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Xu, J., Xu, M., Wang, Y., **Mathena, R. P.**, Wen, J., Zhang, P., ... & Mintz, C. D. (2019). Anesthetics disrupt growth cone guidance cue sensing through actions on the GABAA α2 receptor mediated by the immature chloride gradient. *Neurotoxicology and teratology*, *74*, 106812.

Xu, J., **Mathena, R. P.**, Singh, S., Kim, J., Long, J. J., Li, Q., ... & Mintz, C. D. (2019). Early developmental exposure to repetitive long duration of midazolam sedation causes behavioral and synaptic alterations in a rodent model of neurodevelopment. *Journal of neurosurgical anesthesiology*, *31*(1), 151.

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Xu, J., **Mathena, R.**, Xu, M., Wang, Y., Chang, C., Fang, Y., ... & Mintz, C. (2018). Early Developmental Exposure to General Anesthetic Agents in Primary Neuron Culture Disrupts Synapse Formation via Actions on the mTOR Pathway. *International journal of molecular sciences*, *19*(8), 2183.

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Abstract

Title: "Interest-STING": Inhibition of Innate Immune Signaling by Prostaglandin E_2.

Reilley Paige Mathena, Master of Science, 2020

Thesis Directed By: Stefanie Vogel, PhD, and Darren Perkins, PhD, Professors of Microbiology and Immunology

The cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) pathway senses cytosolic double-stranded DNA from microbial or host cells. cGAS-STING activation elicits an inflammatory response, including production of type I interferons, through activation of TBK1 kinase and the transcription factor IRF3. Therefore, negative regulation of cGAS-STING activity would be predicted to prevent microbial-induced or autoimmune inflammatory damage. Based on prior work, we tested the hypothesis that the inflammatory product prostaglandin E_2 (PGE_2) acts on immune cells to control inflammation induced by cGAS-STING. STING pathway activating agents, 5,6 dimethylxanthenone-4-acetic acid (DMXAA) and cyclic guanosine monophosphate–adenosine monophosphate (cGAMP), were used to initiate STING signaling. Exogenous PGE_2 suppressed DMXAA- and cGAMP-induced STING signaling in murine embryonic fibroblasts and primary murine macrophages. Cells treated with PGE_2, followed by DMXAA or cGAMP, exhibited decreased activation of TBK1 and IRF3, and decreased inflammatory gene expression, arguing that PGE_2 signaling may be a mechanism restricting cGAS-STING activation.

“Interest-STING”: Inhibition of Innate Immune Signaling by Prostaglandin E_2

by
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Table of Contents

List of Figures iv

List of Abbreviations v

Chapter 1: Introduction

1.1 Innate and Adaptive Immune Response to Pathogens 1

1.2 The Role of Pattern Recognition Receptors (PRR) in Innate Immune Response ... 3

1.3 Toll Like Receptor 4 (TLR4) 4

1.4 Discovery of the cGAS-STING Pathway 8

1.5 The cGAS-STING Pathway Activates Some of the Same Signaling Intermediates as TLR4 11

1.6 Prostaglandin E_2 and EP Receptors 12

1.7 Goal and Hypothesis of Thesis 17

Chapter 2: Materials and Methods 19

Chapter 3: Experimental Results

3.1 Endogenous PGE_2 Levels are Increased in Cells After DMXAA Stimulation 23

3.2 PGE_2 Negatively Regulates STING-Dependent Signaling Even in the Presence of DMXAA 25

3.3 PGE_2 in the Presence of cGAMP Mimics the Restricting Effects in STING Signaling Previously Shown with DMXAA 28

3.4 PGE_2 Treatment of Macrophages Blunts DMXAA- and cGAMP-induced STING Dependent Gene Expression 32

Chapter 4: Discussion 39

Chapter 5: Conclusion 46

References 47

List of Figures

Figure 1 TLR4 signaling pathway 7

Figure 2 Model of cGAS-STING pathway 10

Figure 3 Prostaglandin generation pathway 13

Figure 4 Model of PGE_2 signaling via EP receptors 1-4.............................16

Figure 5 DMXAA treatment of MEFs and macrophages increases endogenous PGE_2 levels 24

Figure 6 DMXAA stimulation restricts downstream STING signaling........26

Figure 7 Pre-incubation of macrophages with PGE_2, followed by treatment with DMXAA Western blots 27

Figure 8 PGE_2 pretreatment of macrophages induces decreased STING signaling stimulated by cGAMP...30

Figure 9 PGE_2 decreased signaling stimulated by cGAMP over a time course 31

Figure 10 PGE_2 treatment reduces downstream DMXAA-induced gene expression 33

Figure 11 PGE_2 treatment reduces downstream cGAMP-induced gene expression 36

List of Abbreviations

AC	Adenylate cyclase
AGS	Aicardi-Goutières syndrome
AKT	Protein Kinase B
ALR	AIM2-like receptor
cGAMP	Cyclic guanosine monophosphate–adenosine monophosphate
cGAS	Cyclic GMP-AMP synthase
CLR	C-type lectin receptor
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
Ct	Cycle threshold
DAG	Diacylglycerol
DAMP	Damage-associated molecular pattern
DMXAA	5,6 Dimethylxanthenone-4-acetic acid
dsDNA	Double stranded DNA
EP	Prostaglandin E_2 receptor
ER	Endoplasmic Reticulum
ERK	Extracellular-signal regulated kinases

GSK-3 Glycogen synthase kinase 3

HMGB1 High mobility group box 1

IFN Type I interferons

IKK Inhibitor of kappa-B kinase

IRF Interferon regulatory factor-3

KSHV Kaposi's sarcoma-associated herpesvirus

LPS Lipopolysaccharide

MAPK Mitogen-activated protein kinase

MD2 Myeloid differentiation factor 2

MEF Murine embryonic fibroblast

MRP4 Multi-drug resistance protein-4

MYD88 Myeloid differentiation primary response 88

NF-κB Nuclear factor-κB

NLR NOD-like receptor

NOD Nucleotide-binding oligomerization domain

PAMP Pathogen-associated molecular pattern

PGD_2 Prostaglandin D2

PGE_2 Prostaglandin E_2

PGES Prostaglandin E_2 synthase

$PGF_{2\alpha}$ Prostaglandin $F_{2\alpha}$

PGG_2 Prostaglandin G_2

PGI_2 Prostacyclin

PI3K Phosphatidylinositol 3-kinase

PKA Protein kinase A

PKC Protein kinase C

PRR Pattern recognition receptor

RAGE Receptor for advanced glycation endproducts

RIG-I Retinoic acid-inducible gene-I

RLR RIG-I like receptor

SAVI STING-associated vasculopathy with onset in infancy

SLE Systemic lupus erythematosus

STING Stimulator of interferon genes

TBK1 Tank-binding kinase-1

TIR Toll-Interleukin-1 receptor

TIRAP Toll-Interleukin-1 receptor domain-containing adaptor protein

TLR Toll like receptor

TNF Tumor necrosis factor

TRAM TRIF-related adaptor molecule

TRIF TIR-domain-containing adapter-inducing interferon-β

ULK1 Unc-51-Like Autophagy Activating Kinase 1

VIRF1 Viral interferon regulatory factor 1

VSV Vesicular stomatitis virus

Chapter 1: Introduction

Subchapter 1.1: Innate and Adaptive Immune Response to Pathogens

In vertebrates, the immune system is comprised of an abundance of cells and soluble substances that trigger humoral and cellular responses in order to defend the host against foreign and pathogenic substances. Host immune responses have been categorized into two main categories: innate and adaptive immunity. Innate immunity works as the first and most rapid line of defense for host protection against pathogens through the utilization of physical barriers, as well as innate immune response cells and their mediators to induce a protective response [1]. Such physical barriers include tight junctions between epithelial cells that make up the skin, mucus membranes, and even mucus itself to aide in "external" defense against foreign substances [1,2].

If a pathogen circumvents the physical and chemical barriers of the innate immune response, enzymes and other anti-microbial substances, produced largely by innate immune cells, mediate effector responses that cause downstream cytokine release and inflammation for battling the invading pathogen [2]. Such white blood cells are known as leukocytes and include basophils, neutrophils, eosinophils, monocytes, and lymphocytes [3]. The neutrophils and monocytes are derived from a common bone marrow-derived progenitor cell. In response to a pathogen, circulating neutrophils and monocytes extravasate from the blood vessels and enter into the tissues to the site of infection. The neutrophils are short-lived and the first cells to be attracted to the site of an infection. In their effort to ingest and/or kill the invading pathogen, they are destroyed and release substances that recruit circulating monocytes to the site of infection. While some monocytes move into tissues and become "resident" macrophage populations that

acquire organ-specific functions (*e.g*., osteoclasts in bone, Kupffer cells in liver, etc.), the interaction of monocytes and macrophages with pathogens through receptors on their surface or in the cytoplasm mediates both an effector response that causes downstream cytokine release leading to inflammation, and the ingestion of the pathogen for intracellular killing [4].

In contrast, the adaptive immune response functions as the second line of defense, in which the immune system recognizes foreign antigens, resulting in clonal expansion of antigen-specific lymphocytes, and ultimately, development of memory cells. Memory cells respond more rapidly and robustly against the specific invading pathogen's antigens when the host is re-exposed to the same or similar pathogen [5]. Adaptive immunity relies predominantly on lymphocytes, specifically B and T cells, that work together to eliminate pathogens based on the specificity of antigenic epitopes on the pathogens; however, sometimes the adaptive immune response, while initially directed toward the pathogen, may cross-react with antigens of the host, potentially leading to development of an autoimmune disease [5,6]. The importance of immunological memory is that it enables the adaptive immune system to respond more rapidly and more robustly to the foreign antigens to which they were originally exposed, thus retaining, and often improving, the appropriate antigen-specific responses required to rid the host of a re-infecting pathogen. While the innate immune response responds first and quickly, the adaptive provides long-lasting and more antigen specific responses.

Subchapter 1.2: The Role of Pattern Recognition Receptors (PRR) in Innate Immune Response

Some of the early responses to pathogens via the innate immune system depend on the ability of innate immune cells to recognize unique structural features of pathogens for their rapid destruction [1]. Macrophages, neutrophils, and dendritic cells are the key innate immune cells that must be able to distinguish between molecules found within the host and those not commonly present in the host. Charles Janeway first introduced the idea of how innate immune cell receptors function by proposing they detect conserved microbial structures, and these receptors are referred to as "pattern recognition receptors" (PRRs) [7,8].

PRRs can be either membrane bound or unbound intracellular receptors, each detecting various conserved chemistries on different groups of pathogens. The five classifications of PRRs include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), AIM2-like receptors (ALRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). TLRs and CLRs are found on the cell surface, while ALRs, RLRs, and NLRs are cytoplasmic [9,10]. Regardless if it is CLRs binding fungal species in humans or RLRs binding viral RNA, each PRR performs a critical function in producing an effective innate immune response by detecting pathogen-associated molecular patterns (PAMPs) [11,12]. In some cases, host molecules may interact with PRRs and these have been referred to as "danger-associated molecular patterns" (DAMPs) [13]. Ligand binding to PRRs results in the recruitment of downstream adaptors and activation of kinases and transcription factors that lead to cytokine release and the inflammatory response [1,2,14].

PAMPs are molecules with conserved chemical motifs that help aid the host in distinguishing "self *vs*. non-self" to promote innate immune signaling in response to pathogens [15]. In vertebrate species, as well as some invertebrate species, these PAMPs are often found both intracellularly or extracellularly, in the form of DNA fragments, lipopolysaccharide (LPS), and peptidoglycan [16,17]. TLRs serve as some of the most integral PRRs in innate immune response and their interactions with PAMPs have been well studied.

DAMP binding to PRRs alerts the immune system of dangers beyond pathogen invasion, and may include host molecules released upon untimely cell death, stress, cancer, and other causes of tissue damage [15]. DAMPs are typically released from both the extracellular and intracellular spaces of damaged cells and are recognized by PRRs, such as numerous TLRs, as well as by other non-PRRs, including the receptor for advanced glycation endproducts (RAGE) [18]. High mobility group box 1 (HMGB1) is a chromatin-associated protein that, when found outside of the cell, functions as a DAMP and induces inflammation via Nuclear Factor-κB (NF-κB) by using both TLRs and RAGE [19,20]. As with all immune response signaling, overactivation of DAMPs has been linked with human diseases, such as organ failure, Alzheimer's disease, and cystic fibrosis [21].

