Extracellular cyclic dinucleotides induce polarized responses in barrier epithelial cells by adenosine signaling

Denis Chang[a], Aaron T. Whiteley[b], Katlynn Bugda Gwilt[a], Wayne I. Lencer[a,c], John J. Mekalanos[c,d,1], and Jay R. Thiagarajah[a,c,1]

[a]Division of Gastroenterology, Hepatology and Nutrition, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115; [b]Department of Biochemistry, University of Colorado Boulder, Boulder, CO 80309; [c]Harvard Digestive Disease Center, Harvard Medical School, Boston, MA 02115; and [d]Department of Microbiology, Harvard Medical School, Boston, MA 02115

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Cyclic dinucleotides (CDNs) are secondary messengers used by prokaryotic and eukaryotic cells. In mammalian cells, cytosolic CDNs bind STING (stimulator of IFN gene), resulting in the production of type I IFN. Extracellular CDNs can enter the cytosol through several pathways but how CDNs work from outside eukaryotic cells remains poorly understood. Here, we elucidate a mechanism of action on intestinal epithelial cells for extracellular CDNs. We found that CDNs containing adenosine induced a robust CFTR-mediated chloride secretory response together with cAMP-mediated inhibition of Poly I:C-stimulated IFNβ expression. Signal transduction was strictly polarized to the serosal side of the epithelium, dependent on the extracellular and sequential hydrolysis of CDNs to adenosine by the ectonucleosidases ENPP1 and CD73, and occurred via activation of A_{2B} adenosine receptors. These studies highlight a pathway by which microbial and host produced extracellular CDNs can regulate the innate immune response of barrier epithelial cells lining mucosal surfaces.

cyclic dinucleotide | intestine | epithelial | adenosine

Cyclic dinucleotides (CDNs) were originally discovered as bacterial second messengers that play a central role in critical bacterial processes, including virulence, motility, metabolism, and survival (1). CDNs consist of two nucleotide monophosphates interlinked by phosphodiester bonds to form a cyclic structure (1). Well-known examples of important bacterial CDNs include cGMP-GMP (c-di-GMP), cAMP-AMP (c-di-AMP), and 3′3′ cGMP-AMP (3′3′ cGAMP). Mammalian cells also produce a CDN; however, unlike bacterial CDNs which have two 3′–5′ bonds, they produce 2′–5′/3′–5′ cGMP-AMP (2′3′ cGAMP). Synthesis of 2′3′ cGAMP occurs by the cytosolic enzyme cGMP-AMP synthase (cGAS), upon detection of mislocalized or microbial DNA (2). Subsequently, 2′3′ cGAMP activates the endoplasmic reticulum-associated transmembrane protein STING (stimulator of IFN gene), resulting in the production of type I IFN and a potent innate immune response (3). Although bacterial CDNs can also activate STING, 2′3′ cGAMP binds with a greater affinity (4) and is therefore considered a key messenger in detecting pathogen DNA and activation of the host cell antiviral response.

The diversity of biologically active CDNs and their proposed roles in both microbial and host physiology have rapidly expanded over the past few years. A CDN target protein, the oxidoreductase RECON (reductase controlling NF-κB), was recently identified (5) and found to bind specifically to bacterial CDNs with subsequent action on NF-κB signaling. Unlike specific bacterial CDNs, host 2′3′ cGAMP does not bind RECON (5). More recently, a number of bacterial CDNs were discovered including the pyrimidine-containing CDN, cyclic UMP-AMP (cUA), as well as cyclic trinucleotides, such as cAMP-AMP-GMP (cAAG) (6). Functional studies suggested that these CDNs can signal through the RECON pathway, expanding the range of bacterial CDNs capable of impacting host responses.

Although the host signaling mechanisms involved in CDN action inside cells via activation of the STING pathway in the innate immune response have been widely explored (2, 4, 7, 8), the pathways involved in the biological activity of extracellular CDNs remain a new and evolving field. A number of lines of evidence suggest that mammalian cells release (9) or secrete (10) CDNs into the extracellular environment positioning CDNs as potentially important paracrine or autocrine signaling molecules. Recent studies suggest that extracellular 2′3′ cGAMP can be transported into or between cells by specific pathways including via the folate transporter SLC19A1 (11, 12), gap junctions (13), endocytosis (9), or volume-activated LRRC8A anion channels (14).

The gastrointestinal tract is a unique environment where host cells and the surrounding microbial environment exist in close proximity, constantly interfacing via a single layer of barrier epithelial cells. Although the ability of intestinal epithelial cells to respond to many extracellular pathogen- or danger-associated molecular pattern molecules (PAMPs and DAMPs) such as LPS or TNFα are well described (15, 16), there are little data on their ability to detect and/or respond to extracellular CDNs. Here, in human colon epithelial cells, we find that both bacterial and

Significance

Cyclic dinucleotides (CDNs) are important signaling molecules that are involved in many microbial processes and in the host cell response to intracellular pathogens. Intracellular CDN signaling is mediated by well-described sensor proteins; however, much less is known about how CDNs signal in the extracellular environment. Here we discover, in intestinal epithelial cells, that extracellular CDNs are hydrolyzed by enzymes present in the cell membrane to form adenosine and activate cell-surface adenosine receptors. This stimulates epithelial chloride secretion and inhibits cellular antiviral responses. Signaling originates exclusively from the serosal tissue-facing side of the epithelium. Our study implicates adenosine signaling as an important mechanism by which extracellular CDNs can modulate host defense at mucosal surfaces.

