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Articles

c-di-GMP Induces COX-2 Expression in Macrophages in a STING-Independent Manner

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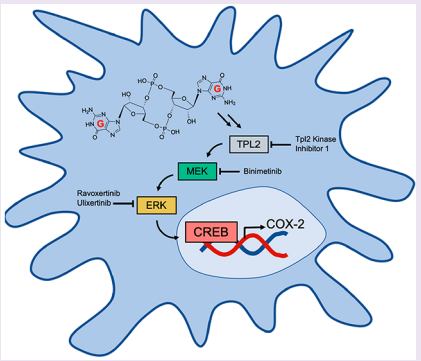
ABSTRACT: Many pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and lipoteichoic acid, are potent immunostimulatory molecules and promote the expression of cyclooxygenase 2 (COX-2). While the production of COX-2, and ultimately prostaglandin E_2, could be protective, persistent induction of COX-2 leads to inflamed environments that can result in septic shock and death. Bacterial derived cyclic dinucleotides (CDNs), c-di-GMP and c-di-AMP, are also PAMPs and have been shown to produce inflamed environments via the production of pro-inflammatory cytokines such as type I interferons. The well-characterized CDN immunostimulatory mechanism involves binding to stimulator of interferon genes (STING), which ultimately results in the phosphorylation of IRF3 or release of NF-κB to promote expression of type I IFN or pro-inflammatory cytokines. In this study, we sought to investigate if CDNs promote COX-2 expression. Using RAW macrophages as a model system, we reveal that c-di-GMP, but not c-di-AMP or the host-derived 2′,3′-cGAMP, promotes COX-2 expression. Using analogues of CDNs, we show that the presence of two guanines and two 3′,5′-phosphodiester linkages are requirements for the promotion of COX-2 expression by cyclic dinucleotides. Both c-di-GMP and LPS inductions of COX-2 expression in RAW macrophages are STING-independent and are regulated by Tpl2-MEK-ERK-CREB signaling; inhibitors of Tpl2, MEK, and ERK could attenuate COX-2 expression promoted by c-di-GMP. This work adds to the growing body of evidence that cyclic dinucleotides regulate pathways other than the STING−TBK1−IRF3 axis. Additionally, the differential COX-2 induction by c-di-GMP but not c-di-AMP or cGAMP suggests that the type and level of inflammation could be dictated by the nucleotide signature of the invading pathogen.



INTRODUCTION

Cyclooxygenase (COX) is the rate-determining enzyme in the prostaglandin synthesis pathway.[1] There are two kinds of COX enzymes (COX-1 and COX-2), and they are encoded by different genes.[1] COX-1 is constitutively expressed in many cells and produces prostanoids to maintain cell functions. The expression of COX-2 is maintained at a very low level under physiological conditions. COX-2 expression can, however, be induced by a variety of stimulants. For example, robust COX-2 inductions upon CD3 antibody (OKT3) or lipopolysaccharide (LPS) stimulation have been shown in Jurkat T cells[2] or RAW macrophages,[3,4] respectively. LPS triggers COX-2 expression through a Tpl2-dependent pathway and results in the activation of ERK-p90RSK and p38 MAPK-Msk1 pathways.[3] The activations of the aforementioned pathways result in the phosphorylation and activation of cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), which is essential for the transactivation of COX-2 (Figure 1).[3]

Overexpression of COX-2 has emerged as a crucial determinant of cell death associated with inflammation.[5,6] For example, COX-2 is upregulated in amyotrophic lateral sclerosis (ALS) patients.[7] COX-2 is also expressed in many types of cancers to promote carcinogenesis and chemotherapy resistance.[8] Moreover, COX-2 produces prostaglandin E_2 (PGE_2), which is found at high levels in tumor cells and induces aromatase activity in human breast cancer,[9] and the PGE_2 concentration is correlated to high metastatic potential in clinical studies.[10,11] It was reported that an increased level of expression of COX-2 leads to angiogenesis, while inhibition of COX-2 activity decreases the abnormal blood vessel growth in tumors, thereby inhibiting tumor growth and metastasis.[12] Due to the important roles played by COX-2 in inflammation and cell death, many studies have investigated the effects of pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs, respectively) on COX-2 levels. For example, bacterial derived PAMPs, LPS,[3,4] lipoteichoic acid (LTA),[13] unmethylated CpG motifs[14] (found more frequently in bacterial pathogens than human hosts), and DAMPs, such

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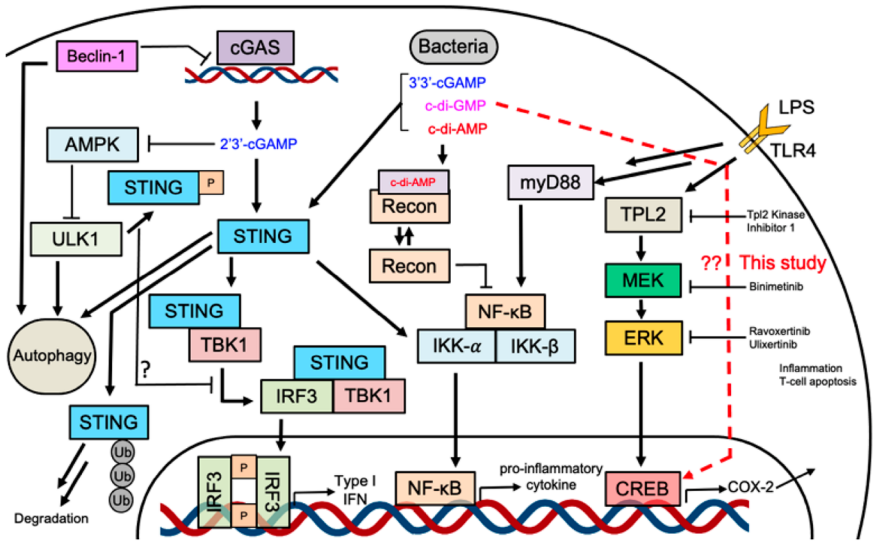

Figure 1. Schematic diagram illustrating the cGAS−STING pathway in response to cyclic dinucleotides and COX-2 upregulation by LPS. cGAMP has negative feedback to cGAS and the activating DNA via Beclin-1.[31] ULK1, Beclin-1, and STING all promote autophagy.[32−36] Further work is needed to clarify the role of STING phosphorylation by ULK1 and IRF3 binding. c-di-AMP but not cGAMP binds to Recon to relieve the inhibition of NF-kB by Recon.[37]