Subchapter 1.3: Toll Like Receptor 4 (TLR4)

TLRs are a family of closely related transmembrane receptors and some of the most studied PRRs in innate immunity, in which there are ten functional TLRs that have been identified in humans and thirteen in mice [22]. In humans, TLR1, 2, 4, 5, 6, and 10 are found on the cell surface for detection of microbial cell wall and membrane structures,

while the remaining TLRs are considered endosomal that recognize nucleic acids, mainly from viruses [23]. The Drosophila protein Toll was first identified in the mid-1980's in Drosophilia embryos, and in 1996 was identified as essential for innate immune responses when researchers discovered that loss-of-function Toll mutations caused Drosophila to become susceptible to fungal and bacterial infections [24,25]. By comparing the genome sequence of Toll to known sequences of the human genome, researchers were able to identify homologous sequences and ultimately cloned human "hToll", which later became known as "Toll-like Receptor 4" or TLR4 [26].

Further studies led researchers to better understand the mechanisms behind the role TLR4 plays in innate immunity. We now know that TLR4 acts as a cell surface PRR in mammals. TLR4 senses a Gram negative bacterial PAMP, known as LPS, after this prototype ligand binds to a non-covalently bound TLR4 co-receptor, myeloid differentiation factor 2 (MD-2) [27]. Binding of this prototype ligand to MD-2 causes a conformational change in TLR4 causing it to dimerize and become activated [27,28]. Once dimerized, the Toll-Interleukin-1 receptor resistance (TIR) domains, located intracellularly on each TLR4 monomer, form a "signaling platform" that recruits downstream adaptors Toll-Interleukin-1 receptor domain-containing adaptor protein (TIRAP) and myeloid differentiation primary response 88 (MyD88), which then activate a kinase cascade that leads to NF-κB activation for transcription of downstream genes to produce proinflammatory cytokines [29].

TLR4 is unique among the TLRs in that it can also be endocytosed and signal from the endosome [30]. In this case, the TLR4-TIR domain recruits TRIF-related adaptor molecule (TRAM), rather than TIRAP, that in turn recruits TIR-domain-containing

adapter-inducing interferon-β (TRIF) for downstream activation of Tank-binding kinase-1 (TBK1) [30,31]. TBK1 subsequently phosphorylates interferon regulatory factor-3 (IRF3), a transcription factor required for induction of type I interferons (IFNs) [32]. Other TLRs utilize either the MyD88-dependent signaling pathway or the TRIF-signaling pathway; however, TLR4 is unique in that it activates both signaling pathways and, perhaps, this accounts for why this PRR is such a potent inducer of inflammation. A model for TLR4 signaling is shown in Figure 1.

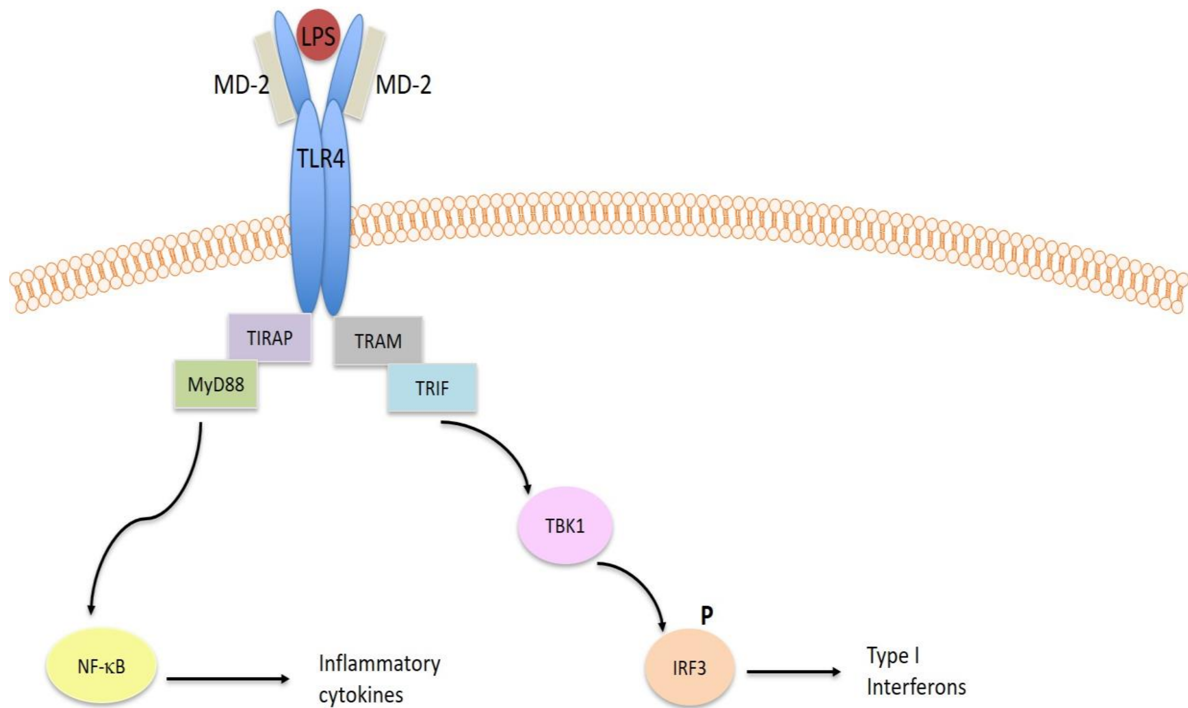


Figure 1. TLR4 signaling pathway. LPS is bound by MD-2, which is an co-receptor protein, to activate TLR4 and initiate intracellular signaling. When this occurs at the cell surface, adaptors TIRAP and MyD88 are recruited to the intracellular TIR domains of TLR4, leading to downstream signaling and subsequent transcription of NF-κB to yield proinflammatory cytokines. TLR4 can also be internalized into endosomes (not pictured), and when it signals from the endosome, it recruits TRAM. TRAM then enlists TRIF and TRIF activates TBK1 for further downstream phosphorylation of IRF3. IRF3 is a transcription factor that is necessary for induction of IFNs.

Subchapter 1.4: Discovery of the cGAS-STING Pathway

Although it was previously recognized that PAMPs detected by PRRs during the innate host response led to activation of genes used to fight pathogens, it was unknown how detection of viral intracellular DNA or RNA initiates an innate immune response. In 2008, Ishikawa and Barber first identified stimulator of interferon genes (STING) as another endoplasmic reticulum adaptor that mediates the signaling in innate immunity [33]. They determined that STING was expressed in numerous tissues during an ongoing immune response to infection and aided in activating NF-κB and IRFs for transcription of IFNs [33]. They also found that mice deficient in STING had higher infection rates with Vesicular Stomatitis Virus (VSV), but when STING function was restored, susceptibility to the virus decreased [33].

In 2013, other studies determined that in response to DNA transfection into the cytosol of mammalian cells, cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) was produced, which was shown to bind and activate STING [34]. The synthase for cGAMP, known now as cyclic GMP-AMP synthase (cGAS), proved to be a cytosolic DNA sensing protein that, upon activation by binding nucleic acid, triggers a cascade of signaling for downstream immune responses. This was identified by fractionation and mass spectrometry, and overexpression studies revealed that STING activation was accompanied by activation of IRF3 and induction of IFN-β [34]. This study also showed that knockdown of cGAS decreased IRF3 activation and subsequent IFN-β production in response to stimulation by transfected DNA and virus infection [34]. Ultimately, researchers determined that cGAS acts as a "PRR" for DNA in the cytosol and leads to

the induction of cGAMP [35]. cGAMP binds to STING and initiates downstream signaling leading to IFN-β transcription via the transcription factor IRF3 [34].

cGAS has a high affinity for binding double stranded DNA (dsDNA) and, upon interaction with dsDNA, cGAS undergoes a conformational change. This rearrangement allows cGAS to catalyze ATP and GTP into cGAMP, a second messenger. cGAMP binds to STING, which is located on the endoplasmic reticulum (ER) [36]. STING consists of two C-terminal domains that form a V-shaped pocket, and once cGAMP binds, these domains enclose and cover the cGAMP binding site. This conformational change causes STING to translocate from the ER to the Golgi apparatus. In murine cells, an anti-tumor drug, 5,6-dimethylxanthenone-4-acetic acid (DMXAA), binds directly to murine STING and elicits the conformational change normally mediated by cGAMP [37]. While DMXAA is a very good activator of murine STING, it does not activate human STING well; however, other structural analogs of DMXAA work similarly in human cells [38].

Once in the Golgi, STING stimulates phosphorylation of TBK1, which subsequently mediates the binding of IRF3 to the C-terminal portion of STING [36]. TBK1 then directly phosphorylates IRF3, which translocates to the nucleus for transcription of IFN. STING translocation to the Golgi also recruits IκB kinase (IKK) to phosphorylate the NF-κB inhibitor IκBα [39]. IκBα becomes phosphorylated and dissociates from NF-κB, allowing NF-κB to move to the nucleus where it activates the transcription of proinflammatory cytokines [39]. These cytokines work to mediate the inflammatory response in immune cells by binding to cytokine-specific receptors to provide an effective response to viral pathogens. Figure 2 illustrates a model for STING activation.

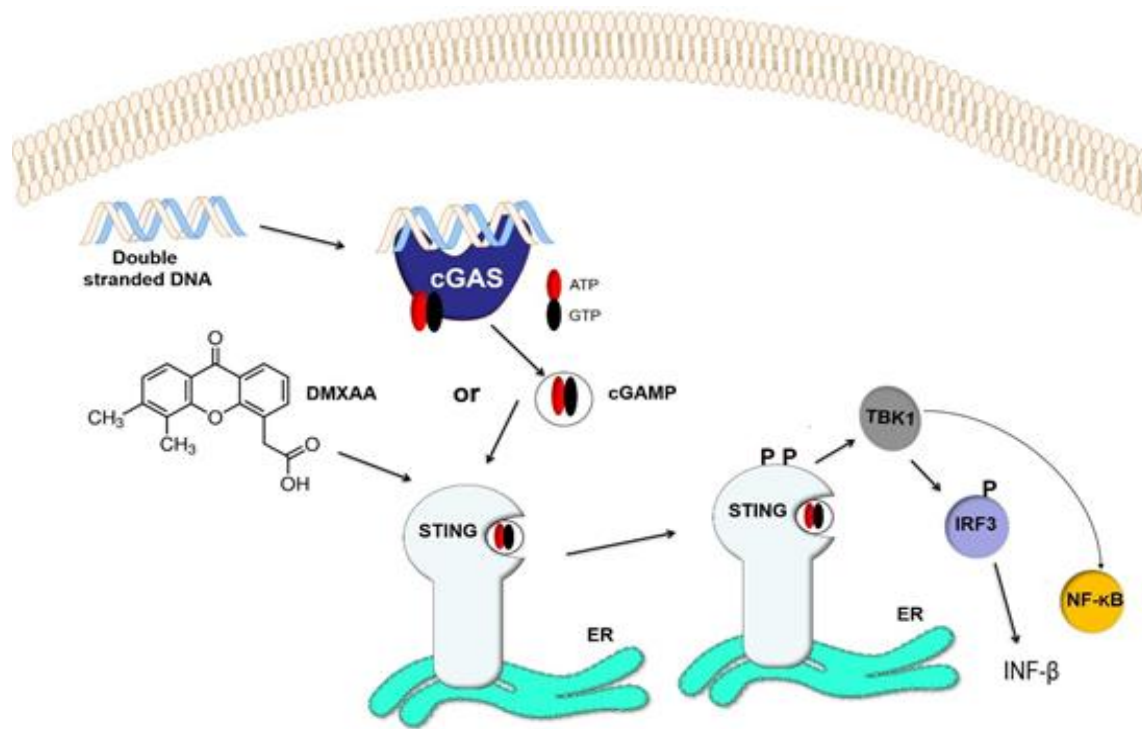


Figure 2. Model of cGAS-STING pathway. When dsDNA enters the cytosol, cGAS recognizes and binds the dsDNA, causing a conformational change in cGAS. This conformational change catalyzes ATP and GTP into cGAMP, which binds to the STING protein (located in the ER). STING then undergoes a conformational change and translocates to the Golgi apparatus (not depicted in the figure). Following this, STING phosphorylates TBK1, which directly phosphorylates IRF3 and indirectly phosphorylates NF-κB via IκB kinase. Phosphorylated IRF3 and NF-κB translocate to the nucleus for transcription of cytokines. DMXAA overcomes the need for DNA sensing and cGAMP generation and is shown binding directly to STING, thereby prompting the signaling cascade organically caused by cGAMP.