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[1]To whom correspondence may be addressed. Email: jay.thiagarajah@childrens.harvard.edu or john_mekalanos@hms.harvard.edu.

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mammalian extracellular CDNs induce rapid and polarized ion secretion. The action of extracellular CDNs in this context occurs extracellularly and independent of the canonical intracellular CDN recognition pathways involving STING or RECON. Rather, signal transduction occurs through extracellular hydrolysis of CDNs to adenosine via the ectonucleotidases ENPP1 and CD73, followed by activation of the adenosine A_{2B} receptor.

Results

Extracellular Host and Bacterial Cyclic Dinucleotides Induce Polarized Responses in Intestinal Cells. To measure the effect of extracellular CDN on transepithelial ion transport, polarized human colonic cells were grown as a monolayer on porous inserts. Extracellular CDNs were added to either the apical or basolateral compartment and short-circuit current (Isc) was measured.

To assess whether CDNs affect epithelial ion transport responses, we initially tested the mammalian CDN, 2′3′ cGAMP, which is synthesized by a variety of host cells (3). CDNs were applied at micromolar concentrations as suggested by previous studies (9, 10, 17). We found that 2′3′ cGAMP added to the apical surface did not elicit any changes in short-circuit current (Fig. 1 *A* and *B*). In contrast, basolateral 2′3′ cGAMP resulted in an increase in short-circuit current within seconds (Fig. 1 *A* and *B*). This response was dose dependent (EC_{50} = 4.3 μM) with a response seen with doses as low as 100 nM (Fig. 1*B*).

To test whether polarization of the short-circuit current signal is specific to host CDNs or if bacterial CDNs result in a similar response, we applied the canonical bacterial CDNs c-di-AMP, c-di-GMP, and 3′3′ cGAMP. c-di-GMP is produced by diverse bacteria, whereas c-di-AMP is associated with mainly gram-positive bacteria (1). *Vibrio cholerae* (1) is a major source of 3′3′ cGAMP and it differs structurally from 2′3′ cGAMP by the presence of two 3′–5′ phosphodiester bonds. Similar to the polarized response elicited by 2′3′ cGAMP, both c-di-AMP and 3′3′ cGAMP caused a robust increase in short-circuit current only when added basolaterally (Fig. 1*C*). In contrast, c-di-GMP did not induce any current change either apically or basolaterally (Fig. 1*C*). Basolateral CDN-induced currents were reflective of classical cystic fibrosis transmembrane conductance regulator (CFTR)-mediated chloride secretion as shown by the dose-dependent and near-complete inhibition of responses by the CFTR inhibitor, $CFTR_{inh}$-172 (Fig. 1*D*).

Extracellular CDN-Induced Chloride Secretion in Colonic Epithelial Cells Is STING Independent. Recent studies exploring bystander cell signaling via 2′3′ cGAMP, notably in the context of tumor cells, have shown a number of transport pathways that enable CDNs to enter the cytosol, activate STING, and promote subsequent responses (11, 12, 18). Given these results, we investigated whether extracellular CDN-mediated chloride secretion, in intestinal epithelial cells, may be mediated via a STING-dependent pathway. Recent studies (6) have shown that the bacterial cyclic trinucleotide cAAG and the pyrimidine containing CDN cUA are exclusively agonists for the RECON pathway, in contrast to 2′3′ cGAMP which signals exclusively via STING (5) (Fig. 2*A*). Basolateral administration of cAAG produced a similar response and dose dependency to 2′3′ cGAMP implicating a common pathway of signal transduction, suggesting that neither the STING nor RECON pathways are likely involved (Fig. 2*B*). To test this interpretation, we used H-151, a small molecule inhibitor of STING (19), followed by basolateral 2′3′ cGAMP administration. In this study, the eukaryotic 2′3′ cGAMP was used as it has a much stronger binding affinity to STING (K_d ~4 nM) than bacterial CDNs (3). STING inhibition with H-151 (Fig. 2*C*) was found to have no effect on extracellular 2′3′ cGAMP-induced chloride secretion. To test this another way, we used the linearized form of