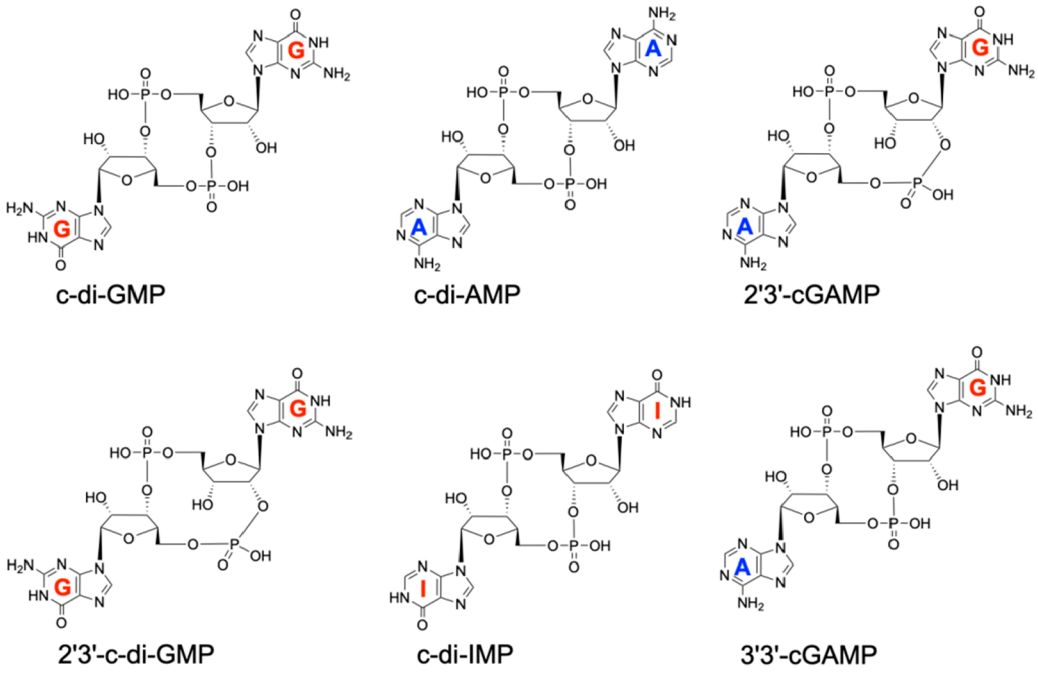

Figure 2. Chemical structures of cyclic dinucleotides used in this study.

as serum amyloid A (SAA)[15,16] and uric acid,[17] have been shown to promote COX-2 production.

Bis(3′−5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) is a bacterial second messenger molecule, which regulates many processes in bacteria, including biofilm formation and motility in several Gram-negative bacteria.[18] As expected for a bacterial derived molecule, c-di-GMP is also an immunostimulatory molecule, which triggers the host immune response via the well-characterized STING-TBK1 pathway.[19] Upon binding of c-di-GMP to stimulator of interferon genes (STING), structural changes to STING and translocation of STING to endoplasmic reticulum−Golgi intermediate compartment (ERGIC) leads to the phosphorylation of IRF3 by TBK1. Phosphorylated IRF3 then promotes the expression of pro-inflammatory cytokines, such as type I interferons, for pathogen elimination (Figure 1).[20,21] The cyclic dinucleotide signaling axis has emerged as an important target for cancer immunotherapy and neurodegenerative disease.[22−26] Various STING agonists, including hydrolytically resistant cyclic dinucleotides, are now in various stages of clinical trials as cancer immunotherapy and are thought to operate via IRF3 activation.[27] The effects of cyclic dinucleotides on the other inflammatory pathways, such as COX-2 induction, are not well characterized. On the contrary, the mechanistic details of how cyclic mononucleotides, such as cGMP or cAMP, affect COX-2 expression are known. For

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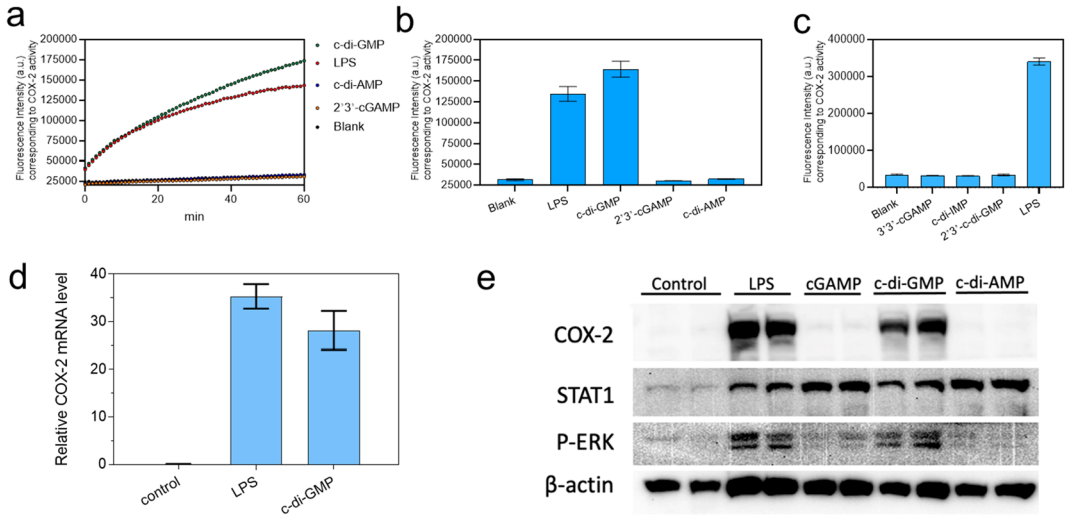

Figure 3. c-di-GMP, but not other dinucleotides, induces COX-2 expression. RAW ISG cells were treated with 1 μg/mL LPS, 100 μM c-di-GMP, 100 μM 2′,3′-cGAMP, and 100 μM c-di-AMP for 24 h. The COX-2 activity was measured by the COX-2 fluorescent assay (a) in a kinetic mode and (b) via the fluorescence intensity at 60 min. (c) RAW ISG cells were treated with 1 μg/mL LPS, 100 μM 3′,3′-cGAMP, 100 μM 2′,3′-c-di-GMP, and 100 μM c-di-IMP for 24 h. The COX-2 activity was measured by the COX-2 fluorescent assay (fluorescence at t_{assay} = 60 min). (d) RAW ISG cells were treated with either 100 μM c-di-GMP or 1 μg/mL LPS for 24 h, and then COX-2 mRNA levels were measured by the qPCR assay. COX-2 transcript levels were normalized with actin. The relative change in COX-2 mRNA levels was 353-fold upon LPS treatment, whereas a 282-fold change was observed upon c-di-GMP treatment, compared to controls. (e) RAW ISG cells were treated with 1 μg/mL LPS, 100 μM c-di-GMP, 100 μM 2′,3′-cGAMP, and 100 μM c-di-AMP for 24 h. The protein levels of COX-2, STAT1, and p-ERK were examined by western blot analysis. Experiments were performed in two biological replicates. Error bars indicate the mean ± the standard error of the mean of two independent measurements.

example, it has been shown that cGMP induces COX-2 via cGMP-dependent protein kinase[28] while cAMP increases the level of COX-2 expression via mitogen-activated kinase.[29] Herein, we fill the gap in knowledge regarding the relationship among COX-2, cyclic dinucleotides, and inflammation.

