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# A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP

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The innate immune system responds to unique molecular signatures that are widely conserved among microbes but that are not normally present in host cells. Compounds that stimulate innate immune pathways may be valuable in the design of novel adjuvants, vaccines, and other immunotherapeutics. The cyclic dinucleotide cyclic-di-guanosine monophosphate (c-di-GMP) is a recently appreciated second messenger that plays critical regulatory roles in many species of bacteria but is not produced by eukaryotic cells. In vivo and in vitro studies have previously suggested that c-di-GMP is a potent immunostimulatory compound recognized by mouse and human cells. We provide evidence that c-di-GMP is sensed in the cytosol of mammalian cells via a novel immunosurveillance pathway. The potency of cytosolic signaling induced by c-di-GMP is comparable to that induced by cytosolic delivery of DNA, and both nucleic acids induce a similar transcriptional profile, including triggering of type I interferons and coregulated genes via induction of TBK1, IRF3, nuclear factor  $\kappa$ B, and MAP kinases. However, the cytosolic pathway that senses c-di-GMP appears to be distinct from all known nucleic acid-sensing pathways. Our results suggest a novel mechanism by which host cells can induce an inflammatory response to a widely produced bacterial ligand.

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Abbreviations used: c-di-GMP, cyclic-di-GMP; DAI, DNA-dependent activator of IFN regulatory factors; HSA, human serum albumin; MEF, mouse embryonic fibroblast; TLR, Toll-like receptor.

To sense infection, the innate immune system preferentially responds to conserved molecular signatures of microbes that are absent from normal host cells. There is currently great interest in determining what molecular features of pathogens are detected, and how these features are sensed by the innate immune system. A better understanding of the fundamental principles of how immune responses are stimulated is critical for the design of more effective vaccines, adjuvants, and immune therapeutics.

One important class of ligands sensed by the innate immune system is nucleic acids. Several distinct nucleic acid sensors have been described and have been found to be distributed to distinct subcellular locations and exhibit distinct specificities for different nucleic acid ligands. A common theme is that nucleic acid sensors tend to induce expression of type I IFN genes and, thus, appear to be particularly important for initiating immune responses to viruses.

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Toll-like receptor (TLR) 3, 7, 8, and 9 are transmembrane nucleic acid receptors that reside in intracellular compartments, where they detect endocytosed or autophagocytosed nucleic acids (Kawai and Akira, 2007; Medzhitov, 2007). TLR7, 8, and 9 all require the signaling adaptor MyD88 to initiate downstream signaling, whereas TLR3 requires the signaling adaptor TRIF. Thus, *MyD88*<sup>-/-</sup>*Trif*<sup>-/-</sup> double-knockout cells are deficient in signaling through all known TLRs that sense nucleic acids (Hoebe et al., 2003; Yamamoto et al., 2003).

Interestingly, recent work has established that *MyD88*<sup>-/-</sup>*Trif*<sup>-/-</sup> cells are capable of responding to nucleic acid ligands via cytosolic immunosurveillance pathways. Cytosolic RNA appears to be sensed by two RNA helicase-containing proteins, RIG-I and Mda5 (Yoneyama et al., 2004). Interestingly, RIG-I and Mda5 do not perform redundant functions and appear to respond to distinct classes of viruses (Gitlin et al., 2006; Hornung et al., 2006; Kato et al., 2006; Pichlmair et al., 2006; Saito and Gale, 2008). Signaling by RIG-I and Mda5 requires a common adaptor molecule called MAVS (also known as IPS-1, Cardif, or VISA; Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005; Sun et al., 2006). MAVS recruits the TBK1 kinase that phosphorylates and activates the IRF3 transcription factor. Other transcription factors such as ATF2/c-Jun and NK-κB form a coordinated DNA-bound complex with IRF3, and are together required for robust activation of the IFN-β promoter (Maniatis et al., 1998; Panne, 2008).

Many cell types also appear to respond to the cytosolic presence of DNA (Ishii et al., 2006; Stetson and Medzhitov, 2006a). The cytosolic response to DNA does not require MAVS, but does require TBK1 and IRF3 in most cell types (Ishii et al., 2006; Stetson and Medzhitov, 2006a; Sun et al., 2006). Recently, a putative cytosolic sensor of DNA was identified, and was named DNA-dependent activator of IFN regulatory factors (DAI; previously known as DLM-1 or ZBP1; Takaoka et al., 2007). Knockdown experiments indicated that DAI was involved in sensing cytosolic DNA in the L929 fibroblast-like and RAW macrophage-like cell lines (Takaoka et al., 2007; Wang et al., 2008). DAI was expressed in other cell types, such as mouse embryonic fibroblasts (MEFs), but was dispensable for the response to cytosolic DNA in these cell types, suggesting that additional uncharacterized nucleic acid sensor proteins also exist (Wang et al., 2008). Indeed, cells from DAI knockouts appear to respond normally to cytosolic DNA (Ishii et al., 2008), possibly because of redundancy with other cytosolic DNA sensors.