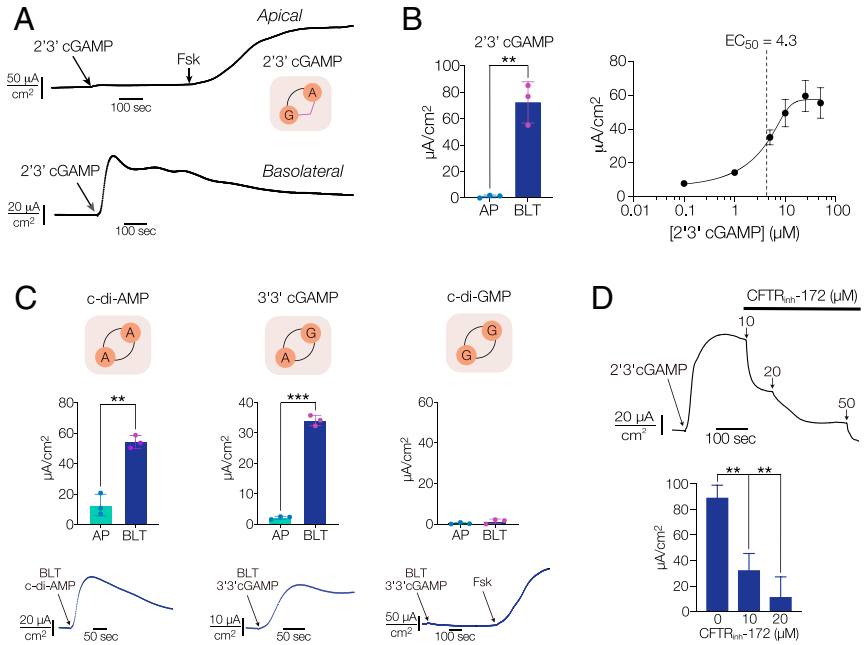

Fig. 1. Extracellular CDNs induce polarized chloride secretion in T84 cells. (*A*) Short-circuit current (Isc) tracings following application of 2′3′ cGAMP either in the apical (*Top*) or basolateral (*Bottom*) compartment of T84 cells. Forskolin (Fsk) (20 μM) was applied as indicated. (*B*) Maximal ΔIsc following addition of apical or basolateral 2′3′ cGAMP (20 μM). Error bars represent means ± SD, n = 3 (*Left*). Dose–response for basolateral 2′3′ cGAMP. Error bars represent means ± SEM, $n \geq 3$ (*Right*). (*C*) Maximal ΔIsc and short-circuit current tracings for c-di-AMP (*Left*), 3′3′ cGAMP (*Middle*), and c-di-GMP (*Right*). All CDNs were used at a concentration of 20 μM. Fsk (20 μM) was applied as indicated. Error bars represent means ± SD, n = 3. (*D*) Isc tracings following application of 2′3′ cGAMP (20 μM) in the basolateral compartment of T84 cells, followed by addition of the CFTR inhibitor, $CFTR_{inh}$-172, at the concentrations indicated (*Top*). Maximal ΔIsc for basolateral 2′3′ cGAMP (20 μM) followed by dose escalation of $CFTR_{inh}$-172 (*Bottom*). Error bars represent means ± SD, n = 3. $^{**}P < 0.01$, $^{***}P < 0.001$.

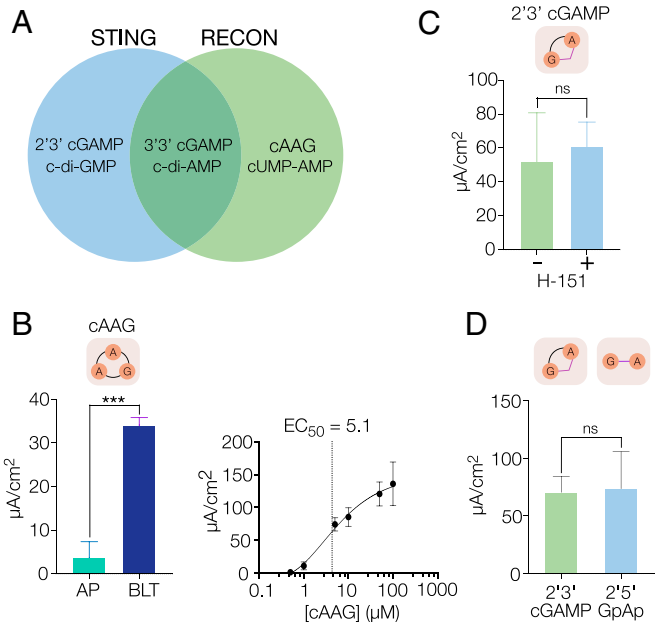

Fig. 2. Extracellular CDN-induced chloride secretion is STING independent. (*A*) Schematic of CDN interaction with STING or RECON. (*B*) Maximal ΔIsc following addition of apical or basolateral cAAG (20 μM). Error bars represent means ± SD, $n = 3$ (*Left*). Dose–response for basolateral cAAG. Error bars represent means ± SEM, $n \geq 3$ (*Right*). (*C*) Maximal ΔIsc following addition of basolateral 2′3′ cGAMP (20 μM) ± STING inhibitor H-151 (10 μM). Error bars represent means ± SD, $n = 3$. (*D*) Maximal ΔIsc following addition of basolateral 2′5′ GpAp (20 μM) (linearized 2′3′ cGAMP). Error bars represent means ± SD, $n = 3$. ***$P < 0.001$, ns, nonsignificant.