In our previous study, we demonstrated via global proteomics that c-di-GMP differentially affected the expression of various proteins in RAW macrophages, including COX-2, when compared to that of cGAMP.[30] In this previous study, the focus was on global protein modulation by cyclic dinucleotides so details of the many individual proteins that were affected by both c-di-GMP and cGAMP were not pursued at that time. In this more detailed study, we selected COX-2 due to its importance in inflammation. First, this study sought to confirm the prediction by global proteomics that c-di-GMP but not cGAMP induced COX-2 expression, this time using other traditional techniques such as immunoblotting and the COX-2 specific fluorogenic assay to confirm COX-2 induction. Second, using cyclic dinucleotide analogues, we sought to establish the determinants of COX-2 induction by cyclic dinucleotides [such as nucleobase and phosphodiester linkage requirements (see Figure 2 for the analogues used)]. STING-negative RAW macrophages are available from InvivoGen, so we were also able to investigate if COX-2 induction by cyclic dinucleotides channeled through the STING-TBK1 axis pathway or other kinase networks, such as the MEK-ERK-CREB pathway reported for the LPS induction of COX-2. Finally, this new study has identified small molecules (kinase inhibitors) that could be used to inhibit the induction of COX-2 by c-di-GMP.

RESULTS AND DISCUSSION

The COX-2 activity was evaluated by the cyclooxygenase activity fluorescent assay with the treatment of COX-1 specific inhibitor SC560. The cell lysates of RAW macrophages were collected following stimulation of LPS, c-di-GMP, cGAMP and c-di-AMP for 24 h (Figure 2). An increasing fluorescence intensity was observed for the cells upon stimulation of LPS, while no significant fluorescence intensity increase was observed for the cells without any stimulation (Figure 3a,b). A similar increasing trend was observed for the cells with c-di-GMP treatment. In mammals, c-di-GMP is recognized by STING and triggers the inflammatory response by the production of type I IFNs and inflammatory cytokines (Figure 1).[38] To provide structure−activity relationship information for COX-2 induction by cyclic dinucleotides, we investigated other cyclic dinucleotides (such as c-di-AMP and 2′,3′-cGAMP), which can also bind and activate STING.[39] Similar to c-di-GMP, bis(3′−5′)-cyclic dimeric adenosine monophosphate (c-di-AMP) is also a bacterial second messenger that mediates cell wall metabolism and the stress response of bacteria.[40] 2′,3′-cGAMP is produced by cGAS (cGAMP synthase) in mammalian cells when the cytoplasmic DNA is recognized by cGAS.[21,41] However, no increase in COX-2 activity was observed upon the stimulation of RAW macrophages with 2′,3′-cGAMP and c-di-AMP (Figure 3a & b). It appears that unlike IRF3-promoted cytokine induction, which is activated by different types of cyclic dinucleotides, irrespective of the nature of the nucleobase or phosphodiester linkage, the nature of the nucleobase and/or phosphodiester linkage in the cyclic dinucleotide could be important for COX-2 induction. To better understand the selective recognition of c-di-GMP in this COX-2 upregulation pathway, we further evaluated if other cyclic dinucleotide analogues (Figure 2) could trigger the increase in COX-2 activity. 2′,3′-c-di-GMP is a synthetic analogue of the bacterial second messenger c-di-GMP. 3′,3′-cGAMP {cyclic [G(3′,5′)pA(3′,5′)p]} is a cyclic dinucleotide produced by bacteria. Cyclic di-inosine mono-

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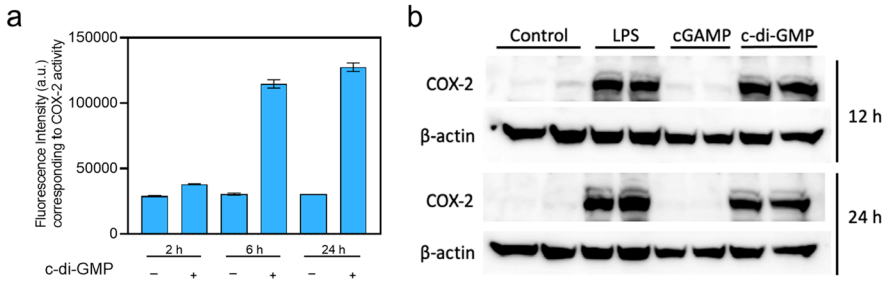

Figure 4. c-di-GMP induces COX-2 expression in a time-dependent manner. (a) RAW ISG cells were treated with 100 μM c-di-GMP for 2, 6, and 24 h. The COX-2 activity was measured by the COX-2 fluorescent assay (fluorescence at t_{assay} = 60 min). (b) RAW ISG cells were treated with 1 μg/mL LPS, 100 μM 2′,3′-cGAMP, and 100 μM c-di-GMP for 12 and 24 h. The protein level of COX-2 was examined by western blot analysis. Experiments were performed in biological replicates. Error bars indicate the mean ± the standard error of the mean of two independent measurements.