Although type I IFNs are primarily considered to be antiviral cytokines (Stetson and Medzhitov, 2006b), there is growing appreciation for their complex role in bacterial infections as well. Indeed, it is clear that type I IFNs are induced by many, if not all, bacterial pathogens, and can contribute to diverse outcomes in vivo (Coers et al., 2000; O'Riordan et al., 2002; Opitz et al., 2006; Stetson and Medzhitov, 2006a; Henry et al., 2007; Roux et al., 2007; Stanley et al., 2007; Charrel-Dennis et al., 2008). For several bacterial pathogens,

including *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Legionella pneumophila*, *Francisella tularensis*, group B *Streptococcus*, and *Brucella abortus*, it appears that induction of type I IFN is via a novel cytosolic pathway and is independent of TLRs. It has been suggested that these pathogens trigger the cytosolic DNA-sensing pathway, but neither the host sensors nor the bacterial ligands that trigger cytosolic induction of type I IFNs by these pathogens have been identified. Intriguingly, evidence from *L. monocytogenes* suggests that bacterial multidrug efflux pumps are required for induction of type I IFN (Crimmins et al., 2008), and raises the possibility that a small molecule substrate of these pumps, rather than DNA, is the bacterial trigger of type I IFN gene expression.

Nucleotide second messengers are critical transmitters of signaling in all living things. cAMP is used by bacteria and eukaryotes alike, whereas certain nucleotide second messengers are unique to bacteria. For example, guanosine tetraphosphate (ppGpp) is a key regulator of the stringent response in bacteria (Srivatsan and Wang, 2008). Another nucleotide second messenger, cyclic-di-GMP (c-di-GMP), is a relatively recently appreciated cyclic ribonucleotide (Ross et al., 1987) synthesized by bacteria from two GTP precursors that are hydrolyzed and ultimately circularized via 5'-to-3' monophosphate linkages. The bacterial diguanylate cyclases that synthesize c-di-GMP all contain a characteristic GGDEF domain, whereas the phosphodiesterases that specifically degrade c-di-GMP to pGpG contain an EAL domain. The GGDEF domain, and presumably c-di-GMP, appears to be limited to Bacteria (Galperin et al., 2001) and is not found in Archaea or Eukarya. c-di-GMP appears to play complex roles as a second messenger in most bacterial species, and regulates diverse processes, such as motility, biofilm formation, and virulence gene expression (Tamayo et al., 2007). The molecular mechanisms by which c-di-GMP regulates these biological processes in bacteria are only beginning to be understood. It appears that c-di-GMP is capable of specific binding to regulatory proteins containing the PilZ protein domain (Cotter and Stibitz, 2007). The PelD protein of *P. aeruginosa* is an additional non-PilZ-containing c-di-GMP sensor protein (Lee et al., 2007).

Several recent reports have suggested that c-di-GMP can stimulate a variety of signaling pathways in mammalian cells in vivo. One early report demonstrated that 50 μM of exogenous c-di-GMP inhibited growth of human colon cancer cells (Karaolis et al., 2005a) without toxicity against normal kidney cells. Two additional reports have indicated that c-di-GMP can function as an adjuvant. One group demonstrated a highly significant (>200-fold; P < 0.001) induction of anti-ClfA IgG2a titers when mice were vaccinated with ClfA and c-di-GMP as compared with vaccination with ClfA alone (Karaolis et al., 2007a). Another group found a similar induction of anti-β-Gal titers when c-di-GMP was used as an adjuvant (Ebensen et al., 2007). Both groups also found increased T cell responses in mice injected with c-di-GMP. Karaolis et al. (2007a) also showed that c-di-GMP has immunostimulatory effects in vivo and in vitro on innate cell populations,

including monocytes, macrophages, and granulocytes. c-di-GMP induced dendritic cell maturation and led to the production of various cytokines and chemokines, including TNF, IL-1 $\beta$ , IP-10, Rantes, and CXCR4 (Karaolis et al., 2007a). However, c-di-GMP did not stimulate induction of type I IFNs by plasmacytoid dendritic cells. Impressively, preinjection of 2.5 mg/kg c-di-GMP protected against subsequent lethal challenge with *Klebsiella pneumoniae* or *Staphylococcus aureus* (Karaolis et al., 2005b; Karaolis et al., 2007b). Despite the impressive in vivo biological effects of c-di-GMP, the mechanism by which c-di-GMP stimulates host immunity remains unknown.

In this study, we provide evidence that mammalian cells survey their cytosol for the presence of c-di-GMP. We find that c-di-GMP triggers a transcriptional response virtually indistinguishable from the response triggered by cytosolic DNA. Like the response to DNA, the response to c-di-GMP requires the TBK1 kinase and IRF3 transcription factor. However, the pathway for sensing c-di-GMP can be distinguished from that for sensing DNA in certain cell types and, moreover, appears to be distinct from all other known cytosolic sensing pathways. Our results suggest a novel mechanism by which host cells can induce an inflammatory response to a widely produced bacterial ligand.