Subchapter 1.5: The cGAS-STING Pathway Activates Some of the Same Signaling Intermediates as TLR4

The cGAS-STING pathway utilizes some of the same downstream signaling molecules used by the TLR4 innate immune signaling pathway. cGAS-STING is stimulated by the presence and detection of cytosolic nucleic acids, usually stemming from a virus. Through the signaling cascade of this pathway, TBK1 becomes activated and phosphorylates IRF3 for downstream IFN production [40]. TBK1 can also facilitate activation of NF-κB to promote proinflammatory response in the pathway [40]. Thus, the cGAS-STING pathway utilizes several of the same downstream signaling molecules as the TLR4-TRIF pathway, and thereby induces many of the same immune responses. Yet, less is known about how the cGAS-STING signaling pathway is regulated.

Studies have that shown excessive activation of cGAS-STING can cause overproduction of inflammatory cytokines and lead to the further development of cancer and other autoinflammatory diseases. STING-associated vasculopathy with onset in infancy (SAVI) is caused by a gain of function mutation that leads to continuous STING activation, thereby causing abnormal inflammation throughout the body [41,42]. This leads to cell and tissue damage, and individuals can develop scar tissue in the lungs and other respiratory complications, such as pulmonary fibrosis [41,43].

Perkins et al. demonstrated that TLR4 activation by LPS stimulation of mouse macrophages induces prostaglandin E_2 (PGE_2). Once released from the cell, PGE_2 engages a specific receptor, prostaglandin E_2 receptor 4 (EP4), to elicit a negative feedback loop that inhibits continued TLR4 activation and downstream immune

responses [44]. This response was shown to be TRIF-selective and, therefore, utilizes many of the same downstream molecules in the cGAS-STING pathway.

Subchapter 1.6: Prostaglandin E_2 and Receptors

Prostaglandins are structurally related to lipids, are derived from arachidonic acid, and play a role in inflammatory responses at the site of injury. Phospholipase A2 releases arachidonic acid from membrane phospholipids, which is then catalyzed to prostaglandin G_2 (PGG_2) by cyclooxygenases (COX) COX-1 and COX-2 [45]. COX-2 contains a peroxidase site that produces prostaglandin H_2 (PGH_2) by catalyzing PGG_2 [45]. Various synthase enzymes catalyze PGH_2 to produce the following four main types of prostaglandins: PGE_2, prostacyclin (PGI_2), prostaglandin D2 (PGD_2), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Prostaglandin E_2 synthase (PGES) catalyzes PGH_2 to generate PGE_2 [46]. PGE_2 is the most abundant prostaglandin found endogenously in mammalian cells and aids in regulating immune responses and inflammation [47]. Figure 3 illustrates the generation of various prostaglandin species.

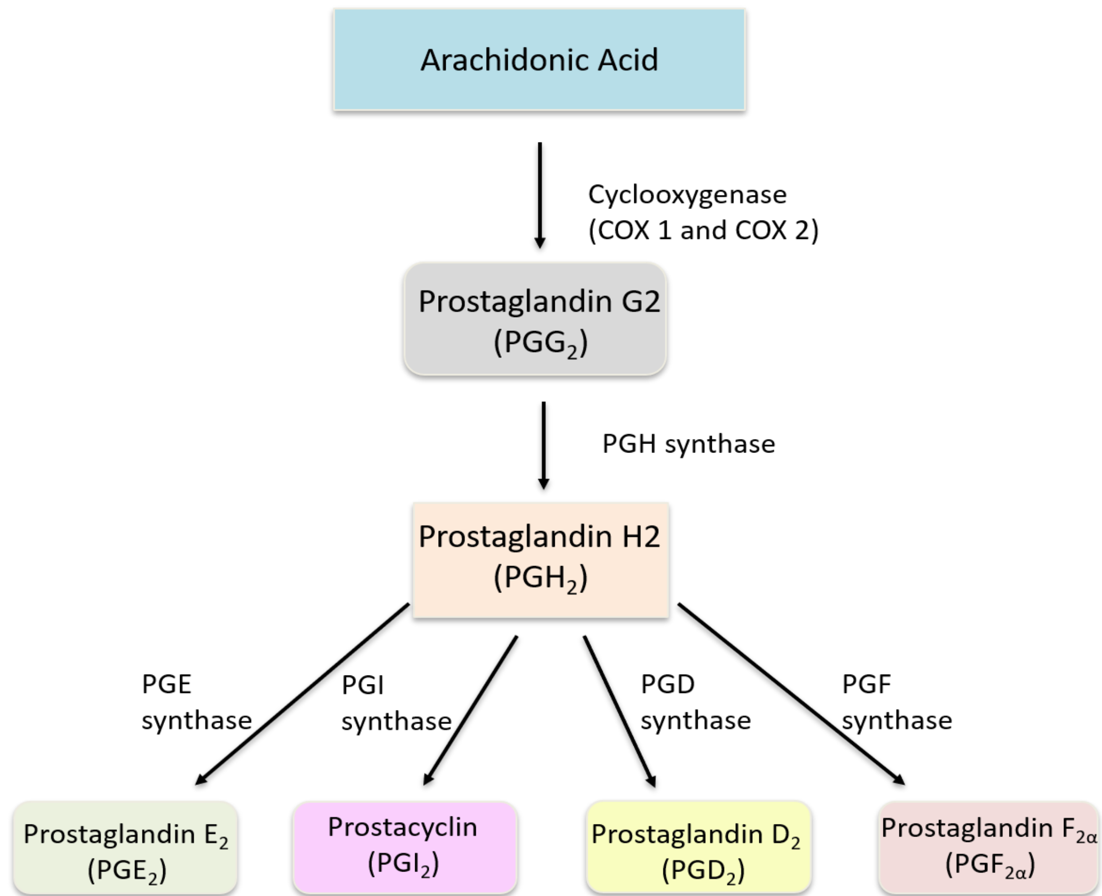


Figure 3. Prostaglandin generation pathway. Arachidonic acid is released from phospholipase A2 (not pictured) and is catalyzed by COX-1 and COX-2 to form PGG_2. COX-2 uses a peroxidase site to induce catalysis on PGG_2 and produce PGH_2. Prostaglandin synthases breakdown PGH_2 further until one of four prostaglandins is produced: PGE_2, PGI_2, PGD_2, or $PGF_{2\alpha}$.

Endogenous prostaglandins are transported outside of the cell using a prostaglandin transporter and in all cells using the multi-drug resistance protein-4 (MRP4) [48]. Once outside of the cell, PGE_2 can bind to any of four known receptors, which include EP1, EP2, EP3, and EP4, to initiate numerous functions [49,50]. PGE_2 has been shown to be involved in autocrine-paracrine signaling while utilizing any of the four EP receptors, which further adds to the extensive recruitment of adaptors, messengers, and outcomes in terms of downstream signaling by these immune pathways [51].

The four EP receptors are classified as G protein-coupled receptors and all have varying degrees of affinity for PGE_2 as well as the outcomes produced after binding. EP2 and EP4 have shown the greatest affinity for PGE_2 and its involvement in mediating its effects in both adaptive and immune cells [47]. These receptors utilize $G\alpha_s$ subunits to transduce signaling, causing an increase in the activation of adenylate cyclase (AC), that in turn, increases the amount of cAMP present inside the cell [52]. Both receptors also play a role in inhibition of glycogen synthase kinase 3 (GSK-3). While EP2 and EP4 both activate cAMP, they each independently interact with molecules that lead to similar downstream and diverging signaling cascades. In particular, EP4 inhibits GSK-3 based on the presence of activated phosphatidylinositol 3-kinase (PI3K) [47]. Direct activation of PI3K, triggered by bound PGE_2, causes extracellular-signal regulated kinases (ERKs) to become activated, to help control downstream signal transduction and expression of genes such as tumor necrosis factor (TNF-α) and COX-2 [47].

EP1 is coupled with Gq to help increase intracellular levels of calcium and, as a consequence, increases activation of protein kinase C (PKC). PKC triggers mitogen-activated protein kinase (MAPK) stimulation, which plays a major role in inducing

immune responses to combat viral infections [53], but EP1 itself has yet to show any direct roles in innate immune responses. EP3 exists in multiple forms, each with nearly equal affinity for binding and utilizing PGE_2, and it has been shown to play a role in inhibition of cAMP, while also increasing levels of cAMP, much like EP2 and EP4. A model for PGE_2 signaling through EP receptors is presented in Figure 4.

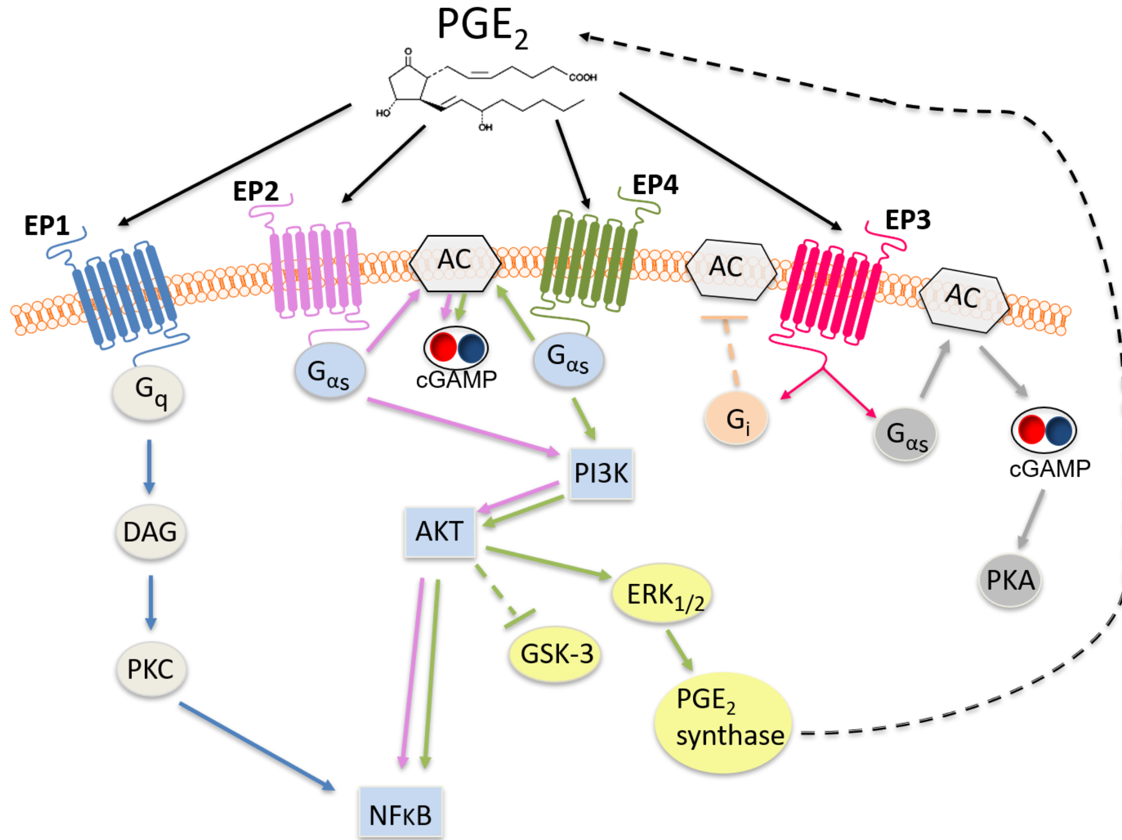


Figure 4. Model of PGE_2 signaling via EP receptors 1 - 4. PGE_2 - EP1 pathway: PGE_2 binds to EP1, which is bound to Gq, causing activation of diacylglycerol (DAG), and subsequent activation of PKC. Activated PKC triggers cascade signaling for NF-κB and downstream transcription of cytokines. **PGE_2 – EP2 pathway:** Once bound to EP2, PGE_2 utilizes the $G\alpha_s$ and raises adenyl cyclase (AC) levels, which in turn, stimulates cGAMP production. Activation of the $G\alpha_s$ through EP2 also increases phosphatidylinositol 3-kinase (PI3K) production, which then activates Protein Kinase B (AKT), and leads to downstream NF-κB signaling. **PGE_2 – EP4 pathway:** EP4 utilizes the $G\alpha_s$ similarly to EP2, in which cGAMP production is increased due to AC and follows the PI3K signaling for downstream production of NF-κB. EP4 can also inhibit glycogen synthase kinase 3 (GSK-3) by activating extracellular signal-regulated protein kinase 1/2 ($ERK_{1/2}$) to produce PGE synthase, which exits the cell and eventually becomes PGE_2. **PGE_2 – EP3 pathway:** EP3 also plays a role in increasing AC and cGAMP due to its interaction with $G\alpha_s$ when PGE_2 is bound, and this leads to activation of protein kinase A (PKA). EP3 and PGE_2 interactions can also inhibit AC production, leading to decreased cellular levels of cGAMP.