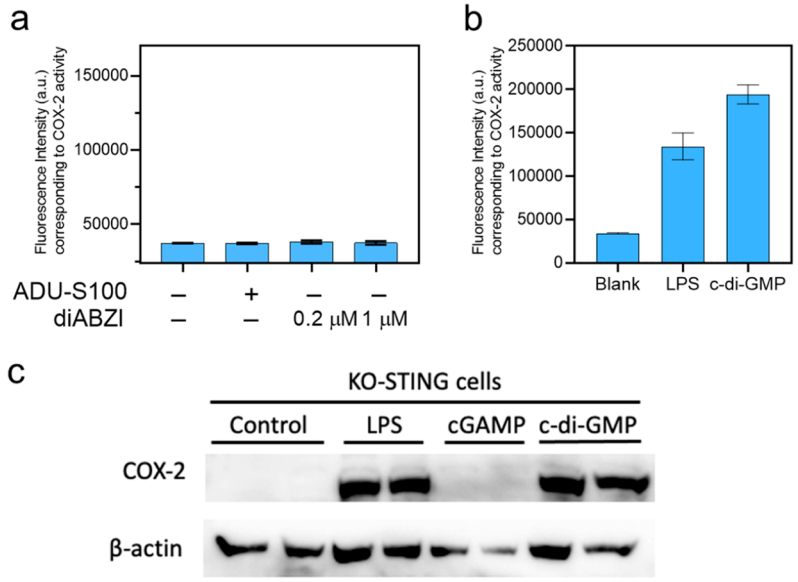

Figure 5. COX-2 expression is independent of STING activation. (a) RAW ISG cells were treated with STING agonist ADU-S100 (100 μM) and diABZI (0.2 and 1 μM) for 24 h. The COX-2 activity was measured by the COX-2 fluorescent assay (fluorescence at t_{assay} = 60 min). (b) STING knockout RAW macrophages were treated with 1 μg/mL LPS and 100 μM c-di-GMP for 24 h, and the COX-2 activity was measured by the COX-2 fluorescent assay. (c) STING knockout RAW macrophages were treated with 1 μg/mL LPS, 100 μM 2′,3′-cGAMP, and 100 μM c-di-GMP for 24 h. The protein levels of COX-2 was examined by western blot analysis. Experiments were performed in two biological replicates. Error bars indicate the mean ± the standard error of the mean of two independent measurements.

phosphate (c-di-IMP) is a synthetic analogue of the bacterial second messengers c-di-AMP and c-di-GMP. All of these cyclic dinucleotide analogues are reported to enter immune cells, when added extracellularly, bind STING, and activate IRF3, albeit to varying degrees.[20,38,42,43] However, these cyclic dinucleotide analogues could not induce COX-2 activity in RAW macrophages (Figure 3c and Supplementary Figure 1a). These results show the unique COX-2 upregulating ability of c-di-GMP and the recognition of c-di-GMP for COX-2 induction (yet to be identified) are quite selective.

To provide another validation of the differential induction of COX-2 by the natural cyclic dinucleotides, we also examined expression of COX-2 by cyclic dinucleotides via immunoblotting (Figure 3e). Stimulation by LPS, c-di-GMP, cGAMP, and c-di-AMP upregulated STAT1 expression in RAW macrophages (Figure 3e), in agreement with previous studies.[44] On the contrary, only c-di-GMP (and not c-di-AMP or cGAMP) could induce COX-2 expression when probed by immunoblotting, confirming the COX-2 specific fluorescence assay (Figure 3a). RT-PCR revealed that both LPS and c-di-GMP increased the relative level of COX-2 mRNA (Figure 3d).

COX-2 is not expressed under physiological conditions in many cells, and its level increases upon stimulation in a time-dependent manner. We also examined the time dependence of COX-2 upregulation by LPS or cyclic dinucleotides. Upon treatment of RAW macrophages with c-di-GMP, the level of COX-2 was low at 2 h but increased to near maximum after 6 h (Figure 4 and Supplementary Figure 1b).

Other STING agonists, ADU-S100[45] and diABZI[46] (a non-nucleotide agonist of STING), also did not induce COX-2 expression (Figure 5a and Supplementary Figure 1e). Because many STING-activating cyclic dinucleotides as well as non-nucleotide agonists such as diABZI could not induce COX-2 expression, we hypothesized that the induction by COX-2 is probably not via the STING−TBK1 axis. To validate this, we examined the COX-2 induction by LPS and c-di-GMP in a STING deficient system, STING knockout (KO) macrophage cells. A similar trend was obtained in STING-KO macrophages

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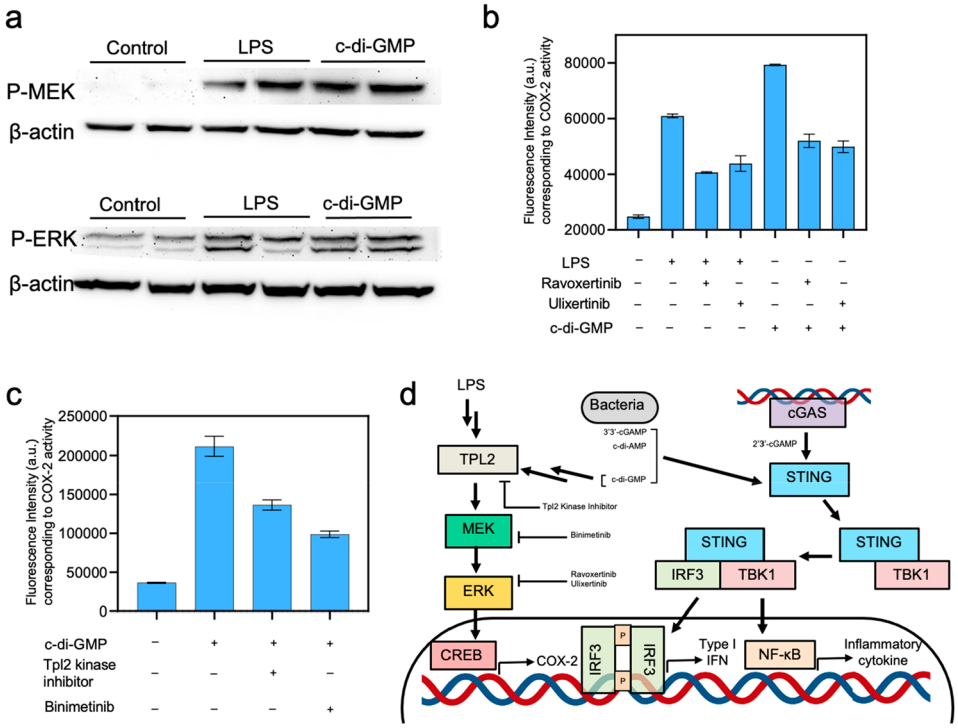

Figure 6. c-di-GMP induced COX-2 expression channels through the Tpl2−MEK−ERK signaling axis. (a) RAW macrophages were treated with 1 μg/mL LPS and 100 μM c-di-GMP for 2 h. The protein levels of P-MEK and P-ERK were examined by western blot analysis. (b) RAW ISG cells were pretreated with ERK inhibitors ravoxertinib (5 μM) and ulixertinib (5 μM) for 2 h and then treated with 1 μg/mL LPS or 100 μM c-di-GMP for 6 h. (c) RAW ISG cells were pretreated with DMSO, 4-[(3-chloro-4-fluorophenyl)amino]-6-[(3-pyridinylmethyl)amino]-1,7-naphthyridine-3-carbonitrile, Tpl2 kinase inhibitor (10 μM), or binimetinib (10 μM) for 2 h and then with 100 μM c-di-GMP for 6 h. The COX-2 activity was measured by the COX-2 fluorescent assay (fluorescence at t_{assay} = 60 min). Experiments were performed with two biological replicates. Error bars indicate the mean ± the standard error of the mean of two independent measurements. (d) Schematic diagram illustrating the cGAS−STING pathway in response to cyclic dinucleotides and the proposed model of COX-2 transactivation in response to c-di-GMP.

whereby LPS and c-di-GMP stimulation induced COX-2 expression while no COX-2 expression was induced by cGAMP stimulation (Figure 5b,c and Supplementary Figure 1f). These results indicate that STING activation is not critical for LPS and c-di-GMP induction of COX-2 activity.