2′3′ cGAMP, 2′5′-GpAp. STING activation requires the circularized form of 2′3′ cGAMP; and the linearized form 2′5′-GpAp is not active (20). Induction of the short-circuit current response by the linearized 2′5′-GpAp was equivalent to the Isc induced by 2′3′ cGAMP (Fig. 2*D*). Therefore, extracellular CDNs induce chloride secretion independently of intracellular STING or RECON activation.

Polarized Epithelial Responses to Extracellular CDNs Occur via Membrane Adenosine Receptors and Require Hydrolysis by ENPP1 and CD73. Our finding that induction of chloride secretion only occurs upon application of adenine-containing dinucleotides led to the hypothesis that extracellular CDN responses may be mediated via cell-surface adenosine signaling. Adenosine is an extracellular signaling molecule involved in a wide array of pathways in all tissues (21). Extracellular adenosine binds to and activates any one of several isoforms (A_1AR, A_{2A}AR, A_{2B}AR, or A_3AR) of the adenosine receptor (21). A_{2B}AR is the predominant isoform expressed in the colon (22). Activation of the A_{2B} receptor results in an increase in intracellular cAMP, which subsequently activates protein kinase A resulting in the activation of CFTR channels and chloride secretion (23). There are a number of hydrolysis pathways that can lead to the production of adenosine at cell surfaces. ATP and 5′-AMP, which can be produced during inflammation or hypoxia (24), can be hydrolyzed by two cell-surface ectonucleotidases, CD39 and CD73, resulting in the formation of adenosine (25) (Fig. 3*A*). We therefore investigated whether the polarized responses to CDNs may be mediated by cell-surface adenosine receptor signaling.

Both apical and basolateral administration of adenosine caused a robust increase in short-circuit current (Fig. 3*B*). We used the A_{2B}AR-specific inhibitor, PSB603, to test whether adenosine-induced currents were due to activation of A_{2B} receptors in our cell monolayers. Addition of PSB603 resulted in significant inhibition of the adenosine-induced current both apically and basolaterally (Fig. 3*B*). PSB603 also strongly inhibited currents induced by basolateral addition of 2′3′ cGAMP, 3′3′ cGAMP, and cAAG (Fig. 3*B*, *Right*). To confirm that the inhibitor did not have unintended inhibition of the CFTR channel or nonspecific toxicity, cells were subsequently treated with forskolin, which induces increases in cAMP via direct activation of adenylate cyclase (therefore bypassing A_{2B}AR) (26). In all cases, forskolin induced a robust increase in Isc (*SI Appendix*, Fig. S1).

These results suggest that either hydrolysis of CDNs to adenosine by nucleosidases or direct action of CDNs on the adenosine receptor is responsible for activation of epithelial chloride secretion. We therefore investigated the likely enzymes that could hydrolyze extracellular CDNs in the intestine. CDNs are comprised of at least two nucleotides bound by a 3′–5′ or 2′–5′ phosphodiester bond (1) (Fig. 3*A*). Intestinal cells express the ectonucleotidase CD73, which is required for the hydrolysis of 5′-AMP to adenosine (27), and also for the linear dinucleotide, diadenosine tetraphosphate (Ap4a), which is found in both bacterial and mammalian cells (28). Both apical and basolateral 5′-AMP induce robust increases in short-circuit current (Fig. 3*C*). The stimulatory effect of 5′-AMP on short-circuit current can be blocked by the CD73 inhibitor, α,β-methylene adenosine diphosphate (APCP) (27), confirming that hydrolysis of 5′-AMP to adenosine is a required step, and that APCP had no effect on adenosine-mediated stimulation (Fig. 3*C*). Ap4a is also known to be hydrolyzed to 5′-AMP (29) and ultimately to adenosine by the action of CD73 (27). Addition of basolateral Ap4a elicited a robust current that was fully inhibited by APCP. Currents induced by 2′3′ cGAMP were similarly abolished by APCP (Fig. 3*C*), suggesting that hydrolysis by CD73 is required for extracellular CDN-induced stimulation of epithelial chloride secretion.

To confirm that hydrolysis is required for signal transduction by the extracellular CDNs, we used a nonhydrolyzable form of 2′3′ cGAMP, 2′3′ cGsAsMP. This analog contains two phosphothioate diester linkages that are resistant to enzymatic hydrolysis and thus confers increased stability of the CDN (30). Consequently, 2′3′ cGsAsMP is a more potent activator of STING compared to 2′3′ cGAMP (30). In contrast to 2′3′ cGAMP, basolateral addition of 2′3′ cGsAsMP led to minimal increases in current, implicating hydrolysis as a necessary step (Fig. 3*D*).