Subchapter 1.7: Goal and Hypothesis of Thesis

The major goal of this thesis research was to determine if PGE_2 exerted an inhibitory effect on signaling through its action on the cGAS-STING pathway. We know that PRRs function to prevent foreign substances from wreaking havoc within the body by detecting and binding these foreign substances and triggering the release of proinflammatory cytokines, as well as IFNs. The cGAS-STING pathway operates by recognizing and binding cytosolic nucleic acids to trigger the immune response. Typically, these nucleic acids originate from a viral source. Occasionally, cellular processes malfunction such that the nucleus is damaged, or replication of damaged DNA goes awry. This can cause self-DNA to leak into the cytosol, tricking cGAS into thinking it is encountering foreign material and activating the pathway. Should such damage occur continuously, the body will continue to attack these “self” substances, leading to chronic release of inflammatory response and may result in autoimmune disease

There have been a number of inflammatory diseases described that are caused by over-production of IFNs. Aicardi-Goutières syndrome (AGS) is a hereditary encephalopathy that affects newborns, in which mental capabilities are severely impacted and is a direct result of an over-abundance of IFNs [54]. The loss of TREX1, an exonuclease that digests cytosolic DNA, allows for the overproduction of IFNs, leading to overactivation of astrocytes in AGS [55,56].

Systemic lupus erythematosus (SLE) in humans is also caused by malfunction of TREX1. SLE is an autoimmune disease in which the body attacks its own tissue, causing inflammation and damage in the afflicted areas. While there are existing treatments,

there are no known cures for either AGS or SLE currently. However, researchers were able to rescue the autoimmune phenotype in TREX1$^{-/-}$ mice by eliminating cGAS and, in turn, lowering the levels of IFNs [57].

These studies, along with the work conducted by Perkins et al. inspired the work conducted in this thesis, which focused on the potential role of PGE_2-induced signaling in mitigating STING activation as a novel approach, and potential therapeutic target, to limit autoimmune responses mediated by STING**.** Therefore, the central hypothesis for this thesis is PGE_2 provides an inhibitory signal that limits the inflammatory response of macrophages and murine embryonic fibroblasts (MEFs) induced by cGAS-STING.

Chapter 2: Materials and Methods

Cells and Cell Culture

Six to eight-week old C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Intraperitoneal injection of sterile 3% fluid thioglycollate broth (3 ml) (Thermo Fisher, Waltham, MA) was administered to mice to elicit murine peritoneal exudate macrophages. Four days following the injection, mice were euthanized, and macrophages were harvested by lavaging the peritoneal cavity with sterile saline. Macrophages represented >90% of the peritoneal exudate cells and were cultured using RPMI 1640 media (1x, Corning, Corning, NY) with 2% fetal bovine serum (Hyclone, Logan, UT), 1% penicillin and streptomycin (Gibco, Carlsbad, CA), and 2mM L-glutamine (Gibco, Carlsbad, CA). Macrophages were plated in 12-well (2 x 10^6 cells/well) tissue culture plates in supplemented RPMI media and incubated at 37°C in the presence of 5% CO_2 overnight. The next day, cells were washed to remove non-adherent cells and the remaining macrophages were treated as described in the text and figure legends.

MEFs were kindly provided by Dr. Genhong Cheng (University of California, Los Angeles, Los Angeles, CA). MEFs were obtained from frozen stocks, passaged up to 5 passages in DMEM (BioWhittaker, Walkersville, MD) with 10% fetal bovine serum (Hyclone, Logan, UT), 1% penicillin and streptomycin, and 2 mM L-glutamine. MEFs were plated at 2 x 10^6 cells/well in 12 well tissue culture plates and were kept at 37° C in 5% CO_2 until the cells were ~80% confluent. The next day, cells were washed to remove non-adherent cells and the remaining MEFs were treated as described in the text and figure legends.

Reagents

DMXAA (Sigma Aldrich, St. Louis, MO) was used at a concentration of 0.01 μg/ml in DMSO (Sigma Aldrich). PGE_2 (Tocris, Bristol, UK) was used at a concentration of 50 μM. cGAMP (Tocris) was prepared at a working concentration of 2 μg/μl in DEPC water. Primary antibodies obtained from Cell Signaling Technology (Danvers, MA) used for Western blot analyses include rabbit anti-phospho-IRF3 (1:1000), rabbit anti-phospho-TBK1/NAK (1:1000), rabbit anti-p38 (1:1000), rabbit anti-phospho-NF-κB p65 (1:1000), and rabbit anti-GAPDH (1:1000). For all antibodies that detect phosphorylated proteins, 5% bovine serum albumin (Sigma Aldrich) was added to minimize non-specific binding of antibodies. Peroxidase-conjugated goat anti-rabbit IgG (1:5000, Jackson Laboratories) diluted in TRIS-buffered saline (1x, Bio-Rad, Hercules, CA) with 0.1% Tween 20 (TBST, Bio-Rad) and non-fat dried milk was used during incubation as the secondary antibody. For protein detection, the Pierce ECL Plus Western Blotting Substrate kit (Thermo Fisher) was used per the manufacturer's instructions immediately before exposure and development.

Western Blot Analysis

Following treatment, cells were washed with 1X PBS, then lysed in the plate by addition of 50 μl Lysis buffer/well (DEPC water, 20 mM HEPES pH 6.8, 1% TRITON X-100, 0.1% SDS, 150 mM NaCl, 1 PhosSTOP tablet (Roche, Basel, Switzerland), and 1 Complete Mini tablet (Roche)) and stored at -80° C. Following thawing, cells were scraped from the wells and transferred into centrifuge tubes. The lysates were centrifuged at 6,000 rpm for 5 minutes at 4° C. Eight μl of each lysate was added to 7 μl of β-mercaptoethanol in Laemmli buffer and electrophoresed using 12% polyacrylamide gels

(Bio-Rad). Tris/Glycine/SDS (1X, Bio-Rad) was used as the running buffer and the gels were electrophoresed at 33 volts for ~1 hour. Following this, transfer buffer (compromised of 100 ml of 10X Tris/Glycine buffer (Bio-Rad), 700 ml deionized water, and 200 ml methanol) was used to transfer the proteins in the gel to extra thick blot paper (Bio-Rad) at 20 volts for 38 minutes. TBST + non-fat dried milk was used to block the membrane, followed by overnight rotating incubation of TBST + primary antibody (1:1000) at 4° C. Membranes were washed for 1 hour at room temperature on a rotator using fresh TBST every 15 minutes. The secondary antibody (1:5000) was added in TBST for 1 hour at room temperature, followed by another round of washing as done previously, and developed using the ECL plus kit.

ELISA

To detect PGE_2, ELISAs were performed using the PGE_2 ELISA Kit (Enzo LifeSciences, Farmingdale, NY) following the manufacturer's instructions.

Quantitative RT-PCR (qRT-PCR)

Total RNA was harvested from macrophages using TriPure Isolation Reagent (Roche, Basel, Switzerland), following the manufacturer's protocols. The RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher). Using the iScript Reverse Transcription Kit (Bio-Rad), 0.5 μg of total RNA was reversed transcribed following the manufacturer's instructions. Total cDNA was used for qRT-PCR using SYBR Green (Applied Biosystems, Foster City, CA), and amplified using primer pairs for the genes encoding the chemokine IFN-β stimulated protein-10 (IP-10 (Cxcl10), 5'-GTG TTG AGA TCA TTG CCA CGA-3'; 5'- TTT TTG GCT AAA CGC TTT CAT

TAA-3') , interferon-β (IFN-β, 5'- CAC TTG AAG AGC TAT TAC TGG AGGG-3'; 5'-CTC GGA CCA CCA TCC AGG-3'), the pro-inflammatory cytokine TNF-α (Sigma, 5'-GAC CCT CAC ACT CAG ATC ATC TTCT -3'; 5'- CCA CTT GGT GGT TTG CTA CGA-3'), and GAPDH (Millipore, Burlington, MA, 5'- AGC CTC GTC CCG TAG ACA AAA T-3'; 5'- TGG CAA CAA TCT CCA CTT TGC-3') were used for detection. Cycle threshold (Ct) values were compared using the -*Δ*Ct method using *Gapdh* as the housekeeping gene [58]. In addition, the "fold increase" in gene expression was also derived from the -*ΔΔ*Ct data to allow an estimate of the relative increase over background levels for each gene examined.

Statistics

Statistics for all data was analyzed using GraphPad Prism (v.8.0, San Diego, CA). Statistics for the data in each figure are described in the corresponding figure legend. For statistical analysis, the -*Δ*Ct data was analyzed and transformed to "fold induction" based on a comparison of the arithmetic value of the treated cultures to the medium-treated control value. Unless otherwise indicated, graphs represent the mean ± SEM. Comparisons for Figure 5 were performed using unpaired Student's *t* test for both graphs. Comparisons for Figures 10 and 11 were performed using One-Way ANOVA with Holm-Sidak multiple comparison post-hoc analysis. Outliers were excluded as needed, in which case Mixed Effects Analysis was completed instead of One-Way ANOVA. On all graphs, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Chapter 3: Experimental Results

Subchapter 3.1: Endogenous PGE_2 Levels are Increased in Cells After DMXAA Stimulation

Many non-myeloid-derived cells (*e.g*., fibroblasts, epithelial cells, endothelial cells) provide the front line innate immune defense by providing physical barriers, producing anti-microbial substances, and activating certain signaling pathways to combat pathogenic invaders, making them ideal for host-pathogen interaction studies [59]. Macrophages are recruited to the site of infection and play an important role in removing bacteria and initiating inflammation through cytokine release. PGE_2 is known to mediate inflammatory responses and modulates the activity of immune cells, such as macrophages, and particularly, by regulating cytokine secretion of these cells [60]. Therefore, we analyzed the responses of both MEFs and macrophages in our study.

We compared the levels of endogenous PGE_2 with and without STING activation by DMXAA in both MEFs and primary peritoneal murine macrophages. PGE_2 ELISA was carried out on cell supernatants after treatment, and the data collected and analyzed. The results showed a significant increase in PGE_2 levels in cells stimulated with DMXAA in comparison to their untreated controls (Figure 5), suggesting the possible existence of an underlying relationship between STING and PGE_2 in the signaling pathway.