## RESULTS

### Cytosolic recognition of c-di-GMP leads to potent induction of type I IFN

We hypothesized that c-di-GMP might be sensed by a cytosolic sensor leading to the induction of type I IFNs. To test this hypothesis, we compared the ability of overlaid versus transfected c-di-GMP to induce type I IFN in bone marrow macrophages. At the concentrations used (up to 25  $\mu$ g/ml), overlay of c-di-GMP did not elicit a significant response (Fig. 1 A), although IFN- $\beta$  production could be detected at higher concentrations (50 or 100  $\mu$ g/ml; not depicted). In contrast, delivery of c-di-GMP to the cytosol by transfection elicited robust activation of IFN- $\beta$  in a dose-dependent manner (Fig. 1 A). A significant response was detected with a concentration of c-di-GMP as low as 2.5  $\mu$ g/ml (equivalent to 3.6  $\mu$ M). These results suggested that c-di-GMP signals via a cytosolic immunosurveillance pathway.

We tested whether compounds related to c-di-GMP were capable of stimulating IFN- $\beta$  expression when delivered to the cytosol. We measured induction of endogenous type I IFN protein by bioassay (Fig. 1 B), and induction of IFN- $\beta$  and IFN- $\alpha$ 5 mRNA levels by quantitative RT-PCR (Fig. 1, C and D). No detectable IFN- $\beta$  production was observed upon transfection with GTP, cGMP, ppGpp (a different bacterial nucleotide second messenger), or pGpG (hydrolyzed c-di-GMP), whereas the cytosolic response to 3.3  $\mu$ g/ml of transfected c-di-GMP was of a similar magnitude to that of 3.3  $\mu$ g/ml of transfected DNA (pdA:dT) and RNA (pI:C), which are known cytosolic inducers of type I IFN. We do not believe that the cytosolic induction of type I IFNs in response to c-di-GMP is caused by a contaminant (such as LPS) because

(a) the c-di-GMP compound was chemically synthesized and >98% pure by HPLC (not depicted), (b) phosphodiesterase treatment of c-di-GMP resulted in a product that is no longer stimulatory (Fig. 1 E), and (c) macrophages deficient in all TLR signaling and unable to respond to LPS still responded to c-di-GMP (see below). Thus, these observations suggest that c-di-GMP is sensed in the cytosol, resulting in a potent induction of type I IFN in bone marrow macrophages.

### Cytosolic c-di-GMP and DNA induce similar transcriptional responses

We performed microarray experiments to compare the global transcriptional response induced by cytosolic c-di-GMP with that induced by cytosolic DNA (Fig. 1 F and Table S1). Whole transcriptome spotted MEEBO microarrays (Verdugo and Medrano, 2006) were used in these experiments. The results indicated that the transcriptional profile of cells stimulated by cytosolic c-di-GMP was very similar to the transcriptional profile of cells stimulated with cytosolic DNA. Induction of type I IFNs and known type I IFN-inducible genes dominated the transcriptional response, but other genes (e.g., *Il6*, *Cd86*, and *Cxcl9*) were also strongly induced (Fig. 1 F and Table S1). Although the transcriptional profiles of cells stimulated with c-di-GMP and DNA were highly similar, a small number of genes were reproducibly preferentially induced by c-di-GMP as compared with DNA (e.g., *HtrA* serine peptidase 4 and *claudin 23*; Table S1). Collectively, these results suggested that similar but not identical downstream signaling pathways are triggered by DNA and c-di-GMP.

### Cytosolic c-di-GMP signals independently of TLRs but requires TBK1 and IRF3

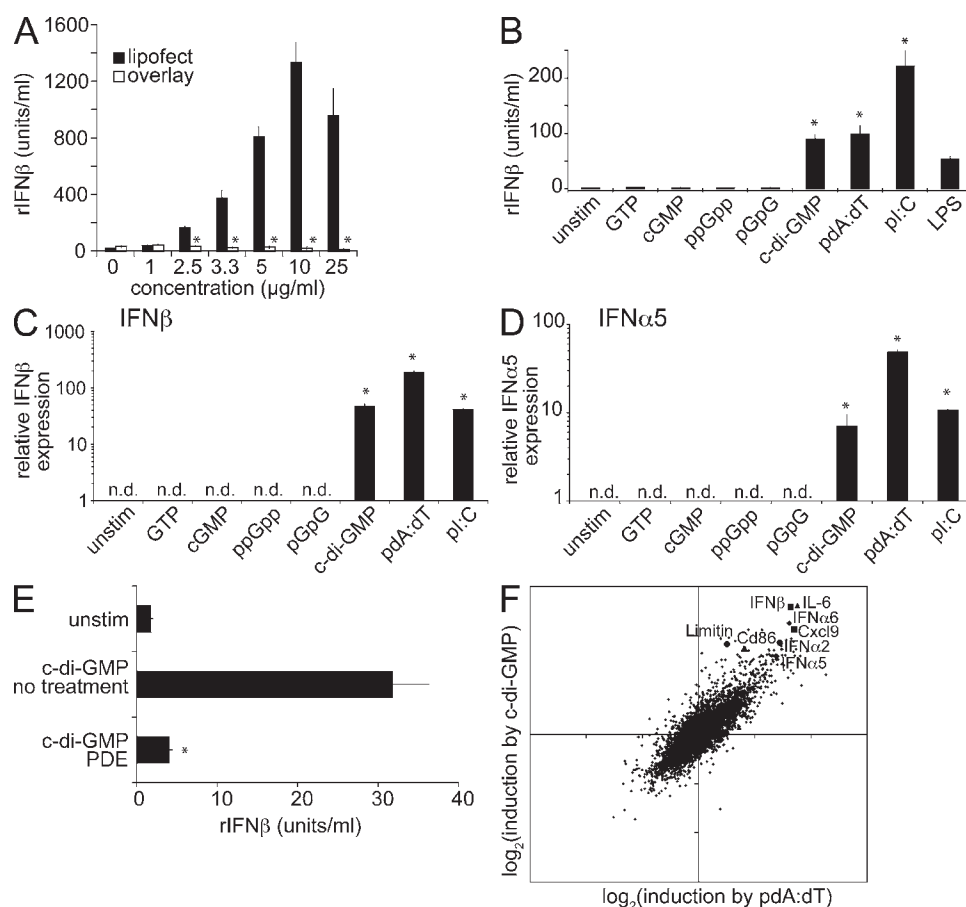
It was previously reported that HEK293 cells stably transfected with various TLRs failed to respond to c-di-GMP (Karaolis et al., 2007a), but the reason for this failure was unclear. For example, HEK293 cells might lack an essential accessory protein. To rule out a role for TLRs in sensing of c-di-GMP, we tested responses in *Myd88*<sup>-/-</sup>*Trif*<sup>-/-</sup> double-knockout macrophages, which are deficient in all TLR signaling (Yamamoto et al., 2003). Both wild-type and *Myd88*<sup>-/-</sup>*Trif*<sup>-/-</sup> macrophages showed a robust induction of IFN- $\beta$  after c-di-GMP stimulation (Fig. 2 A). As expected, the *Myd88*<sup>-/-</sup>*Trif*<sup>-/-</sup> macrophages did not respond to LPS (Fig. 2 A). These results indicated that TLR signaling is not required for responsiveness to c-di-GMP, and are consistent with c-di-GMP signaling via a cytosolic surveillance pathway.