We next sought to identify the phosphodiesterase involved in the initial hydrolysis of the phosphodiester bond present in CDNs. Although there are several phosphodiesterases known to degrade 3′–5′ phosphodiester bonds (30), ENPP1 is the only known eukaryotic hydrolase that acts to degrade the 2′–5′ bonds present in CDNs (30). ENPP1 is widely expressed in a variety of cell types and tissues including in the intestine (31). To test the involvement of ENPP1 in CDN-mediated stimulation of epithelial cells, we used a recently validated ENPP1-specific inhibitor, STF-1084 (18). Upon treatment with STF-1084, there was significant inhibition of the current change previously seen with 2′3′ cGAMP and 3′3′ cGAMP (Fig. 3*E*). To confirm that STF-1084 did not impact CD73 action, adenosine receptors, or CFTR directly, we tested 5′-AMP-mediated stimulation, which was unchanged in the presence of STF-1084 as expected (*SI Appendix*, Fig. S1). Taken together, these findings suggest that extracellular adenine containing CDNs are hydrolyzed to adenosine via the sequential action of ENPP1 and CD73 in intestinal epithelial cells.

Polarized Epithelial Antiviral Responses Are Modulated by Extracellular CDNs. Conventional cytosolic STING-mediated signaling by CDNs results in IFN stimulation. We therefore wondered whether extracellular CDNs may also potentiate IFNs in intestinal epithelial cells. Stimulation of the pattern-recognition receptor toll-like receptor 3 (TLR3) in intestinal epithelial cells by the canonical ligand polyinosinic:polycytidylic acid (Poly I:C) results in an increased expression of type I IFNs (32). As expected, basolateral