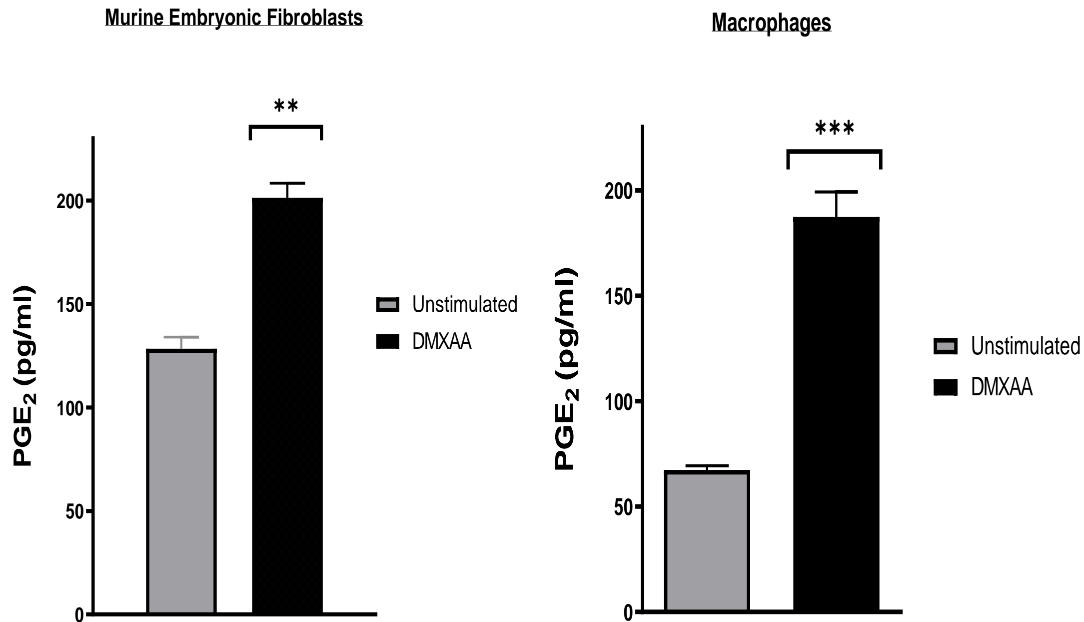
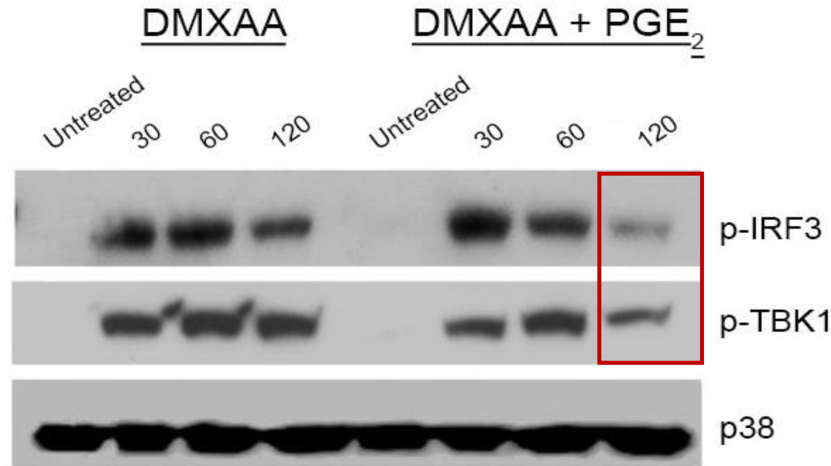


Figure 5. DMXAA treatment of MEFs and macrophages increases endogenous PGE_2 levels. PGE_2 levels (mean±SEM) in MEFS and primary murine macrophages. MEFS from C57BL/6J mice were stimulated with DMXAA (0.01 µg/ml) for 6 hours or left unstimulated. Peritoneal macrophages from C57BL/6J mice were stimulated with DMXAA (0.01 µg/ml) overnight or left unstimulated. An ELISA was performed to measure levels of endogenous PGE_2 found in supernatants of both types of cells. Data shown are from two separate experiments from each cell type (MEFs and macrophages). MEFs: unpaired Student's *t* test, $p < 0.01$. Macrophages: unpaired Student's *t* test, $p < 0.001$.

Subchapter 3.2: PGE_2 Negatively Regulates STING-Dependent Signaling Even in the Presence of DMXAA

To elucidate a possible role of PGE_2 in the regulation of STING-dependent signaling, select MEFs and macrophages were first pre-treated with medium only or exogenous PGE_2 for 30 minutes, followed by stimulation with DMXAA for the indicated times to evaluate time-dependent activation of the STING pathway. The remaining MEFs and macrophages were treated only with DMXAA for the indicated time points. Phospho-specific antibodies against activated IRF3 and TBK-1 were used in Western blots to measure the effect of PGE_2 on downstream STING signaling. The images in Figure 6 show that treatment of both MEFs and macrophages with PGE_2, followed by DMXAA stimulation, decreased phosphorylation of IRF3 and TBK-1 when compared to cells lacking pretreatment with exogenous PGE_2 prior to DMXAA stimulation. Decreased levels of phosphorylation indicate reduced activation of both IRF3 and TBK-1. These findings support the hypothesis that PGE_2 potentially plays a role in regulation of STING signaling by restricting STING activation of TBK-1 and IRF3.

To better understand the interaction between PGE_2 and STING signaling, the duration of the suppressive effects of PGE_2 exposure on STING activation was determined. To approach this question, primary murine macrophages were pre-treated with PGE_2 for 2, 5, 7, or 20 hours, followed by treatment with DMXAA for an additional 2 hours. The Western blots showed that pre-treatment of cells with PGE_2 decreased DMXAA-induced phosphorylation of TBK1 and IRF3 that was most striking at 7 and 20 hours (Figure 7). This further supports the results of Figure 6 showing that PGE_2 restricts STING downstream activation.



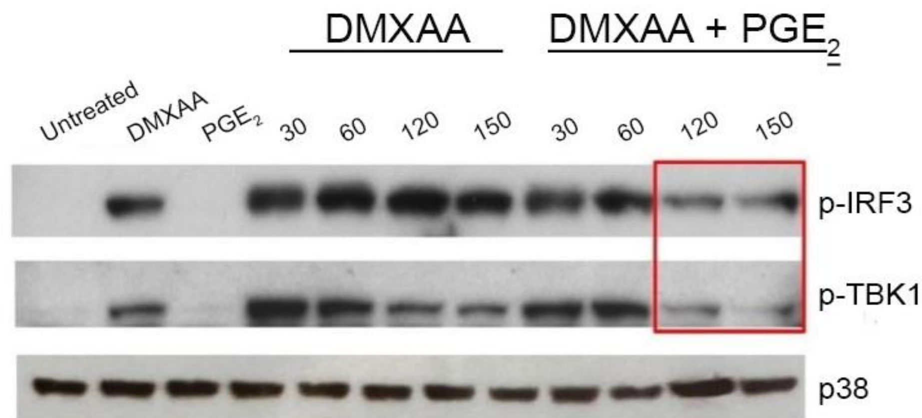


Figure 6. DMXAA stimulation restricts downstream STING signaling. MEFs and macrophages from C57BL/6J mice were either pre-treated with PGE_2 (50 µM) or left untreated in media for a 30-minute incubation period. Following this incubation period, cells were stimulated with DMXAA (0.01 µg/ml) for a total of either 30, 60, 120, or 150 (macrophages only) minutes. Cells were harvested and cell lysates were collected for Western blot analysis for phosphorylated IRF-3 and phosphorylated TBK1, with total p38 used as a loading control. The red box indicates the most noticeable difference in signaling in the MEFs and macrophages. Results are representative of two independent experiments for each Western Blot.

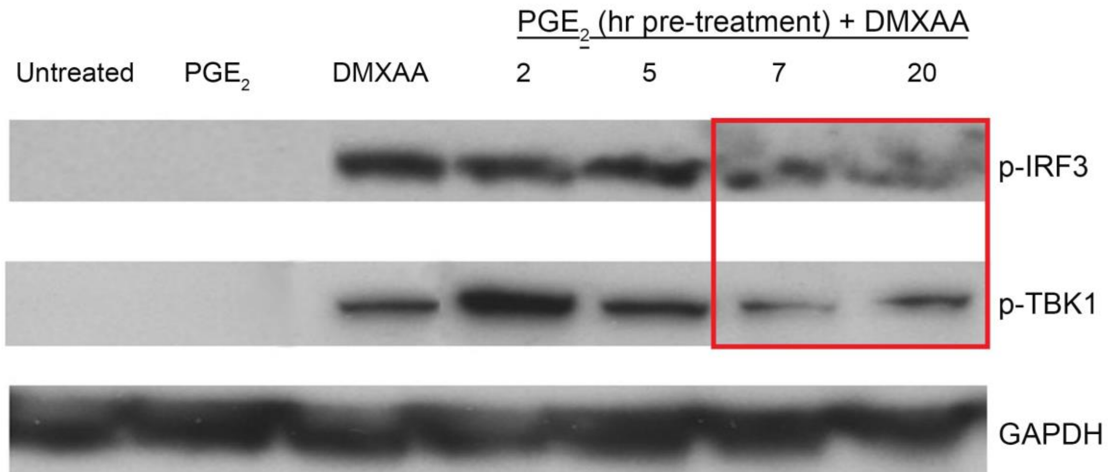


Figure 7. Pre-incubation of macrophages with PGE_2, followed by treatment with DMXAA Western blots. Murine primary macrophages from C57BL/6 mice were pre-treated with PGE_2 (50 µM) for a 2, 5, 7, or 20-hour incubation period ("Untreated" represents media only cells). Following this incubation period, all cells were stimulated with DMXAA (0.01 µg/ml) for an additional 2 hours. Cells were harvested and lysates were used for Western blot analysis for phosphorylated IRF-3 and phosphorylated TBK1, with GAPDH used as a loading control. The red box indicates the most noticeable difference in signaling. Results are representative of two independent experiments.

Subchapter 3.3: PGE_2 in the Presence of cGAMP Mimics the Restricting Effects in STING Signaling Previously Shown with DMXAA

After identifying a reoccurring pattern of restricted STING signaling in DMXAA-stimulated cells induced by PGE_2, we sought to confirm our findings using cGAMP, the upstream activator of STING [61]. While DMXAA is a useful pharmacologic tool that directly binds STING and activates downstream signaling [62], it is not a naturally occurring molecule and obviates upstream signaling events that are normally activated by pathogens that initiate the STING signaling pathway. Within the cell, STING is normally activated by cGAMP, acting as a PRR that binds directly to the STING protein to activate the downstream signaling cascade [61]. To better understand the role that PGE_2 might be playing in the regulation of STING activation, we examined the effect of PGE_2 treatment on cGAMP-induced STING activation. Primary murine macrophages were left untreated in media or were pre-treated with PGE_2 (50 μM) for 30 minutes prior to treatment with a broad range of cGAMP concentrations (1.25, 2.5, 5, 10, and 20 μg/ml) for 3 hours. Medium-pretreated macrophages received cGAMP only at those concentrations. The Western blots showed that pre-treatment with PGE_2 decreased cGAMP-induced phosphorylation of IRF3, most notably at 20 μg/ml (Figure 8). There was also significant reduction in signaling at 20 μg/ml for phosphorylated p65 in the cGAMP + PGE_2 macrophages compared to those receiving cGAMP only (Figure 8).

We further investigated the effect PGE_2 pretreatment on STING signaling in the presence of cGAMP over time. Designated wells containing the primary murine macrophages were pre-treated with PGE_2 (50 μM) or left untreated in media for 30 minutes prior to receiving 20 μg/ml of cGAMP for either 60, 120, or 180 minutes. The remaining macrophages received only 20 μg/ml of cGAMP at those same time points.

Western blot analysis showed reduced phosphorylated IRF3 and phosphorylated p65 signaling in cGAMP + PGE_2 cells compared to macrophages receiving cGAMP alone (Figure 9).