Robust transcription of the IFN- $\beta$  gene requires the coordinate activation of several transcription factors, including IRF3 and NF- $\kappa$ B (Panne, 2008). We therefore expected that the c-di-GMP-induced signaling pathway would also require these factors to induce type I IFN. IRF3 activation requires phosphorylation by the TBK1 kinase, which leads to IRF3 dimerization and nuclear translocation (Fitzgerald et al., 2003; Sharma et al., 2003; Hemmi et al., 2004; McWhirter et al., 2004; Perry et al., 2004). To address whether TBK1 is required for activation of type I IFN by c-di-GMP, we tested

c-di-GMP responses in *Tbk1*<sup>-/-</sup> macrophages (Fig. 2 B). We observed very little IFN- $\beta$  induction by c-di-GMP in cells lacking *Tbk1*, suggesting that TBK1 is required for c-di-GMP signaling. We also observed that *Tbk1* is required for the induction of IFN- $\beta$  by pdA:dT (Fig. 2 B), in agreement with a recently published report (Miyahira et al., 2009). Although the data indicate a key role for *Tbk1* in the response to c-di-GMP, it is formally possible, albeit unlikely, that *Tbk1* protein rather than *Tbk1* kinase activity is required for the response. We also tested the requirement for the TBK1-related kinase *Ikk $\epsilon$*  (also called *IKK $\epsilon$*  or *IKK-i*), which plays

a role in IFN- $\beta$ -mediated antiviral immunity (Tenover et al., 2007), and found that *Ikk $\epsilon$* <sup>-/-</sup> macrophages responded normally to c-di-GMP (Fig. 2 C).

To examine the role of IRF3 in c-di-GMP induction of IFN- $\beta$ , we tested the ability of c-di-GMP to induce signaling in macrophages lacking *Irf3*. We observed that *Irf3*<sup>-/-</sup> macrophages failed to induce IFN- $\beta$  in response to c-di-GMP (Fig. 2 D). We also tested *Irf7*<sup>-/-</sup> macrophages and observed a partial requirement for IRF7 by the c-di-GMP pathway (Fig. 2 D). Loss of *Irf7* is likely compensated for by *Irf3* in macrophages. The partial requirement for *Irf7* may reflect a