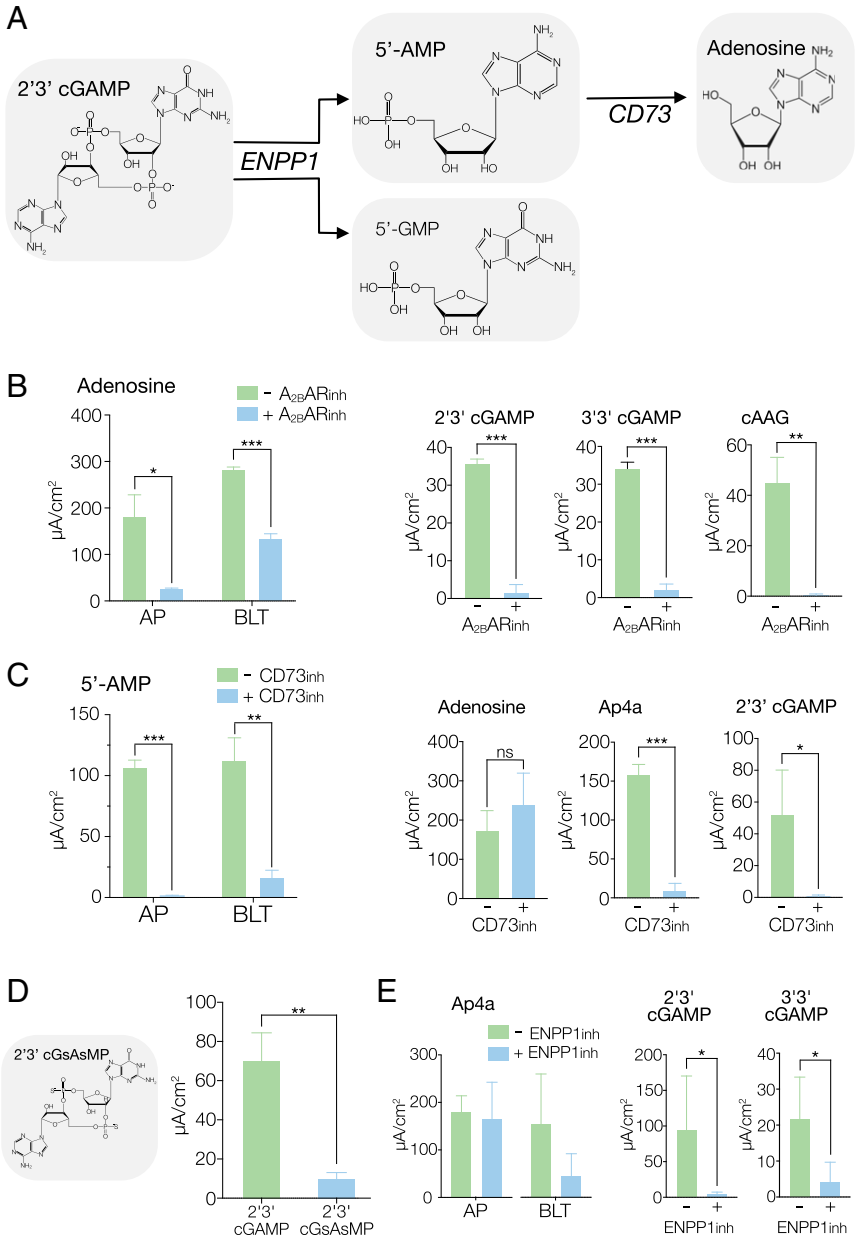

Fig. 3. Extracellular CDNs are hydrolyzed to adenosine via ENPP1 and CD73. (*A*) Schematic of hydrolysis of 2′3′ cGAMP to adenosine. (*B*) Maximal ΔIsc following addition of apical or basolateral adenosine (20 μM) ± A_{2B}AR inhibitor, PSB603 (10 μM) (*Left*). Basolateral 2′3′ cGAMP, 3′3′ cGAMP, and cAAG (20 μM each) ± PSB603 (10 μM) (*Right*). Error bars represent means ± SD, $n = 3$. (*C*) Maximal ΔIsc following addition of apical or basolateral 5′-AMP (20 μM) ± CD73 inhibitor, α,β-methylene adenosine diphosphate (APCP) (1 mM) (*Left*). Basolateral adenosine, Ap4a, and 2′3′ cGAMP (20 μM each) ± APCP (1 mM) (*Right*). Error bars represent means ± SD, $n = 3$. (*D*) Structure of nonhydrolyzable 2′3′ cGAMP (2′3′ cGsAsMP) (*Left*). ΔIsc following apical or basolateral 2′3′ cGsAsMP (20 μM) (*Right*). Error bars represent means ± SD, $n = 3$. (*E*) Maximal ΔIsc following addition of apical or basolateral Ap4a, basolateral 2′3′ cGAMP, and basolateral 3′3′ cGAMP (20 μM each) ± ENPP1 inhibitor, STF-1084 (10 μM). Error bars represent means ± SD, $n = 3$ to 5. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns, nonsignificant.

addition of Poly I:C induced a robust up-regulation of the IFN response as measured by increased expression of IFNβ (Fig. 4*A*). Extracellular CDNs by themselves did not induce any initial IFNβ expression; however surprisingly, the Poly I:C-stimulated IFN response was significantly inhibited either by concurrent addition of 2′3′ cGAMP, cAAG, or adenosine itself (Fig. 4*A*). Regulation of IFNβ expression by extracellular CDNs requires hydrolysis to adenosine, as shown by elevated expression with concurrent inhibition of ENPP1, and likely mediated by cAMP, as significant inhibition was also seen following addition of the direct adenylate cyclase agonist forskolin (Fig. 4*A*).

Discussion

In this study, we report a mechanism for signaling by extracellular cyclic dinucleotides in intestinal epithelial cells (Fig. 4*B*). Our findings reveal that hydrolysis and subsequent activation of adenosine receptors by extracellular CDNs encountering the serosal (basolateral) surface of barrier epithelial cells may operate importantly in innate defense of mucosal surfaces. Signal transduction proceeds independently of the canonical cytosolic binding partners of CDNs—STING and RECON—and the pathway may be generally important for signaling by CDNs in other cell types throughout the body (Fig. 4*B*).

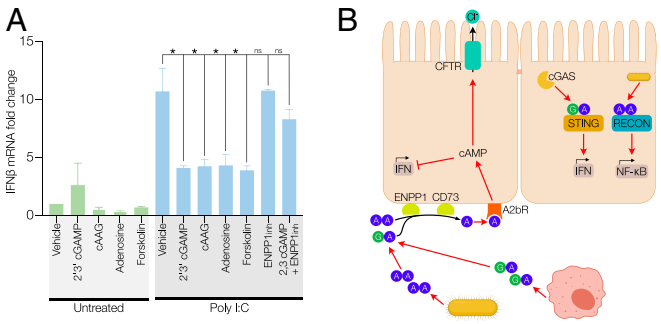

Fig. 4. Extracellular CDN-induced adenosine signaling inhibits epithelial IFN responses. (*A*) Normalized IFNβ expression in polarized T84 cells following basolateral administration of Poly I:C (10 μg/mL) with addition of 2′3′ cGAMP (14 μM), cAAG (10 μM), adenosine (20 μM), forskolin (20 μM), and ENPP1 inhibitor, STF-1084 (20 μM) as indicated. Error bars represent means ± SEM, $n = 3$. $^*P < 0.05$, ns, nonsignificant. (*B*) Summary schematic showing differential signaling in barrier epithelial cells between extracellular (*Left*) and intracellular CDNs (*Right*). Extracellular CDNs generated by either microbes or host immune cells are hydrolyzed to adenosine by cell-surface enzymes. Adenosine binds and activates adenosine receptors (A_{2B} in the case of colonic cells) and induces increased cytosolic cAMP which activates chloride secretion and alters agonist-induced IFN expression. In contrast, intracellular CDNs, either endogenous generated by the host cell via cGAS or produced by intracellular pathogens, activate the canonical sensors STING and RECON, leading to different downstream effects on host immune responses.