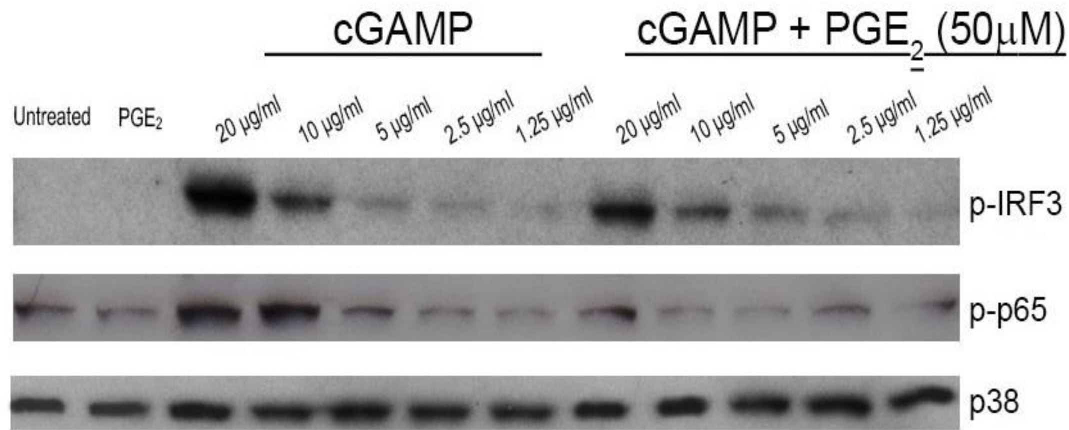


Figure 8. PGE_2 pretreatment of macrophages induces decreased STING signaling stimulated by cGAMP. Primary murine macrophages from C57BL/6J mice were pre-treated with PGE_2 (50 μM) or left untreated in media for 30 minutes prior to addition of cGAMP at the indicated doses for 3 hours. Cell lysates were collected for Western blotting for phosphorylated IRF-3 and phosphorylated p65, with total p38 used as a loading control. Results shown are representative of two independent experiments.

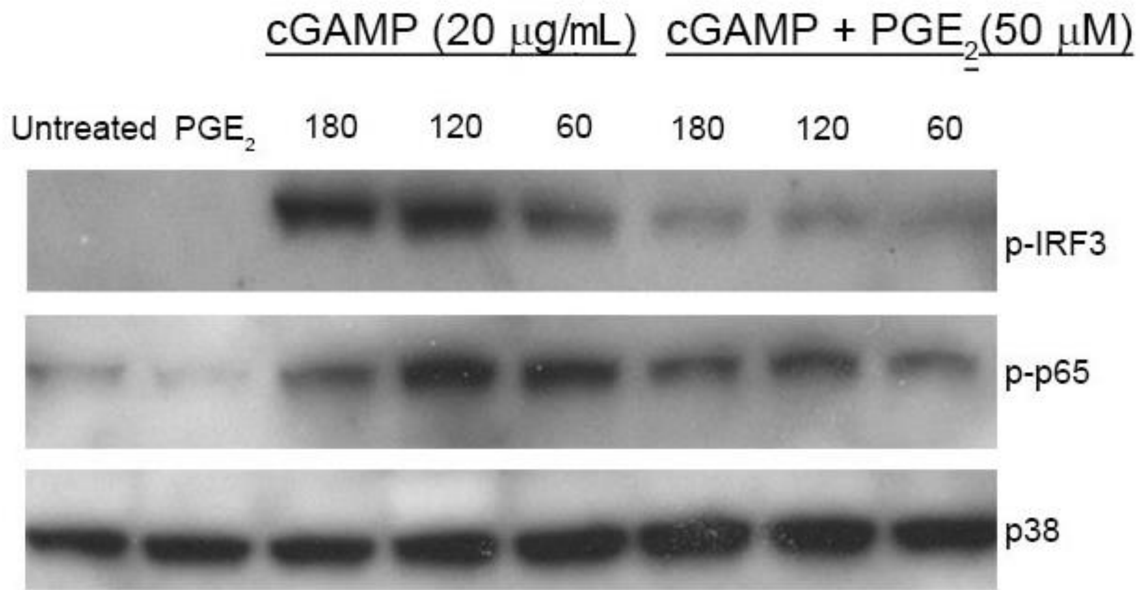


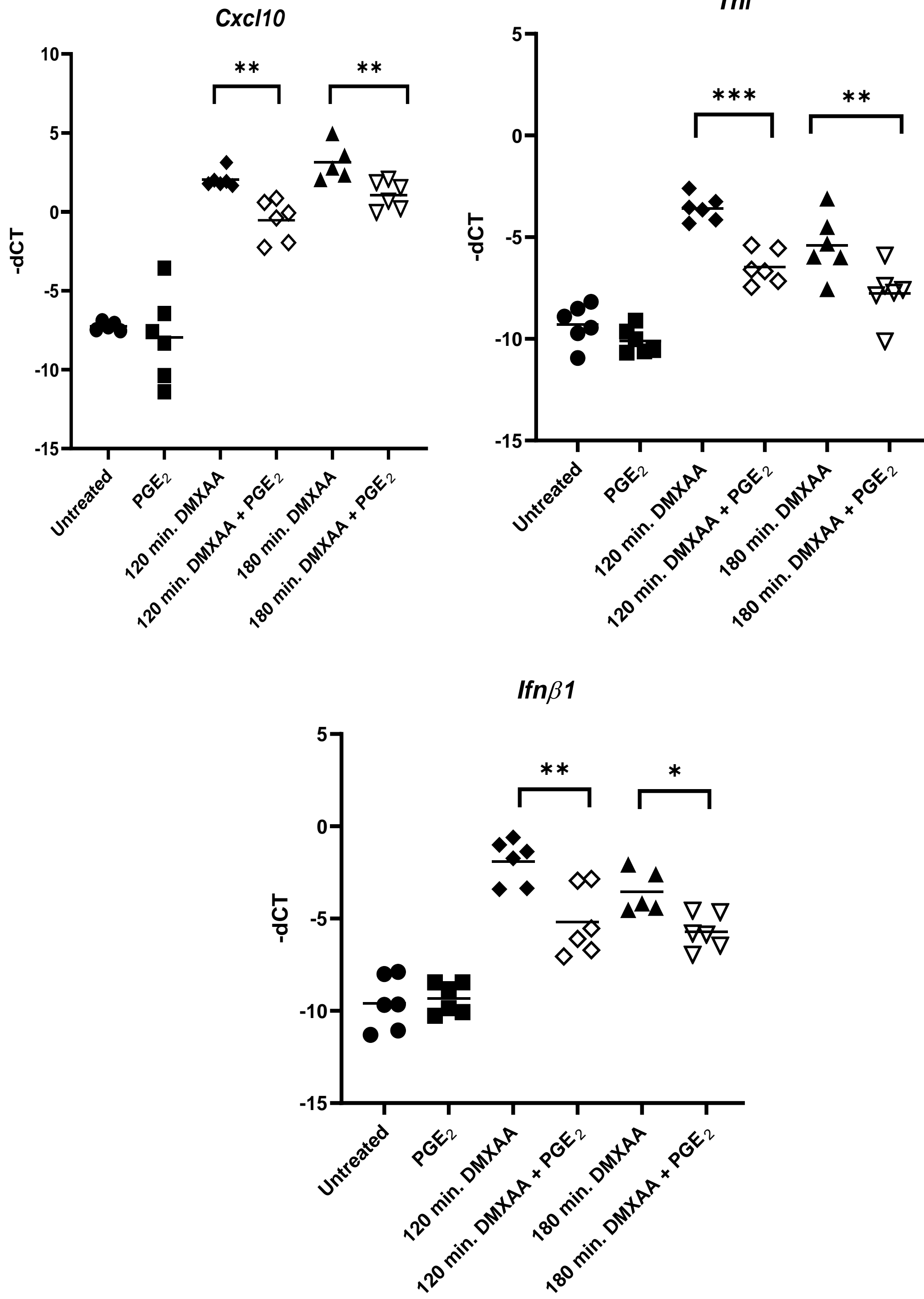
Figure 9. PGE_2 decreased signaling stimulated by cGAMP over a time course. Primary murine macrophages from C57BL/6J mice were pre-treated with PGE_2 (50 μM) or left untreated in media for 30 minutes prior to addition of cGAMP at 20 μg/mL for 180, 120, or 60 minutes. Cell lysates were collected for Western blot analysis for phosphorylated IRF-3 and phosphorylated p65, with total p38 used as a loading control. Results shown are representative of two independent experiments.

Subchapter 3.4: PGE_2 Treatment of Macrophages Blunts DMXAA- and cGAMP-induced STING-Dependent Gene Expression

The inhibition of STING-induced signaling in macrophages caused by exposure to PGE_2 led to the hypothesis that PGE_2 may also restrict STING-dependent transcriptional activation. To test this hypothesis, we utilized qRT-PCR to determine changes in IRF3-dependent gene expression. Macrophages were co-stimulated with DMXAA alone or in combination with PGE_2. Total RNA was isolated from treated cells and used for qRT-PCR analysis (Figure 10). Data were analyzed as -ΔCt (Figure 10A) and transformed as “fold induction” (Figure 10B). At both 120 min and 180 min of DMXAA stimulation, decreased induction of two IRF3-dependent transcripts, *Cxcl10* mRNA (the gene that encodes IP-10, an IFN-stimulated chemokine), and *Ifnb1* (the gene that encodes IFN-β) was observed in macrophages stimulated with DMXAA + PGE_2 compared to macrophages stimulated with DMXAA only. *Tnf* (that encodes TNF-α, a pro-inflammatory cytokine) mRNA was also diminished in cells treated with both DMXAA and PGE_2.

To confirm these findings, similar studies were conducted using cGAMP to activate STING, rather than DMXAA, to mimic the naturally occurring signaling that occurs within the signaling pathway. qRT-PCR was again used to measure downstream gene expression. Macrophages were co-stimulated cGAMP alone or in combination with PGE_2 at 4 or 6 hours. Total RNA was isolated from these cells for qRT-PCR analysis (Figure 11). At both 4 and 6 hours, there is reduced induction in *Cxcl10* and *Ifnb1*, as well as in *Tnf*, mRNA in macrophages stimulated with PGE_2 and cGAMP compared to cGAMP alone.

Figure 10. PGE_2 treatment reduces downstream DMXAA-induced gene expression. Murine primary macrophages from C57BL/6J mice were left untreated in media or stimulated with PGE_2 (50 µm) for 30 minutes prior to addition of DMXAA treatment (0.01 µg/ml) for an additional 120 or 180 min. Controls included cells that were untreated, treated with "DMXAA" only, or treated with "PGE_2" only, and the results were compared to "DMXAA + PGE_2" combined treatment at the indicated time point. Total RNA was isolated. qRT-PCR was conducted to measure the relative induction of *Cxcl10*, *Tnf*, and *Ifnβ1* mRNA, with *Gapdh* as the housekeeping gene.
A. -ΔCt expression for each gene, analyzed using one-way ANOVA with post-hoc analysis, revealed a significant decrease for each gene (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). **B.** The data in A were transformed to permit evaluation of "fold induction" for each gene examined (Mean $\pm$ SEM). Results shown are derived from 3 independent experiments.


A.
Cxcl10
**
**
-dCT
10
5
0
-5
-10
-15
Untreated
PGE2
120 min. DMXAA
120 min. DMXAA + PGE2
180 min. DMXAA
180 min. DMXAA + PGE2
Tnf
***
**
-dCT
5
0
-5
-10
-15
Untreated
PGE2
120 min. DMXAA
120 min. DMXAA + PGE2
180 min. DMXAA
180 min. DMXAA + PGE2
Ifnβ1
**
*
-dCT
5
0
-5
-10
-15
Untreated
PGE2
120 min. DMXAA
120 min. DMXAA + PGE2
180 min. DMXAA
180 min. DMXAA + PGE2