Mechanistic details for the induction of COX-2 in macrophages by LPS have been elucidated by the seminal work of Tsichlis[3] and validated by others in other cell types.[47−49] LPS induces COX-2 expression in macrophages via the MEK−ERK pathway, which promotes the phosphorylation of CREB.[3] Both c-di-GMP and LPS promoted the phosphorylation of MEK and ERK (Figure 6a), so we postulated that inhibitors of kinases in the Tlp2−MEK−ERK axis could attenuate the production of COX-2 by c-di-GMP, similar to what has been shown for LPS.[3] Ravoxertinib and ulixertinib are ERK inhibitors;[50] treating RAW cells with 5 μM ravoxertinib or ulixertinib led to an ∼2-fold reduction in LPS- or c-di-GMP-induced COX-2 activity (Figure 6b and Supplementary Figure 1c,d). A Tpl2 kinase inhibitor (a naphthyridine-3-carbonitrile-based compound)[51] and MEK1/2 inhibitor, binimetinib, could also attenuate COX-2 induction by c-di-GMP (Figure 6c and Supplementary Figure 2). It therefore appears that the upregulation of COX-2 by c-di-GMP channels through the Tpl2−MEK−ERK−CREB pathway, similar to LPS.[3]

CONCLUSION

This study has revealed differential regulation of COX-2 by cyclic dinucleotides. c-di-GMP induces COX-2 expression in RAW macrophages via the Tpl2−MEK−ERK−CREB pathway, but other natural cyclic dinucleotides (2′,3′-cGAMP and c-di-AMP), synthetic analogues (2′,3′-c-di-GMP, c-di-IMP, 3′,3′-cGAMP, and ADU-S100), or non-nucleotide-based STING agonist diABZI do not activate COX-2 expression. Using STING knockout RAW macrophages, we show that c-di-GMP still induced COX-2 expression, and therefore, the induction of COX-2 by c-di-GMP is STING-independent. COX-2 activation by c-di-GMP can be inhibited by Tpl2, MEK, or ERK inhibitors (Figure 6d). The differential COX-2 induction by c-di-GMP but not c-di-AMP or cGAMP indicates that the type of nucleotide signature of an invading pathogen could affect the types and quantities of inflammatory molecules released by immune cells. This work also adds to the growing number of reports of phenotypes modulated by different cyclic dinucleotides, which are not mediated via STING.

MATERIALS AND METHODS

Chemicals. c-di-GMP was purchased from Kerafast (Boston, MA). 2′,3′-cGAMP, ADU-S100, ulixertinib, and ravoxertinib were purchased from Chemietek (Indianapolis, IN). 3′,3′-cGAMP, 2′,3′-c-di-GMP, and c-di-IMP were purchased from InvivoGen (San Diego, CA). Lipopolysaccharides from *Escherichia coli* O111:B4 (catalog no. L2630) were purchased from Sigma-Aldrich (St. Louis, MO). diABZI3 was purchased from ProbeChem. The Tpl2 kinase inhibitor and binimetinib were purchased from Cayman Chemicals (Ann Arbor, MI).

Cell Lines and Culturing. RAW-Blue ISG cells and RAW-Lucia ISG-KO-STING cells were purchased from InvivoGen. These cell lines were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal

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bovine serum, Pen-Strep (100 units/mL, 100 μg/mL). DMEM was purchased from Corning (Corning, NY), and fetal bovine serum was purchased from R&D Systems (Minneapolis, MN). Cell lines were cultured per InvivoGen recommendations at 37 °C under a 5% CO_2 atmosphere.

Western Blot Analysis. The effect of LPS and cyclic dinucleotides on COX-2 expression was tested on RAW ISG macrophage cells and STING knockout (KO-STING) cells. First, 1 × 10^6 RAW ISG macrophage cells or KO-STING cells were seeded in a plate. Twenty-four hours later, cells were treated with 1 μg/mL LPS, 100 μM c-di-GMP, 2′,3′-cGAMP, and c-di-AMP. After the indicated time periods, cells were collected by centrifugation and total protein extracts were obtained using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) supplemented with protease inhibitor cocktail (Roche). The cell lysates were centrifuged at 14000*g* for 10 min at 4 °C, and the protein supernatant was collected. The protein concentration of each sample was determined by the Pierce Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of proteins (≤30 μg) were separated on a sodium dodecyl sulfate−polyacrylamide gel electrophoresis gel and transferred to a nitrocellulose membrane. The membrane was then blocked with 5% bovine seum albumin (BSA) in 1× TBST {Tris-buffered saline and 0.1% Tween 20 [20 mM Tris (pH 7.5), 150 mM NaCl, and 0.1% Tween 20]} for 1 h at room temperature. After being blocked, the membrane was incubated with primary antibodies (COX-2, STAT1, phospho-MEK 1/2, phospho-ERK 1/2, and β-actin) overnight at 4 °C. The primary antibodies COX-2 and STAT1 were purchased from ABclonal (Woburn, MA). The primary antibodies p-ERK (#9101), p-MEK (#9154), and β-actin were purchased from Cell Signaling (Danvers, MA). The membrane was washed three times with 1× TBST and incubated with a secondary anti-rabbit or anti-mouse antibody (Cell Signaling) for 1 h at room temperature. The membrane was washed again, and the signal was detected by SuperSignal West Pico PLUS Chemiluminescent Substrats (Thermo Fisher Scientific). Experiments were performed with two biological replicates.