**Figure 1. c-di-GMP is a potent cytosolic inducer of type I IFNs.** (A) Bone marrow macrophages were overlaid or transfected with the indicated amounts of synthetic, purified c-di-GMP. The amount of type I IFN in the supernatant was assessed after 6 h by use of an L929-ISRE-luc bioassay (Jiang et al., 2005) using recombinant IFN- $\beta$  as a standard. \*,  $P < 0.01$  as compared with transfected c-di-GMP (Student's  $t$  test). (B) Bone marrow macrophages were transfected with 3.3  $\mu$ g/ml of the indicated molecules (or overlaid with 100 ng/ml LPS), and type I IFN was assessed after 6 h by bioassay as in A. \*,  $P < 0.001$  as compared with unstimulated cells (Student's  $t$  test). (C) Bone marrow macrophages were transfected with 3.3  $\mu$ g/ml of the indicated molecules (or overlaid with 100 ng/ml LPS), and transcription of the IFN- $\beta$  gene (*Ifnb*) was assessed by quantitative RT-PCR, with normalization to ribosomal protein *rps17* message. n.d., not detectably induced above background (lipofectamine alone). \*,  $P < 0.001$  as compared with unstimulated cells (Student's  $t$  test). (D) As in C, but transcription of the IFN- $\alpha$ 5 gene was assessed. n.d., not detectably induced above background (lipofectamine alone). \*,  $P < 0.01$  as compared with unstimulated cells (Student's  $t$  test). (E) c-di-GMP, treated or untreated with snake venom phosphodiesterase, was transfected at 3.3  $\mu$ g/ml into B6 bone marrow macrophages and analyzed after 6 h for IFN- $\beta$  production by bioassay, as in A. \*,  $P < 0.02$  as compared with c-di-GMP treatment alone (Student's  $t$  test). Results in A–E are representative of at least three independent experiments. Data are means  $\pm$  SD ( $n = 3$ ). (F) Bone marrow macrophages were transfected with poly dA:dT (DNA) or with c-di-GMP. Total RNA was isolated after 6 h of stimulation. Probes were amplified and hybridized to whole-transcriptome spotted MEEBO microarrays. Each dot represents a single gene, and its position is determined by its induction in response to DNA (x axis) or c-di-GMP (y axis). Most genes lie on the diagonal, indicative of similar induction ratios by both treatments. Selected highly induced genes are labeled. Two independent microarray experiments gave similar results.



role for *Irf7* signaling as part of a positive feedback loop involving signaling through the type I IFN receptor (*Ifnar*). Indeed, *Ifnar*<sup>-/-</sup> macrophages exhibited a diminished response to c-di-GMP (Fig. 2 E). *Irf5* and *Irf1* did not seem to be required for responsiveness to c-di-GMP (Fig. 2 F). In addition, macrophages deficient in *Rip2*, *Nalp3*, or doubly deficient in *Nod1/2* responded normally to c-di-GMP (unpublished data).

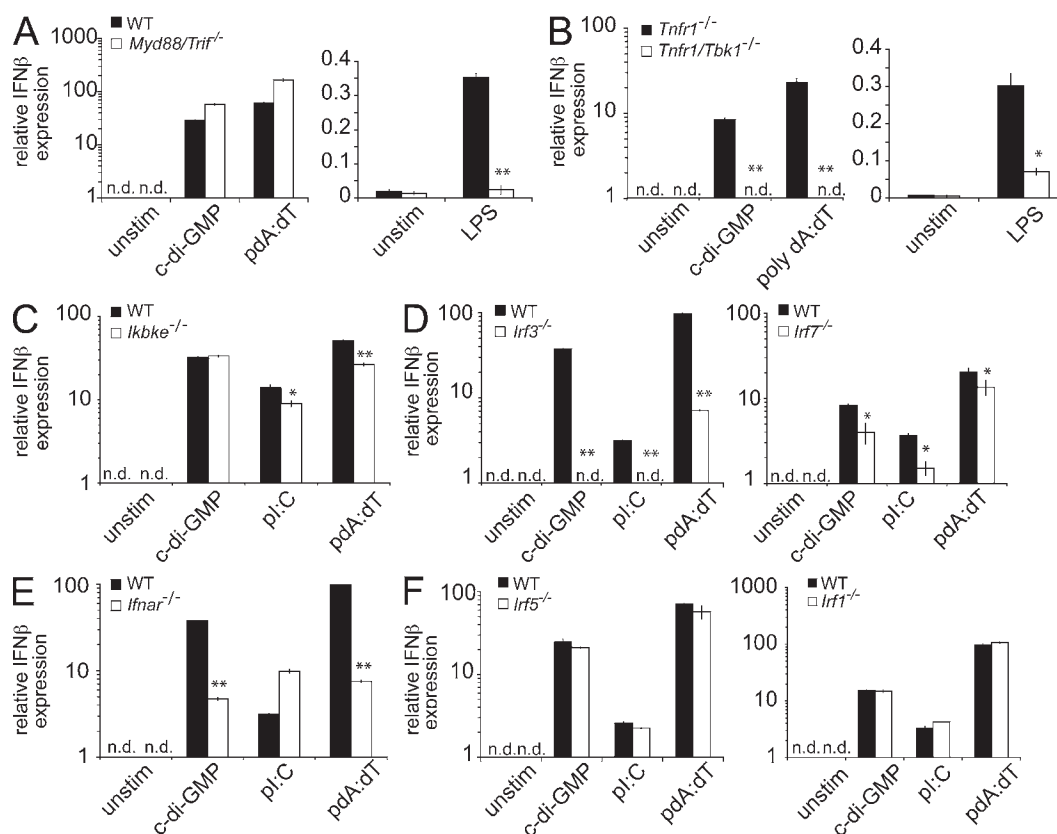
We validated our results by examining production of a second secreted molecule known to be a target of the *Tbk1*–*Irf3* signaling axis, the chemokine Rantes (*Ccl5*). Rantes production was assessed by ELISA (Fig. S1). As expected, we found that Rantes was induced by c-di-GMP in a manner dependent on *Tbk1*, *Irf3*, and *Ifnar*, but largely or entirely independent of *MyD88*/*Trif*, *Irf1*, *Irf5*, *Irf7*, or *Mavs* (Fig. S1).