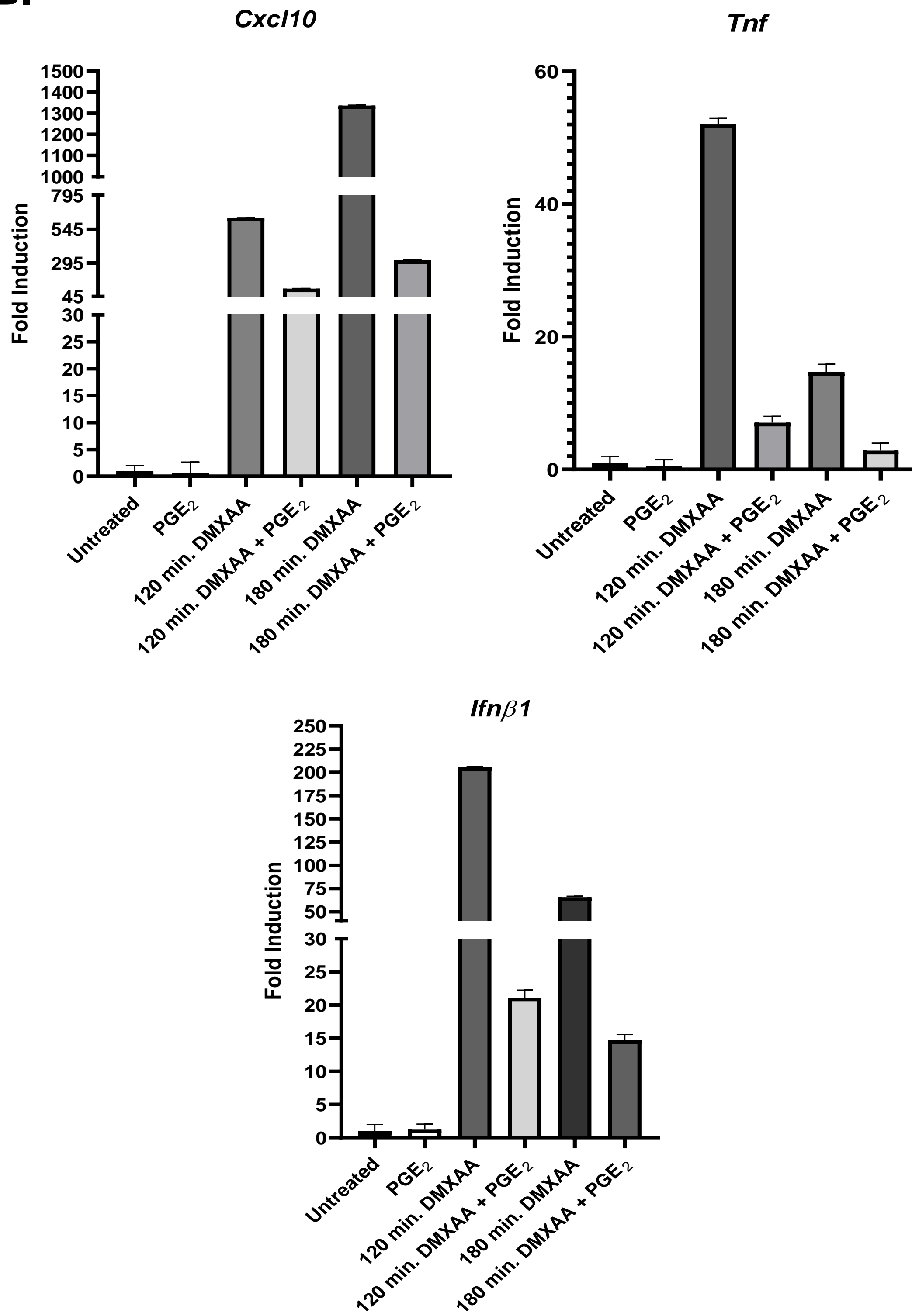
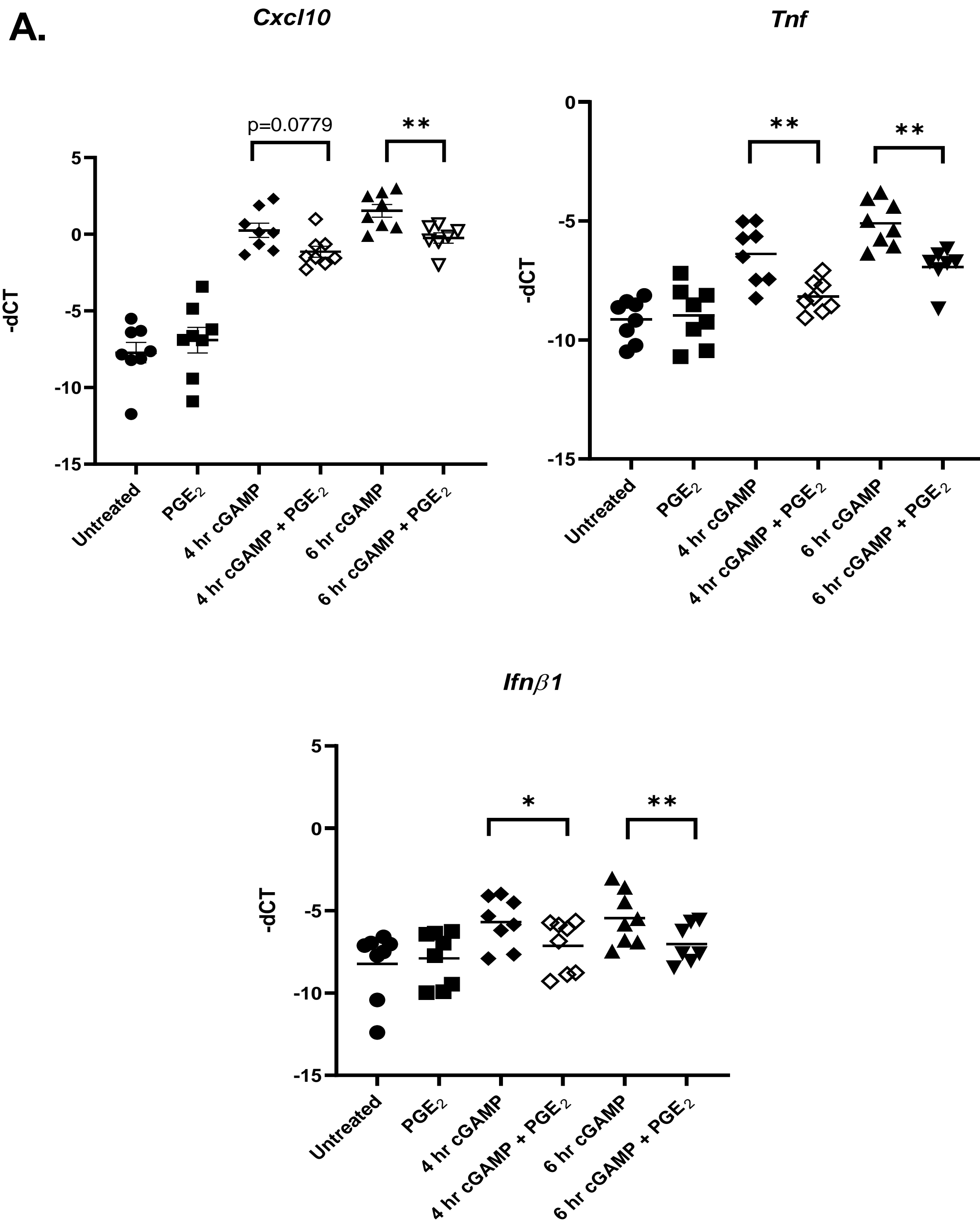
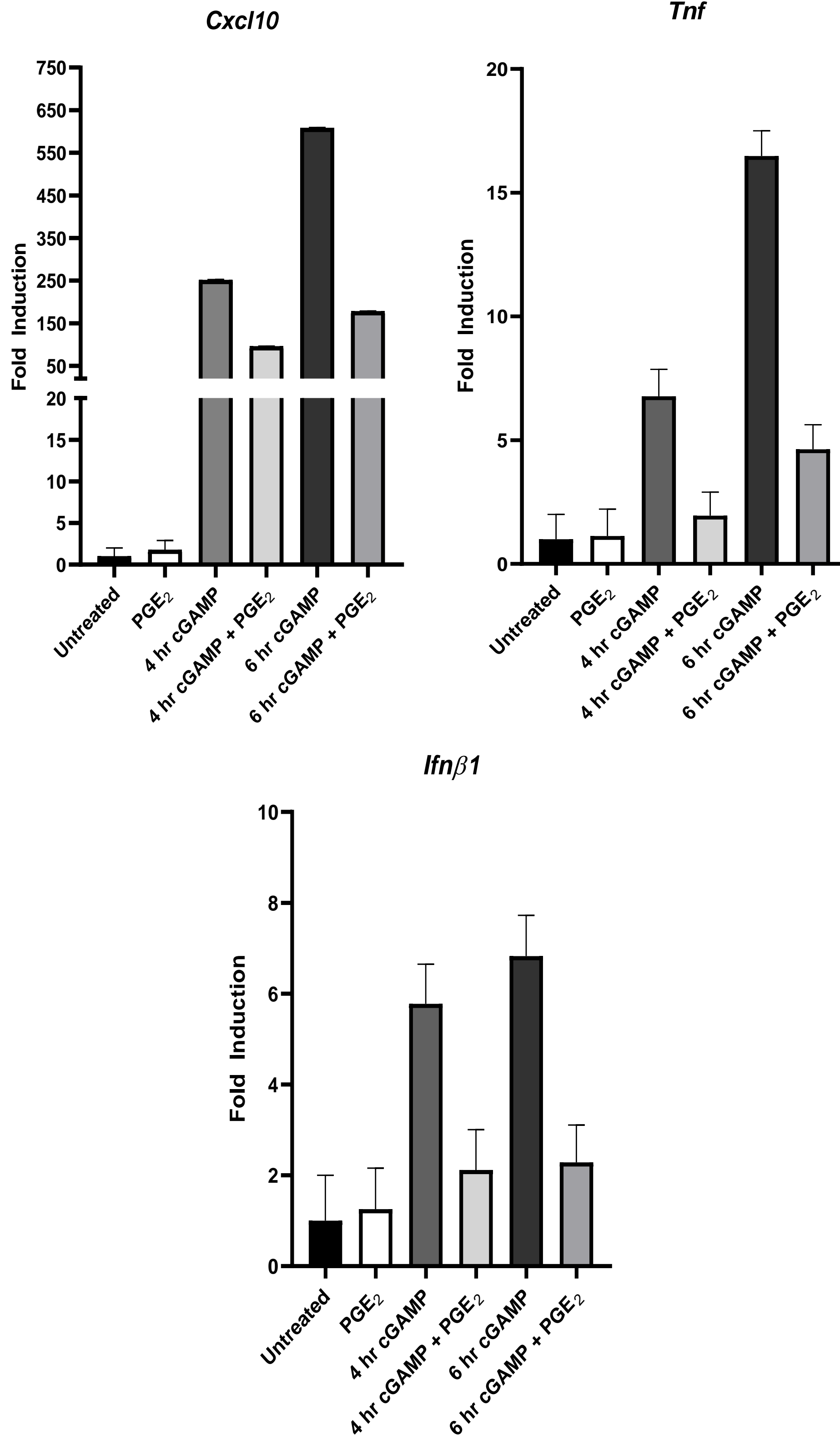

B.
Cxcl10
Fold Induction
1500
1400
1300
1200
1100
1000
795
545
295
45
30
25
20
15
10
5
0
Untreated
PGE2
120 min. DMXAA
120 min. DMXAA + PGE2
180 min. DMXAA
180 min. DMXAA + PGE2
Tnf
Fold Induction
60
40
20
0
Untreated
PGE2
120 min. DMXAA
120 min. DMXAA + PGE2
180 min. DMXAA
180 min. DMXAA + PGE2
Ifnβ1
Fold Induction
250
225
200
175
150
125
100
75
50
30
25
20
15
10
5
0
Untreated
PGE2
120 min. DMXAA
120 min. DMXAA + PGE2
180 min. DMXAA
180 min. DMXAA + PGE2

Figure 11. PGE_2 treatment reduces downstream cGAMP-induced gene expression. Murine primary macrophages from C57BL/6J mice were left untreated in media or stimulated with PGE_2 (50 µm) for 30 minutes prior to addition of cGAMP (0.01 µg/ml) for an additional 4 or 6 hours. Controls included cells that were untreated, treated with "cGAMP" only, or treated with "PGE_2" only, and the results were compared to "cGAMP + PGE_2" combined treatment at the indicated time point. Total RNA was isolated. qRT-PCR was conducted to measure the relative induction of *Cxcl10*, *Tnf*, and *Ifnβ1* mRNA, with *Gapdh* as the housekeeping gene. **A.** -*Δ*Ct for expression of each gene using one-way ANOVA with post-hoc analysis to compare cGAMP treatment alone vs. cGAMP + PGE_2 at each time point (*$p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$), except cGAMP + PGE_2 at 4 hrs for *Cxcl10* mRNA expression ($p = 0.0779$). **B.** The data in A were transformed to permit evaluation of "fold induction" for each gene examined (Mean $\pm$ SEM). Results are derived from 4 independent experiments.