COX-2 Fluorescent Assay. First, 1 × 10^6 RAW ISG macrophage cells or KO-STING cells were seeded in a plate. Twenty-four hours later, cells were treated with 1 μg/mL LPS, 100 μM c-di-GMP, 2′,3′-cGAMP, c-di-AMP, or the synthetic cyclic dinucleotides. After the indicated time periods, cells were collected by centrifugation and total protein extracts were obtained using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) supplemented with protease inhibitor cocktail (Roche). The cell lysates were centrifuged at 14000*g* for 10 min at 4 °C, and the protein supernatant was collected. The protein concentration of each sample was determined by the Pierce Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of proteins (~20 μg unless indicated) were used for the COX-2 fluorescent assay. Forty micrograms of total protein was used for Figure 3c. The COX-2 activity of each sample was determined by the Cyclooxygenase (COX) Activity Assay Kit (Fluorometric) (Abcam) following the manufacturer's recommended protocol. Experiments were performed in two biological replicates. A fluorescence reading (excitation at 535 nm and emission at 587 nm) was taken immediately in a kinetic mode once every minute for 1 h using a BioTek Cytation 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Winooski, VT).

qPCR Assay. The qPCR assay was performed by seeding 2 × 10^6 RAW ISG macrophages in 12-well plates, and after 24 h, cells were treated with either 100 μM c-di-GMP or 1 μg/mL LPS, with sterile water being used as a control. Cells were harvested after 24 h, and RNA isolation was performed using TRIzol reagent (Thermo Fisher Scientific). One thousand nanograms of RNA was used for cDNA synthesis, and the assay was performed using random hexamer primers, dNTPs, and SuperScript II Reverse Transcriptase (Thermo Fisher Scientific). Actin and COX-2 primers were used as previously described.[52] Syber Green Mastermix (Qiagen LLC) was used for qPCR, and the reaction was performed in a QuantStudio 7 Flex System.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.1c00342.

Fluorescence curves measuring COX-2 activity in macrophages in the presence of cyclic dinucleotides or LPS (PDF)

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Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Smith, W. L.; DeWitt, D. L.; Garavito, R. M. Cyclooxygenases: Structural, Cellular, and Molecular Biology. *Annu. Rev. Biochem.* **2000**, *69*, 145−182.

(2) Pablos, J. L.; Santiago, B.; Carreira, P. E.; Galindo, M.; Gomez-Reino, J. J. Cyclooxygenase-1 and −2 are expressed by human T cells. *Clin. Exp. Immunol.* **1999**, *115*, 86−90.

(3) Eliopoulos, A. G.; Dumitru, C. D.; Wang, C.-C.; Cho, J.; Tsichlis, P. N. Induction of COX-2 by LPS in macrophages is regulated by Tpl2-dependent CREB activation signals. *EMBO J.* **2002**, *21*, 4831−4840.

(4) Kaufmann, W. E.; Worley, P. F.; Pegg, J.; Bremer, M.; Isakson, P. COX-2, a synaptically induced enzyme, is expressed by excitatory neurons at postsynaptic sites in rat cerebral cortex. *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93*, 2317.

(5) Seibert, K.; Masferrer, J.; Zhang, Y.; Gregory, S.; Olson, G.; Hauser, S.; Leahy, K.; Perkins, W.; Isakson, P. Mediation of inflammation by cyclooxygenase-2. *Agents Actions Suppl.* **1995**, *46*, 41−50.

(6) O'Banion, M. K. Cyclooxygenase-2: Molecular Biology, Pharmacology, and Neurobiology. *Crit. Rev. Neurobiol.* **1999**, *13*, 45−82.

(7) Almer, G.; Guégan, C.; Teismann, P.; Naini, A.; Rosoklija, G.; Hays, A. P.; Chen, C.; Przedborski, S. Increased expression of the pro-