### c-di-GMP activates IRF3, NF-κB, and MAP kinases

Our genetic data suggested that the cytosolic responses to DNA, RNA, and c-di-GMP share a common downstream signaling pathway. If this were true, we would expect to be able to detect biochemical activation of the NF-κB and IRF3

transcription factors, as well as phosphorylation of the p38, JNK, and ERK MAP kinases, in response to c-di-GMP. Activation of IRF3 involves phosphorylation, dimerization, and translocation into the nucleus. We tested whether treatment with c-di-GMP resulted in significant nuclear accumulation of IRF3. As expected, we found that c-di-GMP resulted in significant translocation of IRF3 to the nucleus (Fig. 3 A).

We also tested whether transfected c-di-GMP is capable of activating NF-κB. This was particularly important to establish in light of a previous report that exogenous (untransfected) c-di-GMP failed to activate NF-κB (Karaolis et al., 2007a). There have also been inconsistent reports in the literature as to whether cytosolic DNA induces NF-κB (Ishii et al., 2006; Stetson and Medzhitov, 2006a; Sun et al., 2006). We found that c-di-GMP treatment led to the activation of NF-κB, as assessed by using an electromobility gel shift assay, with similar kinetics to that of transfected pI:C and pdA:dT (Fig. 3 B). The probe-bound complex was confirmed to contain NF-κB p65 by supershifting with an anti-p65 antibody (Fig. 3 B). We confirmed these results by using a RAW



**Figure 2. Induction of type I IFN by c-di-GMP is independent of TLRs and requires TBK1 and IRF3.** (A) Bone marrow macrophages from *Myd88*<sup>-/-</sup> *Trif*<sup>-/-</sup> mice were transfected with 3.3 μg/ml c-di-GMP or pdA:dT (DNA), or overlaid with 100 ng/ml LPS as indicated. After 6 h of stimulation, induction of IFN-β mRNA was analyzed by quantitative RT-PCR, with normalization to ribosomal protein *rps17* message. n.d., not detectable. (B) As in A, except *Tnfr1*<sup>-/-</sup> or *Tnfr1*<sup>-/-</sup> *Tbk1*<sup>-/-</sup> macrophages were analyzed. *Tbk1* deficiency is embryonic lethal, but viability can be rescued on the TNF receptor 1 (*Tnfr1*)-deficient background. (C) As in A except *Ikbke* (IKKε/IKK-i)-deficient mice were analyzed. (D) as in A, except *Irf3*<sup>-/-</sup> and *Irf7*<sup>-/-</sup> macrophages were analyzed. (E) as in A, except macrophages deficient in the type I IFN receptor (*Ifnar*<sup>-/-</sup>) were analyzed. (F) as in A, except *Irf5*<sup>-/-</sup> and *Irf1*<sup>-/-</sup> macrophages were analyzed. Results are representative of at least three independent experiments. Data are means ± SD (*n* = 3). \*, *P* < 0.05; and \*\*, *P* < 0.001 as compared with wild-type bone marrow macrophages (Student's *t* test).

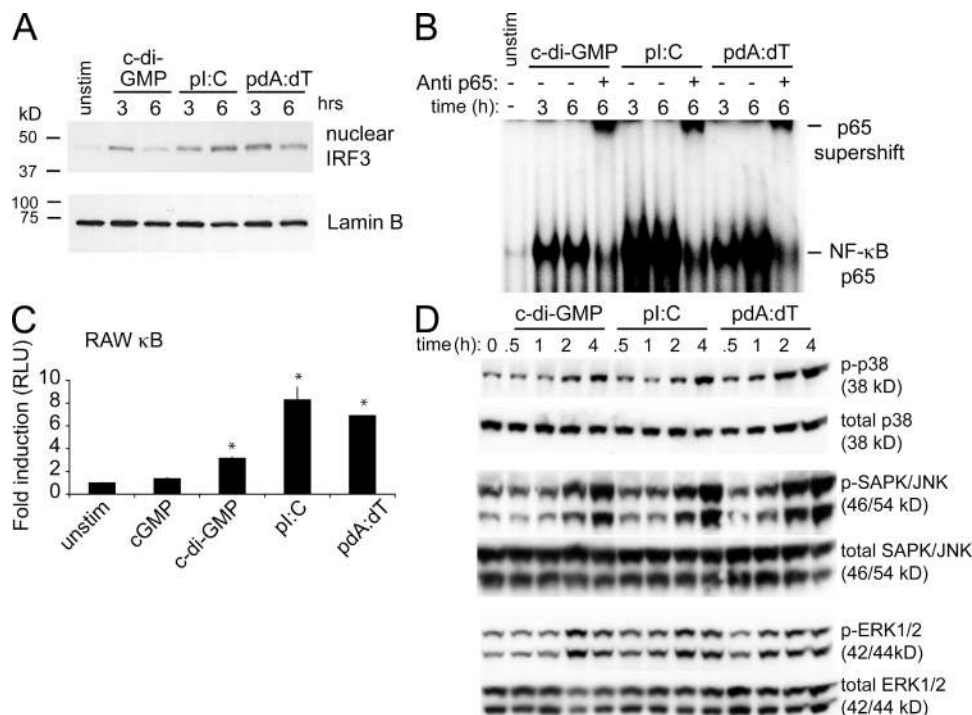
macrophage cell line stably transfected with an NF- $\kappa$ B luciferase reporter. We observed activation of the NF- $\kappa$ B luciferase reporter by transfected c-di-GMP (Fig. 3 C). Both the gel shift and reporter assays showed that the activation of NF- $\kappa$ B by transfected c-di-GMP was not as strong as observed with transfected pdA:dT and pl:C. The subtle difference in NF- $\kappa$ B induction between cytosolic DNA and c-di-GMP may suggest that there are distinct signaling components upstream of NF- $\kappa$ B in the two pathways. Nonetheless, we concluded that transfected c-di-GMP is capable of activating NF- $\kappa$ B. The relatively low levels and late time course of NF- $\kappa$ B induction by c-di-GMP (as compared with induction by LPS), as well as the fact that we transfected c-di-GMP into cells, may explain why NF- $\kappa$ B induction was not previously observed in response to c-di-GMP (Karaolis et al., 2007a).