A.
Cxcl10
Tnf
p=0.0779
**
**
**
-dCT
5
0
-5
-10
-15
Untreated
PGE2
4 hr cGAMP
4 hr cGAMP + PGE2
6 hr cGAMP
6 hr cGAMP + PGE2
Ifnβ1
*


B.
Cxcl10
Fold Induction
750
650
550
450
350
250
150
50
20
15
10
5
0
Untreated
PGE2
4 hr cGAMP
4 hr cGAMP + PGE2
6 hr cGAMP
6 hr cGAMP + PGE2
Tnf
Fold Induction
20
15
10
5
0
Untreated
PGE2
4 hr cGAMP
4 hr cGAMP + PGE2
6 hr cGAMP
6 hr cGAMP + PGE2
Ifnβ1
Fold Induction
10
8
6
4
2
0
Untreated
PGE2
4 hr cGAMP
4 hr cGAMP + PGE2
6 hr cGAMP
6 hr cGAMP + PGE2

Chapter 4: Discussion

Many of the components of the cGAS-STING signaling pathway have been highly studied and are well understood. It is well-established that in nature STING is activated by cytosolic dsDNA derived from pathogens or the host. Cytosolic dsDNA activates and binds to an enzyme, cGAS, that in turn, undergoes a conformational change enabling catalysis of ATP and GTP to form the cyclic dinucleotide cGAMP, which subsequently binds to STING (Figure 2). cGAMP-bound STING, in turn, recruits the serine/threonine kinase TBK1 and elicits inflammatory mediator production through the activation of the transcription factors, IRF3 and NF-κB. However, there still are many unknown aspects as to exactly how STING activation and inhibition are co-regulated to balance the immune response. Overactivation of the STING pathway can be caused by many factors, such as gain of function mutations in STING that can lead to constitutive IFN expression, ultimately causing autoimmune disorders [63], as well as contributing to the development of cancer, likely through increased angiogenesis [64]. Some studies show that negative regulation of STING signaling can occur during phosphorylation of a specific serine (S366) by Unc-51-Like Autophagy Activating Kinase 1 (ULK1), which increases degradation of STING and acts as one potential negative-feedback mechanism to inhibit overactive STING signaling [65,66].

Yet, viruses and other foreign pathogens are often able to evade innate immune detection by cGAS-STING and suppress immune response signaling. Pathogens like Kaposi's sarcoma-associated herpesvirus (KSHV) encode several proteins, including viral interferon regulatory factor 1 (vIRF1), that blocks TBK1 from interacting with STING and ultimately inhibits the immune response associated with STING activation

and signaling in the cGAS-STING pathway [67,68]. This raises the question of how the cGAS-STING signaling pathway successfully recognizes and acts upon harmful pathogens without being constantly overactivated by a pathogen's ability to evade detection and destruction.

The small lipid mediator PGE_2 can be produced in all cell types and triggers a variety of physiological responses when it interacts with PGE_2 specific, cell surface EP receptors present on most cells, often acting as a vasodilator to allow activated cells to reach the site of injury [69]. In hypertension, for example, PGE_2 has been shown to possess both positive and negative functions for maintaining homeostasis for regulating blood pressure [70]. The FDA has also approved a clinical version of PGE_2, known as Dinoprostone, for use in the preparation and promotion of labor. By acting through its various receptors, PGE_2 can modulate both inhibitory and amplifying effects on the uterus [71,72]. The EP1-4 receptors are all G-protein coupled receptors involved in numerous potential signaling pathways, which leads to various downstream outcomes, and can cause both supporting and opposing interactions within cells. Thus, it is possible that engagement of distinct EP receptors may lead to inhibitory versus amplifying activities.

PGE_2 has also been shown to play a role in promoting the progression of metastases in numerous cancers, such as colorectal, gastrointestinal, and lung cancer [73–75]. Studies have shown that signaling of PGE_2 via the COX-2-PGE_2-EP pathway can suppress immune cells while promoting tumor evasion and angiogenesis [76,77]. Yet, little research has focused on the potential role PGE_2 may exert on innate immune signaling to help limit overactivation of inflammatory responses. Previous work by Perkins et al. demonstrated a novel negative feedback loop in which PGE_2 inhibits TLR4 activation

through EP4 by inhibiting internalization of the TLR4/MD-2/CD14 complex and subsequent TRIF signaling in murine macrophages [44]. Based on this work, we formulated a hypothesis to examine that PGE_2 might also regulate the STING signaling pathway, which is functionally similar to TLR4 signaling in that it too activates both TBK1 and IRF3, leading to type I IFN production.

To begin to understand how PGE_2 might be playing a role in negatively regulating the STING pathway, we first looked at the levels of endogenous PGE_2, with and without STING activation by DMXAA, a STING agonist that acts by binding directly to murine STING, thereby obviating the necessity for upstream nucleic acid-cGAS-cGAMP-induced triggering of STING [62]. Both MEFS and macrophages stimulated with DMXAA exhibited a significant increase in secreted PGE_2 levels compared to cells that were unstimulated (Figure 5). This result is significant because it indicates, for the first time, a possible relationship between PGE_2 production and STING activity within these immune cells. PGE_2 is produced when arachidonic acid is released from the phospho-lipid bilayer of the cell membrane in response to inflammatory signals, and subsequently metabolized by an enzyme cascade involving COX-1 and COX-2 enzymes, leading to further catalysis and eventually the synthesis and rapid secretion of PGE_2 (Figure 3). The increased levels of secreted endogenous PGE_2 by the cells stimulated with DMXAA implies that DMXAA activation of STING must induce and/or activate these enzymes, leading to production of PGE_2.

Moving forward, we set out to analyze the effect of PGE_2 on the STING signaling response induced by DMXAA. Previous studies showed that STING is a direct receptor for DMXAA (in murine models only) and that binding of DMXAA by STING induces

phosphorylation of the kinase, TBK1, that, in turn, phosphorylates the transcription factor, IRF3 [62,78]. In both the MEFs and murine primary macrophages, we observed that pre-treatment of cells with PGE_2, for as little as 30 minutes, resulted in a significant decrease in DMXAA-induced phosphorylation of both TBK1 and IRF3 in Western blots (Figures 6 and 7). We also observed that longer PGE_2 pre-treatment times resulted in a more striking inhibition of DMXAA-induced signaling as evidenced by the level of diminished expression of phosphorylation of TBK1 and IRF3 (Figure 7). Signaling induced by treatment of cells with exogenous cGAMP, the natural upstream activator of STING, showed similar findings (Figures 8 and 9), and strongly supports our hypothesis that induced PGE_2 feedback plays an active role in negatively regulating STING signaling.

To determine the effect of PGE_2 on downstream gene expression induced by STING signaling, we assayed expression of *Ifnb1* and *Cxcl10* (genes that encode IFN-β and IP-10, respectively), both of which are known to be dependent on phosphorylated TBK1 and IRF3 for their induction, and *Tnf* (encodes TNF-α, a pro-inflammatory cytokine that is NF-κB-dependent) [79] using qRT-PCR. Our results demonstrate that both DMXAA and cGAMP-stimulated mRNA was diminished significantly for all three genes by the presence of PGE_2. It is important to note that in the studies by Perkins et al. [44], the MyD88 signaling pathway induced by Gram negative LPS, which activates NF-κB primarily, was not inhibited by treatment of macrophages with PGE_2, but rather, PGE_2 selectively inhibited the TRIF-mediated signaling pathway.

Our observation that both NF-κB and IRF3 activation by STING-induced signaling is in contrast with the selective inhibition of the TRIF signaling pathway by PGE_2 published by Perkins et al. (42), suggests that PGE_2 mediates suppression of STING activation by a distinct mechanism rather than PGE_2-mediated suppression of TRIF-mediated signaling. We found that PGE_2 suppressed the activating phosphorylation of TBK1 (Figures 6 and 7), as well as the phosphorylation of p65 (Figures 8 and 9), both of which are involved with NF-κB signaling. PGE_2 also suppressed NF-κB-dependent *Tnf* gene expression when co-incubated with DMXAA and cGAMP (Figures 10 and 11). While Perkins et al. found that PGE_2 blocked LPS-induced TRIF signaling by preventing internalization of the TLR4/MD-2/CD14 complex [44], our data show that the negative regulation of downstream STING signaling in both NF-κB and IRF3 signaling by PGE_2 could possibly result from a mechanism that alters the function of the STING protein by a PGE_2-mediated signaling intermediate. When cGAMP or DMXAA directly binds to STING, the STING protein translocates from ER to the Golgi apparatus and then phosphorylates TBK1 for downstream signaling. One might speculate that PGE_2-induced signaling also alters the translocation of the STING protein from the ER to the Golgi apparatus. Much of the work in this study focused on the downstream effects of PGE_2 on the STING signaling pathway. Further investigation of what role, if any, PGE_2 might play on the upstream factors that lead to STING activation would provide a clearer insight into how the entire STING pathway is affected.

PGE_2 utilizes any of four specific cell surface receptors, known as EP1-EP4, which are G-protein coupled receptors that, with assistance from MRP4, transport PGE_2 in and out of cells [80]. Elevated levels of certain EP receptors are often present during

tumor development and other diseases [81]. We attempted to determine the requirement for each of the four EP receptors in the suppressive actions of PGE_2 to clarify how PGE_2 might be involved in negatively regulating STING signaling. Although we tried in MEFs to block each of the EP receptors pharmacologically during PGE_2 pre-treatment and stimulation with DMXAA, the results were inconsistent from blot to blot with regard to which EP receptor(s) blunted signaling induced by PGE_2 + DMXAA. There were also significant issues with the health of the MEFs at this point that could have contributed to an inability to attain a clear conclusion. These inconsistent results could also be due to the fact that the EP receptors are involved in many different signaling pathways with varying downstream effects, all of which depend on the cell type and binding affinity [82]. EP1 has shown to play a role in calcium regulation, although the exact mechanisms are still unclear, while EP2 and EP4 both are involved with regulating cGAMP concentrations [83]. EP3 is known to exist in multiple isoforms, in which each isoforms perform countless signaling roles, further leading to the intricate web of potential pathways and downstream effects resulting from the utilization of this receptor [83]. In the studies by Perkins et al. [44], EP4 was found to be exclusively involved in the inhibition of LPS-stimulated TRIF signaling. Future studies that explore in depth the upstream interactions of the EP receptors with PGE_2 might further elucidate how PGE_2 plays a role in negatively regulating downstream STING signaling.

Nonetheless, our data have begun to elucidate a novel mechanism by which STING-mediated inflammatory responses are down-regulated. Our data revealed that STING signaling led to the release of PGE_2 from cells (Figure 5), supporting the role of PGE_2 as a possible negative regulator. Future studies with MEFs and primary murine

macrophages to identify if endogenous secreted PGE_2 re-enters the cell and/or signals through one or more specific EP receptors is expected to help define additional signaling pathways that interrupt STING signaling. The data presented herein support the concept that the inhibitory effect of PGE_2 is downstream of cGAMP activation of STING, suggesting that either the translocation of STING or its downstream signaling via TBK1 are the more proximal targets of the observed inhibition.

Chapter 5: Conclusion

With over 80 known autoimmune disorders in humans and a nearly 40% expectancy for Americans to be diagnosed with cancer at some point in their lifetime, there is high demand for the development of treatments and cures for these illnesses [84,85]. While DMXAA was intended to be used to inhibit tumor progression through activation of the STING signaling pathway, the drug has shown little efficacy in human models. However, the results from our study, as well as those from Perkins et al., demonstrate that PGE_2 has restrictive capacities within signaling pathways and the ability to regulate these pathways and, therefore, could serve as an alternative potential therapeutic target in controlling these illnesses. The ability to manipulate and target this naturally occurring prostaglandin could eliminate the need for outside drugs and provide a more organic method of regulating immune pathways possibly through amplifying natural STING responses in tumors. But, it should also be realistically approached with the understanding that PGE_2 is involved in a vast number of pathways, and the role it plays needs to be more clearly defined, especially in terms of upstream STING activation to obtain full knowledge of how it can be used as a tool to combat autoimmune disease and regulate innate immune pathways.

The results presented here provide strong evidence that PGE_2 can be utilized to restrict signaling within innate immune pathways. With further investigation, PGE_2 very well could be the major target for how we manage signaling pathways to induce the ideal immune response in those affected by autoimmune disorders.

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