Cyclic dinucleotides serve critical intracellular functions in both bacterial (1) and mammalian cells (3). Within bacterial cells, CDNs are second messengers that regulate diverse processes including motility, biofilm formation, and pathogenesis (1, 33) as well as programmed cell death via the allosteric activation of toxic enzymes (34–37). CDNs are also known to act extracellularly in bacterial cells to modulate interkingdom environmental signaling (10). This is exemplified by the pathogen *Listeria monocytogenes* where secreted c-di-AMP is critical for growth and the establishment of infection in host cells (38). In mammalian cells, a number of recent studies have reported transport pathways that allow extracellular CDNs to traverse the plasma membrane and activate STING (9, 11–14). CDNs are found in the extracellular environment, deriving from active secretion by invading pathogens (10), release from infected dying cells (17), or efflux from cancer cells (18). Consistent with our current results, a previous study found that release of extracellular CDNs may cause the selective apoptosis of monocytes through adenosine receptor signaling (17). The activity of extracellular 2′3′ cGAMP has garnered particular interest in relation to the microenvironment surrounding malignant tumor cells, and recent studies have suggested that 2′3′ cGAMP may facilitate antitumor cell immunity (39, 40). In this context, our findings of an alternative pathway of CDN action via adenosine signaling may be an important consideration for tumor cell-to-cell communication and antitumor therapies.

In intestinal cells, adenosine activates the predominant receptor A_{2B}AR which results in an increase in intracellular cAMP, followed by activation of CFTR chloride channels, releasing chloride into the lumen (23). ATP or ADP, both precursors of adenosine, are released by immune cells during inflammation (25, 41). We propose CDNs as another source for adenosine production through their hydrolysis by enzymes present in the epithelial membrane (27, 31). This was tested and confirmed using chemical inhibitors of the membrane-bound nucleotidases ENPP1 and CD73. We identified adenosine, the byproduct of their hydrolysis, as the substrate by which extracellular CDNs signal via the A_{2B} adenosine receptor by using a well-characterized A_{2B}AR inhibitor (PSB603) (31). Although administration of the A_{2B}AR inhibitor resulted in near complete inhibition of CDN currents, in our control experiments with basolateral adenosine we did find a consistent residual current. This may be due to incomplete inhibition of the receptor in the setting of high-dose adenosine along with the relatively short preincubation with the inhibitor, or possibly the activation of alternate lower affinity adenosine receptors in this setting. Nevertheless, the near-complete inhibition of extracellular CDN-induced short-circuit currents supports adenosine as the signaling substrate.

Our findings also demonstrate a more robust response by the mammalian CDN 2′3′ cGAMP compared to the bacterial CDNs 3′3′ cGAMP, c-di-AMP, and cAAG. One explanation for this may be the greater binding affinity for ENPP1 to 2′3′ cGAMP (30), and thus more rapid hydrolysis and production of adenosine. We also find that the short-circuit current responses produced by CDNs are smaller than the Isc induced by adenosine or the linear dinucleotide Ap4a, which may reflect incomplete or rate-limiting hydrolysis by either of the ectonucleotidases ENPP1 or CD73 or both.

A striking finding is the strictly polarized response of epithelial cells to extracellular CDNs. Previous studies have shown that in intestinal cells, both A_{2B}AR and CD73 are active on both apical and basolateral membranes (22, 27, 31). Here we find, however, that extracellular CDNs induced epithelial responses only when applied to basolateral cell surfaces, suggesting polarized activity of ENPP1 at the basolateral membrane. In the case of CDN signaling then, such polarization may underlie how epithelial cells distinguish between physiologic commensal microbes restricted to the intestinal lumen and pathologic and invasive microbes that enter the lamina propria (the subepithelial space). In externally facing interfaces such the intestinal mucosa, the presence of extracellular CDNs in the lamina propria on the basolateral side of the epithelium likely occurs in the setting of microbial breach of the barrier or during tissue inflammation or stress. The chloride secretory response to CDNs, as with other pathogenic stimuli such as cholera toxin (42), may represent a similarly conserved host defense mechanism. Cell polarity is thought to be important in compartmentalizing innate immune responses in barrier epithelial cells to a variety of pathogen-associated or host damage-associated molecules, exemplified by the basolateral-specific action of flagellin on its cognate host receptor TLR5 (43). More recently, studies have also shown that IFN responses mediated by TLR3 are polarized to the basolateral membranes of intestinal epithelial cells (32).

In addition to chloride secretion, adenosine can affect the cellular response to inflammation (44, 45). A_{2B}AR activation can result in stimulation of transcription factors up-regulating production of IL-6 (46), and the receptor has been shown to play a proinflammatory role during colitis (47). Conversely, adenosine has been shown to also have antiinflammatory effects through its action on the proteasomal degradation of IκB, and thus inhibiting NF-κB signaling (25), in addition to attenuating mucosal inflammation during acute colitis (48). These divergent effects of adenosine signaling during inflammation may be context dependent (49). Here, we find that extracellular 2′3′ cGAMP, through its hydrolysis to adenosine, down-regulates IFNβ expression induced by Poly I:C, surprisingly in an opposite manner to cytosolic CDNs. This observation is likely STING independent, as this inhibitory effect is also seen with cAAG, which cannot bind STING (6). In this context the action of extracellular CDNs may reflect an immune evasive strategy deployed by pathogens (50). How CDNs affect inflammation or infection in vivo and how this pathway functions during the host response to various pathogen- and damage-associated molecular patterns, particularly related to viral signals given our IFNβ results, will be interesting avenues for further studies.