We also tested whether MAP kinase pathways are activated by c-di-GMP. A previous report found that overlay of c-di-GMP induced ERK but not p38 in human macrophages (Karaolis et al., 2007a). In contrast, we found that cytosolic c-di-GMP stimulated p38, ERK1/2, and JNK phosphorylation in mouse bone marrow macrophages transfected with c-di-GMP (Fig. 3 D). Although a difference between human and mouse macrophages may explain the

discrepancy between our results and those of Karaolis et al. (2007a), it is more likely that we were able to detect MAP kinase activation because transfection of c-di-GMP triggers more robust responses than overlaid c-di-GMP, and because we examined later time points.

#### Cell type-specific responses to cytosolic DNA and c-di-GMP

Collectively, these results suggest that there are strong similarities in the signaling pathways triggered by the cytosolic presence of c-di-GMP and other nucleic acids, such as DNA and RNA. However, c-di-GMP is not structurally similar to these nucleic acid ligands. The DNA molecules capable of provoking cytosolic responses are double stranded and >25 deoxyribonucleotides in length (Ishii et al., 2006; Stetson and Medzhitov, 2006a). In contrast, c-di-GMP is composed of two guanosine ribonucleotides (not deoxyribonucleotides) in an unusual cyclic conformation. c-di-GMP also lacks features consistent with recognition by RIG-I or Mda5, such as 5'-triphosphate, polyuridine, or double strandedness (Saito and Gale, 2008; Saito et al., 2008). Therefore, we considered whether distinct sensors might be responsible for triggering responses to DNA/RNA and c-di-GMP, and if so, whether the responses could be distinguished in certain cell types.



**Figure 3. The host transcription factors IRF3 and NF- $\kappa$ B are induced by c-di-GMP, as are the p38, JNK, and ERK1/2 MAP kinases.**

(A) Nuclear extracts from *Myd88/Trif*<sup>-/-</sup> macrophages stimulated for the indicated times with c-di-GMP, poly dA:dT (pdA:dT, DNA), or poly I:C (pl:C, RNA) were probed with the indicated antibodies. (B) Nuclear extracts from *Myd88/Trif*<sup>-/-</sup> macrophages stimulated with c-di-GMP, poly dA:dT (pdA:dT, DNA), or poly I:C (pl:C, RNA) for the indicated times were used in a gel shift assay with an NF- $\kappa$ B consensus binding sequence probe. (C) RAW264.7 macrophages stably expressing an NF- $\kappa$ B luciferase reporter were stimulated with c-di-GMP, poly dA:dT (pdA:dT, DNA), or poly I:C (pl:C, RNA) for 6 h and analyzed for luciferase activity. (D) Whole-cell extracts from *Myd88/Trif*<sup>-/-</sup> macrophages stimulated for the indicated times with c-di-GMP, poly dA:dT (pdA:dT, DNA), or poly I:C (pl:C, RNA) were probed with the indicated antibodies. Results are representative of at least three independent experiments. Data are means  $\pm$  SD ( $n = 3$ ). \*,  $P < 0.05$  compared with unstimulated cells (Student's  $t$  test).