inflammatory enzyme cyclooxygenase-2 in amyotrophic lateral sclerosis. *Ann. Neurol.* **2001**, *49*, 176−185.
(8) Hashemi Goradel, N.; Najafi, M.; Salehi, E.; Farhood, B.; Mortezaee, K. Cyclooxygenase-2 in cancer: A review. *J. Cell. Physiol.* **2019**, *234*, 5683−5699.
(9) Schrey, M. P.; Patel, K. V. Prostaglandin E2 production and metabolism in human breast cancer cells and breast fibroblasts. Regulation by inflammatory mediators. *Br. J. Cancer* **1995**, *72*, 1412−1419.
(10) Rolland, P. H.; Martin, P. M.; Jacquemier, J.; Rolland, A. M.; Toga, M. Prostaglandin in Human Breast Cancer: Evidence Suggesting That an Elevated Prostaglandin Production Is a Marker of High Metastatic Potential for Neoplastic Cells. *J. Natl. Cancer Inst.* **1980**, *64*, 1061−1070.
(11) Fulton, A. M.; Heppner, G. H. Relationships of Prostaglandin E and Natural Killer Sensitivity to Metastatic Potential in Murine Mammary Adenocarcinomas. *Cancer Res.* **1985**, *45*, 4779−4784.
(12) Davies, G.; Martin, L. A.; Sacks, N.; Dowsett, M. Cyclo-oxygenase-2 (COX-2), aromatase and breast cancer:a possible role for COX-2 inhibitors in breast cancer chemoprevention. *Ann. Oncol.* **2002**, *13*, 669−678.
(13) Lin, C.-H.; Kuan, I.-H.; Lee, H.-M.; Lee, W.-S.; Sheu, J.-R.; Ho, Y.-S.; Wang, C.-H.; Kuo, H.-P. Induction of cyclooxygenase-2 protein by lipoteichoic acid from Staphylococcus aureus in human pulmonary epithelial cells: involvement of a nuclear factor-κB-dependent pathway. *Br. J. Pharmacol.* **2001**, *134*, 543−552.
(14) Chen, Y.; Zhang, J.; Moore, S. A.; Ballas, Z. K.; Portanova, J. P.; Krieg, A. M.; Berg, D. J. CpG DNA induces cyclooxygenase-2 expression and prostaglandin production. *Int. Immunol.* **2001**, *13*, 1013−1020.
(15) Lee, H. Y.; Kim, S. D.; Shim, J. W.; Lee, S. Y.; Lee, H.; Cho, K.-H.; Yun, J.; Bae, Y.-S. Serum Amyloid A Induces CCL2 Production via Formyl Peptide Receptor-Like 1-Mediated Signaling in Human Monocytes. *J. Immunol.* **2008**, *181*, 4332−4339.
(16) Li, W.; Wang, W.; Zuo, R.; Liu, C.; Shu, Q.; Ying, H.; Sun, K. Induction of pro-inflammatory genes by serum amyloid A1 in human amnion fibroblasts. *Sci. Rep.* **2017**, *7*, 693.
(17) Oğuz, N.; Kırça, M.; Çetin, A.; Yeşilkaya, A. Effect of uric acid on inflammatory COX-2 and ROS pathways in vascular smooth muscle cells. *J. Recept. Signal Transduction Res.* **2017**, *37*, 500−505.
(18) Hengge, R. Principles of c-di-GMP signalling in bacteria. *Nat. Rev. Microbiol.* **2009**, *7*, 263−273.
(19) Karaolis, D. K. R.; Means, T. K.; Yang, D.; Takahashi, M.; Yoshimura, T.; Muraille, E.; Philpott, D.; Schroeder, J. T.; Hyodo, M.; Hayakawa, Y.; Talbot, B. G.; Brouillette, E.; Malouin, F. Bacterial c-di-GMP Is an Immunostimulatory Molecule. *J. Immunol.* **2007**, *178*, 2171−2181.
(20) Burdette, D. L.; Monroe, K. M.; Sotelo-Troha, K.; Iwig, J. S.; Eckert, B.; Hyodo, M.; Hayakawa, Y.; Vance, R. E. STING is a direct innate immune sensor of cyclic di-GMP. *Nature* **2011**, *478*, 515−518.
(21) Sun, L.; Wu, J.; Du, F.; Chen, X.; Chen, Z. J. Cyclic GMP-AMP Synthase Is a Cytosolic DNA Sensor That Activates the Type I Interferon Pathway. *Science* **2013**, *339*, 786−791.
(22) Sharma, M.; Rajendrarao, S.; Shahani, N.; Ramírez-Jarquín, U. N.; Subramaniam, S. Cyclic GMP-AMP synthase promotes the inflammatory and autophagy responses in Huntington disease. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117*, 15989.
(23) Jiang, M.; Chen, P.; Wang, L.; Li, W.; Chen, B.; Liu, Y.; Wang, H.; Zhao, S.; Ye, L.; He, Y.; Zhou, C. cGAS-STING, an important pathway in cancer immunotherapy. *J. Hematol. Oncol.* **2020**, *13*, 81.
(24) Zhu, Y.; An, X.; Zhang, X.; Qiao, Y.; Zheng, T.; Li, X. STING: a master regulator in the cancer-immunity cycle. *Mol. Cancer* **2019**, *18*, 152.
(25) Kelly, P. N. Targeting STING for cancer therapy. *Science* **2020**, *369*, 930.17.
(26) Chandra, D.; Quispe-Tintaya, W.; Jahangir, A.; Asafu-Adjei, D.; Ramos, I.; Sintim, H. O.; Zhou, J.; Hayakawa, Y.; Karaolis, D. K. R.; Gravekamp, C. STING Ligand c-di-GMP Improves Cancer Vaccination against Metastatic Breast Cancer. *Cancer Immunol. Res.* **2014**, *2*, 901−910.
(27) Sintim, H. O.; Mikek, C. G.; Wang, M.; Sooreshjani, M. A. Interrupting cyclic dinucleotide-cGAS−STING axis with small molecules. *MedChemComm* **2019**, *10*, 1999−2023.
(28) Fang, L.; Chang, H.-M.; Cheng, J.-C.; Leung, P. C. K.; Sun, Y.-P. Nitric Oxide and cGMP Induce COX-2 Expression and PGE2 Production in Human Granulosa Cells Through CREB Signaling Pathway. *J. Clin. Endocrinol. Metab.* **2015**, *100*, E262−E269.
(29) Chen, L.; Sooranna, S. R.; Lei, K.; Kandola, M.; Bennett, P. R.; Liang, Z.; Grammatopoulos, D.; Johnson, M. R. Cyclic AMP increases COX-2 expression via mitogen-activated kinase in human myometrial cells. *J. Cell. Mol. Med.* **2012**, *16*, 1447−1460.
(30) Sooreshjani, M. A.; Gursoy, U. K.; Aryal, U. K.; Sintim, H. O. Proteomic analysis of RAW macrophages treated with cGAMP or c-di-GMP reveals differentially activated cellular pathways. *RSC Adv.* **2018**, *8*, 36840−36851.
(31) Liang, Q.; Seo, G. J.; Choi, Y. J.; Kwak, M.-J.; Ge, J.; Rodgers, M. A.; Shi, M.; Leslie, B. J.; Hopfner, K.-P.; Ha, T.; Oh, B.-H.; Jung, J. U. Crosstalk between the cGAS DNA Sensor and Beclin-1 Autophagy Protein Shapes Innate Antimicrobial Immune Responses. *Cell Host Microbe* **2014**, *15*, 228−238.
(32) Gui, X.; Yang, H.; Li, T.; Tan, X.; Shi, P.; Li, M.; Du, F.; Chen, Z. J. Autophagy induction via STING trafficking is a primordial function of the cGAS pathway. *Nature* **2019**, *567*, 262−266.
(33) Kim, J.; Kundu, M.; Viollet, B.; Guan, K.-L. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat. Cell Biol.* **2011**, *13*, 132−141.
(34) Chan, E. Y. W.; Longatti, A.; McKnight, N. C.; Tooze, S. A. Kinase-Inactivated ULK Proteins Inhibit Autophagy via Their Conserved C-Terminal Domains Using an Atg13-Independent Mechanism. *Mol. Cell. Biol.* **2009**, *29*, 157−171.
(35) Russell, R. C.; Tian, Y.; Yuan, H.; Park, H. W.; Chang, Y.-Y.; Kim, J.; Kim, H.; Neufeld, T. P.; Dillin, A.; Guan, K.-L. ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. *Nat. Cell Biol.* **2013**, *15*, 741−750.
(36) Liu, D.; Wu, H.; Wang, C.; Li, Y.; Tian, H.; Siraj, S.; Sehgal, S. A.; Wang, X.; Wang, J.; Shang, Y.; Jiang, Z.; Liu, L.; Chen, Q. STING directly activates autophagy to tune the innate immune response. *Cell Death Differ.* **2019**, *26*, 1735−1749.
(37) McFarland, A. P.; Luo, S.; Ahmed-Qadri, F.; Zuck, M.; Thayer, E. F.; Goo, Y. A.; Hybiske, K.; Tong, L.; Woodward, J. J. Sensing of Bacterial Cyclic Dinucleotides by the Oxidoreductase RECON Promotes NF-kB Activation and Shapes a Proinflammatory Antibacterial State. *Immunity* **2017**, *46*, 433−445.
(38) Jin, L.; Hill, K. K.; Filak, H.; Mogan, J.; Knowles, H.; Zhang, B.; Perraud, A.-L.; Cambier, J. C.; Lenz, L. L. MPYS Is Required for IFN Response Factor 3 Activation and Type I IFN Production in the Response of Cultured Phagocytes to Bacterial Second Messengers Cyclic-di-AMP and Cyclic-di-GMP. *J. Immunol.* **2011**, *187*, 2595−2601.
(39) Jenal, U.; Reinders, A.; Lori, C. Cyclic di-GMP: second messenger extraordinaire. *Nat. Rev. Microbiol.* **2017**, *15*, 271−284.
(40) Opoku-Temeng, C.; Zhou, J.; Zheng, Y.; Su, J.; Sintim, H. O. Cyclic dinucleotide (c-di-GMP, c-di-AMP, and cGAMP) signalings have come of age to be inhibited by small molecules. *Chem. Commun.* **2016**, *52*, 9327−9342.
(41) Du, M.; Chen, Z. J. DNA-induced liquid phase condensation of cGAS activates innate immune signaling. *Science* **2018**, *361*, 704−709.
(42) Libanova, R.; Ebensen, T.; Schulze, K.; Bruhn, D.; Nörder, M.; Yevsa, T.; Morr, M.; Guzmán, C. A. The member of the cyclic di-nucleotide family bis-(3′, 5′)-cyclic dimeric inosine monophosphate exerts potent activity as mucosal adjuvant. *Vaccine* **2010**, *28*, 2249−2258.
(43) Davies, B. W.; Bogard, R. W.; Young, T. S.; Mekalanos, J. J. Coordinated Regulation of Accessory Genetic Elements Produces Cyclic Di-Nucleotides for V. cholerae Virulence. *Cell* **2012**, *149*, 358−370.

