaenoic acid (DHA) acting through GPR120 and GPR40 and at least partially by their downstream scaffolding protein \(\beta\)-arrestin-2 decrease NLRP1 and NLRP3 activation. \(\beta\)-arrestin-2 was shown to bind to full-length NLRP3 and its C-terminal leucine-rich repeat domain and NBD (NACH) as well as to NLRP1 in an HEK293T overexpression system, but in Arrb2\(^{+/−}\) knockout mice the reversal of DHA or eicosapentaenoic acid–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 cPLA\(_2\) and induces PGE\(_2\) production in RAW 264.7 cells and human primary macrophages (59). This suggests that the proposed PGE\(_2\)-mediated inhibition of NLRP3 by increase in cAMP might be an additional mechanism of omega-3–derived effects.

The role of PGE\(_2\) on IL-1\(\beta\) 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 PGE\(_2\) inhibits IL-1\(\beta\) 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, PGE\(_2\) enhances IL-1\(\beta\) 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 PGE\(_2\) on inflammasome activation. We found that PGE\(_2\) added after LPS priming decreased NLRP3 inflammasome activation via a cAMP-dependent mechanism as assessed by the decrease in mature IL-1\(\beta\) and mature caspase-1 release and IL-1\(\beta\) 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\(\beta\) mRNA and protein expression, we also noted a nonsignificant decrease at the mRNA level, although reflected by the slight decreases in total pro–IL-1\(\beta\) levels in the cells, pointing out the complexity of PGE\(_2\) in the control of IL-1\(\beta\) release.

Our findings that cPLA\(_2\) or COX2 inhibition resulting in the decrease of PGE\(_2\) led to an increase in NLRP3 inflammasome activation and IL-1\(\beta\) secretion in macrophages are consistent with the several early works studying effects of various nonsteroidal anti-inflammatory drugs on IL-1\(\beta\) production. Indomethacin (61–64, 69) and piroxicam (69) were reported to increase IL-1\(\beta\) production. Alternatively, it was reported that inhibition of cPLA\(_2\), or COX2 leads to a decrease in mature IL-1\(\beta\) 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 expression. Our results of pharmacological inhibition with a low dose of a highly specific COX2 inhibitor, cPLA\(_2\) inhibitor, and cPLA\(_2\) knockdown by siRNA, in conjunction with the observations of the exogenous PGE\(_2\) effects on NLRP3 inflammasome activation, suggest that this axis might play a regulatory role in human macrophages.

In summary, our results suggest that PGE\(_2\) 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.

**Acknowledgments**

We are grateful to Drs. Chong-Shan Shi and Jehad Edwan for input and helpful discussions.

**Disclosures**

The authors have no financial conflicts of interest.
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Supplementary Figure 1. PGE$_2$ inhibits IL-1$\beta$ and IL-18 production. (A, B) Human primary monocyte-derived macrophages (MDM) were treated with/without LPS (100 ng/ml) for 4 h in complete medium, followed by 30 min treatment with PGE$_2$ (0.1 $\mu$M) or vehicle (EtOH), then stimulated with alum (400 $\mu$g/ml for 5 h) in serum-free medium. Supernatants were collected for IL-1$\beta$ (A) or IL-18 (B) ELISA. The dots are the average values from independent experiments performed in duplicate using MDM from 7 (A) or 5 (B) healthy donors. The line presents the median. * P < .05 as indicated, as assessed by RM ANOVA, followed by Sidak post hoc test. As depicted, there were substantial differences in the IL-1$\beta$ or IL-18 release between donors. 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 all experiments is included in the figure legend.
Supplementary Figure 2. EP1-4 receptor expression in primary human monocyte-derived macrophages. (A-C) Primary MDM were treated with/without LPS (100 ng/ml) for 4 h in complete medium. Cells were lysed and total RNA was extracted. Gene expression was assessed using RT PCR. (A) Baseline expression of each receptor is presented as the difference between the gene Ct value and GAPDH. (B and C) gene expression was normalized to GAPDH transcripts and represented as a relative quantification (RQ) compared with the vehicle-treated cells. Data represent the mean ± SEM from 4 independent experiments from 4 healthy donors, performed in duplicate. * P < .05 as compared with vehicle, as assessed by Student t-test.
Supplementary Figure 3. PGE₂ inhibits TNF production via EP4/PKA/Epac-dependent pathway. (A) 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. (B) 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 μM), EP4 inhibitor (GW627368X, 2 μM), both inhibitors or vehicle (DMSO), followed by 30 min treatment with PGE₂ (0.1 μM) or vehicle (EtOH), and stimulation with alum (400 μg/ml for 5 h) in serum-free medium. (C, E) 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-IP-P-cAMP, 20 or 50 μM) (C), EPAC selective agonist (8-(4-Chlorophenylthio)-2′-O-methyladenosine 3′,5′-cyclic Monophosphate . sodium salt, 20 or 50 μM) (E), PGE₂ (0.1 μM) or vehicle, then stimulated with alum (400 μg/ml for 5 h) in serum-free medium. (D, F) 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 μM) (D), Epac inhibitor (4- Cyclopentyl-2- (2, 5- dimethylbenzylsulfonyl)-6- o xo- 1, 6- dihydropyrimidine- 5- carboxitrile, 10 μM) (F) or vehicle (DMSO), followed by 30 min treatment with PGE₂ (0.1 μM) or vehicle (EtOH), and stimulation with alum (400 μg/ml for 5 h) in serum-free medium. TNF data are presented as the percentage of the response after LPS/alum treatment, which ranged from 67.4 to 4285.4 pg/ml. TNF release data represent the mean ± SEM from 2-3 independent experiments from 2-3 healthy donors, each performed in duplicate.* P < .05 as indicated, as assessed by RM ANOVA, followed by Sidak’s post hoc test.