In summary, extracellular CDNs are hydrolyzed by enzymes present in the intestinal membrane to form adenosine. This is observed exclusively along the basolateral compartment, suggesting a

mechanism by which cells respond to microbial invasion or activation of the innate immune system. Along with chloride secretion, we find extracellular CDNs also regulate other epithelial innate immune responses via adenosine signaling. These findings suggest that cellular adenosine signaling is an important STING-independent mechanism by which extracellular CDNs modulate host cell responses, relevant to infection, innate immunity, and cancer biology.

Materials and Methods

Materials and Reagents. Cyclic dinucleotides 2′3′ cGAMP (tlrl-nacga23), 3′3′ cGAMP (tlrl-nacga), c-di-AMP (tlrl-nacda), c-di-GMP (tlrl-nacdg), 2′5′ GpAp (tlrl-nagpap), and 2′3′ cGsAsMP (tlrl-nacga2srs) and the STING inhibitor H-151 (inh-h151) were purchased from Invivogen. cAAG was generated as published previously (6). α,β-Methylene adenosine diphosphate (M3763), PSB603 (SML1983), 5′ adenosine monophosphate (A2252), and adenosine (A4036) were purchased from Sigma-Aldrich. STF-1084 was generously provided by Lingyin Li, Department of Biochemistry, Stanford University, Stanford, CA.

Cell Culture. T84 cells (ATCC CCL-248) were cultured in a 1:1 Dulbecco's modified Eagle medium (DMEM)/Ham's F-12 media supplemented with 10% newborn calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were grown on collagen-coated 0.33-cm^2 Transwell inserts (Costar Corning, CLS3472) and incubated in 95% O_2/5% CO_2 at 37 °C for at least 7 d. The medium was changed every 3 to 4 d. Transepithelial electrical resistance (TEER) was measured using an epithelial volt/ohm meter (EVOM; World Precision Instruments) and a TEER >1,000 Ω/cm^2 was used to determine proper monolayer formation.

Short-Circuit Current Measurement. Following the formation of a monolayer, the medium was removed and the cells were rinsed and bathed in buffer solution (in mM) (130 NaCl, 0.47 KCl, 0.124 $MgSO_4$, 0.33 $CaCl_2$, 10 Hepes, 2.5 NaH_2PO_4, 10 dextrose). Custom made chambers were designed and built to measure short-circuit current in 0.33-cm^2 Transwell inserts (*SI Appendix, Supplementary Methods*). The cells were maintained at 37 °C and short-circuit current was measured using an VCCMC8 multichannel voltage clamp (Physiologic Instruments), and LabChart (ADInstruments) was used to record measurements.

IFNβ Expression Analysis by qPCR. T84 monolayers were rinsed and bathed in serum-free DMEM. Adenosine (20 μM), 2′3′ cGAMP (14 μM), cAAG (10 μM), forskolin (20 μM), or Poly I:C (10 μg/mL) was added directly to the basolateral compartment. For cotreated wells, cells were pretreated for 15 min prior to addition of Poly I:C. Cells were incubated at 37 °C for 6 h, followed by PBS 1× rinse three times.

Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen). Cell pellets were lysed in buffer RLT and processed according to the manufacturer's protocol. Total RNA concentrations were measured by absorbance at 260 nm, and quality was assessed by A260/A280 ratios. cDNA was synthesized from 1 μg of RNA, including a DNA elimination step, using QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer's protocol.

Target transcripts were amplified using the primers listed below (Integrated DNA Technologies, Inc.) and Sso Advanced Universal SYBR Green Supermix according to the manufacturer's protocol (Bio-Rad). All qPCR reactions were assayed in triplicate for each sample, and the average Cq value was used to calculate the mean expression ratio of the test sample compared with the control sample (i.e., stress treated compared with control treated) using the 2-ΔΔCt method. Cq values for targets were analyzed relative to Cq values for the hprt housekeeping gene.

PCR primer sequence. Human *IFNβ1*:

Primer 1: 5′-GAAACTGAAGATCTCCTAGCCT-3′

Primer 2: 5′-GCCATCAGTCACTTAAACAGC-3′

Human *HPRT1*:

Primer 1: 5′-GCGATGTCAATAGGACTCCAG-3′

Primer 2: 5′-TTGTTGTAGGATATGCCCTTGA-3′.

Statistics. Significance was assessed using a two-tailed *t* test or two-way ANOVA with post hoc multiple comparison testing (Tukey–Kramer) and where indicated $P < 0.05$ was considered significant. Graphs were generated using GraphPad Prism 8.

Data Availability. All study data are included in the article and *SI Appendix*.

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