https://doi.org/10.1021/acschembio.1c00342
ACS Chem. Biol. 2021, 16, 1663−1670

(44) Panchanathan, R.; Liu, H.; Xin, D.; Choubey, D. Identification of a negative feedback loop between cyclic di-GMP-induced levels of IFI16 and p202 cytosolic DNA sensors and STING. *Innate Immun.* **2014**, *20*, 751−759.
(45) Corrales, L.; Glickman, L. H.; McWhirter, S. M.; Kanne, D. B.; Sivick, K. E.; Katibah, G. E.; Woo, S.-R.; Lemmens, E.; Banda, T.; Leong, J. J.; Metchette, K.; Dubensky, T. W.; Gajewski, T. F. Direct Activation of STING in the Tumor Microenvironment Leads to Potent and Systemic Tumor Regression and Immunity. *Cell Rep.* **2015**, *11*, 1018−1030.
(46) Ramanjulu, J. M.; Pesiridis, G. S.; Yang, J.; Concha, N.; Singhaus, R.; Zhang, S.-Y.; Tran, J.-L.; Moore, P.; Lehmann, S.; Eberl, H. C.; Muelbaier, M.; Schneck, J. L.; Clemens, J.; Adam, M.; Mehlmann, J.; Romano, J.; Morales, A.; Kang, J.; Leister, L.; Graybill, T. L.; Charnley, A. K.; Ye, G.; Nevins, N.; Behnia, K.; Wolf, A. I.; Kasparcova, V.; Nurse, K.; Wang, L.; Puhl, A. C.; Li, Y.; Klein, M.; Hopson, C. B.; Guss, J.; Bantscheff, M.; Bergamini, G.; Reilly, M. A.; Lian, Y.; Duffy, K. J.; Adams, J.; Foley, K. P.; Gough, P. J.; Marquis, R. W.; Smothers, J.; Hoos, A.; Bertin, J. Design of amidobenzimidazole STING receptor agonists with systemic activity. *Nature* **2018**, *564*, 439−443.
(47) Berthou, F.; Ceppo, F.; Dumas, K.; Massa, F.; Vergoni, B.; Alemany, S.; Cormont, M.; Tanti, J.-F. The Tpl2 Kinase Regulates the COX-2/Prostaglandin E2 Axis in Adipocytes in Inflammatory Conditions. *Mol. Endocrinol.* **2015**, *29*, 1025−1036.
(48) Chae, H.-S.; Kang, O.-H.; Lee, Y.-S.; Choi, J.-G.; Oh, Y.-C.; Jang, H.-J.; Kim, M.-S.; Kim, J.-H.; Jeong, S.-I.; Kwon, D.-Y. Inhibition of LPS-Induced iNOS, COX-2 and Inflammatory Mediator Expression by Paeonol through the MAPKs Inactivation in RAW 264.7 Cells. *Am. J. Chin. Med.* **2009**, *37*, 181−194.
(49) Xia, Q.; Hu, Q.; Wang, H.; Yang, H.; Gao, F.; Ren, H.; Chen, D.; Fu, C.; Zheng, L.; Zhen, X.; Ying, Z.; Wang, G. Induction of COX-2-PGE2 synthesis by activation of the MAPK/ERK pathway contributes to neuronal death triggered by TDP-43-depleted microglia. *Cell Death Dis.* **2015**, *6*, e1702−e1702.
(50) Ward, R. A.; Bethel, P.; Cook, C.; Davies, E.; Debreczeni, J. E.; Fairley, G.; Feron, L.; Flemington, V.; Graham, M. A.; Greenwood, R.; Griffin, N.; Hanson, L.; Hopcroft, P.; Howard, T. D.; Hudson, J.; James, M.; Jones, C. D.; Jones, C. R.; Lamont, S.; Lewis, R.; Lindsay, N.; Roberts, K.; Simpson, I.; St-Gallay, S.; Swallow, S.; Tang, J.; Tonge, M.; Wang, Z.; Zhai, B. Structure-Guided Discovery of Potent and Selective Inhibitors of ERK1/2 from a Modestly Active and Promiscuous Chemical Start Point. *J. Med. Chem.* **2017**, *60*, 3438−3450.
(51) Gavrin, L. K.; Green, N.; Hu, Y.; Janz, K.; Kaila, N.; Li, H.-Q.; Tam, S. Y.; Thomason, J. R.; Gopalsamy, A.; Ciszewski, G.; Cuozzo, J. W.; Hall, J. P.; Hsu, S.; Telliez, J.-B.; Lin, L.-L. Inhibition of Tpl2 kinase and TNF-α production with 1,7-naphthyridine-3-carbonitriles: Synthesis and structure−activity relationships. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 5288−5292.
(52) Won, J.-H.; Im, H.-T.; Kim, Y.-H.; Yun, K.-J.; Park, H.-J.; Choi, J.-W.; Lee, K.-T. Anti-inflammatory effect of buddlejasaponin IV through the inhibition of iNOS and COX-2 expression in RAW 264.7 macrophages via the NF-kappaB inactivation. *Br. J. Pharmacol.* **2006**, *148*, 216−225.

https://doi.org/10.1021/acschembio.1c00342
ACS Chem. Biol. 2021, 16, 1663−1670