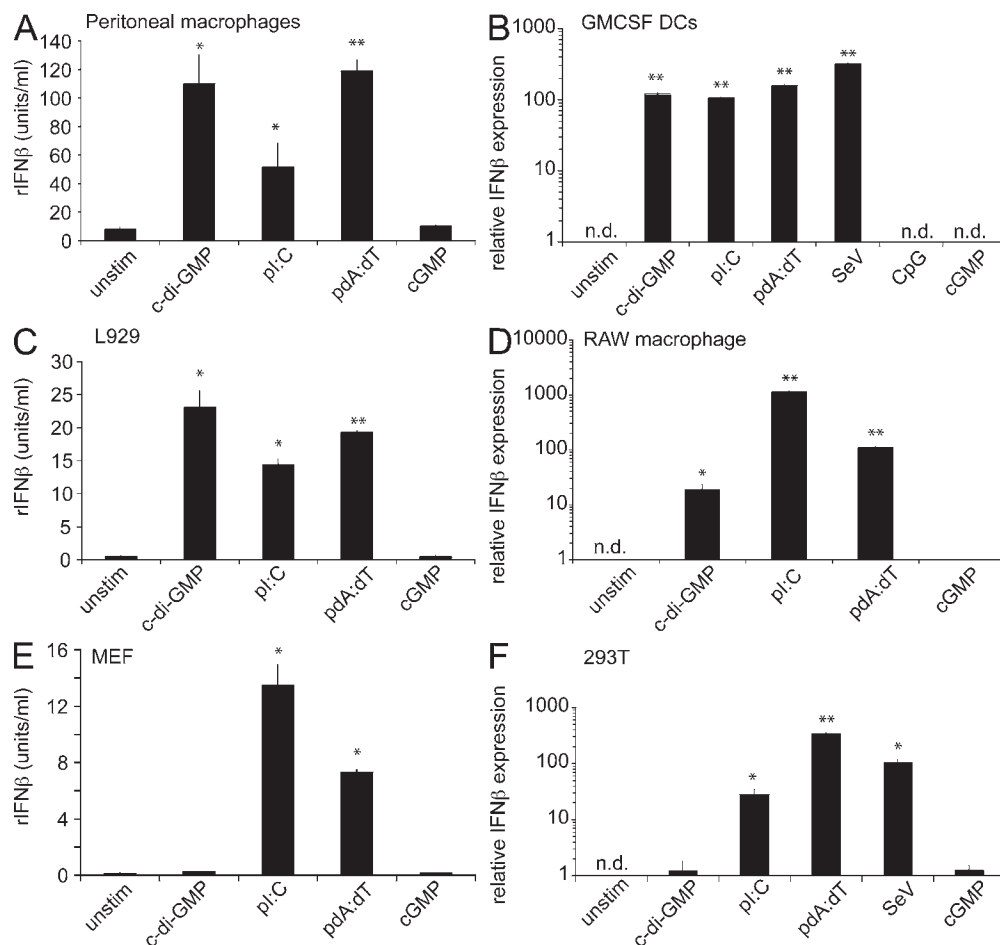
In addition to bone marrow macrophages, we observed induction of IFN- $\beta$  by cytosolic c-di-GMP in a variety of other cell types, including peritoneal macrophages, conventional dendritic cells, L929 cells, and RAW 264.7 macrophages (Fig. 4, A–D). These cells also responded to transfected DNA and RNA. Interestingly, however, we were unable to detect significant induction of type I IFNs by c-di-GMP in MEFs or in 293T cells, even though the cytosolic pathways for detecting DNA and RNA are intact in these cells (Fig. 4, E and F). These results suggest that at least one component of the host signaling pathway responding to c-di-GMP is distinct from that used for responses to cytosolic RNA or DNA, and is differentially expressed in different cell types.

#### Known DNA and RNA sensing pathways are not required for responses to c-di-GMP

MAVS (also known as IPS-1) is an essential adapter protein in the cytosolic RNA pathway leading to type I IFN induc-

tion, and is downstream of two cytosolic viral RNA sensors, RIG-I and Mda5 (Sun et al., 2006). To test if c-di-GMP signals through the cytosolic RNA-sensing pathway, we tested c-di-GMP responses in *Mavs*<sup>-/-</sup> and *Mda5*<sup>-/-</sup> macrophages. Loss of *Mavs* or *Mda5* did not affect the induction of IFN- $\beta$  by c-di-GMP (Fig. 5, A and B; and Fig. S1 C), suggesting that c-di-GMP does not signal through the cytosolic RNA signaling apparatus (Gitlin et al., 2006; Kato et al., 2008; Saito and Gale, 2008).

DAI (encoded by the *Zbp1* gene) is a recently identified molecule that has been proposed to function as a sensor of cytosolic DNA (Takaoka et al., 2007; Wang et al., 2008). We found that bone marrow-derived macrophages from *Zbp1*-deficient mice (Ishii et al., 2008) still responded to c-di-GMP (Fig. 5 C). However, as previously reported (Ishii et al., 2008), *Zbp1*-deficient macrophages also responded normally to DNA (Fig. 5 C). These results imply that macrophages express at least one cytosolic DNA sensor that is distinct from



**Figure 4. Responsiveness to cytosolic c-di-GMP varies among cell types and does not correlate with responsiveness to cytosolic DNA or RNA.** (A) Thioglycollate-elicited peritoneal macrophages were stimulated with 3.3  $\mu$ g/ml c-di-GMP, poly dA:dT (pdA:dT, DNA), or poly I:C (pl:C, RNA), and type I IFN was measured by bioassay as in Fig. 1 A. (B) GM-CSF-differentiated dendritic cells were stimulated as in A. SeV, Sendai virus. (C) L929 cells were stimulated as in A. (D) RAW264.7 macrophage-like cells were stimulated as in A. (E) MEFs were stimulated as in A. (F) 293T cells were stimulated as in A. In B, D, and F, induction of IFN- $\beta$  transcripts was assessed by quantitative RT-PCR. Results are representative of at least three independent experiments. Data are means  $\pm$  SD ( $n = 3$ ). n.d., not detectable. \*,  $P < 0.01$ ; and \*\*,  $P < 0.001$  as compared with unstimulated cells (Student's  $t$  test).

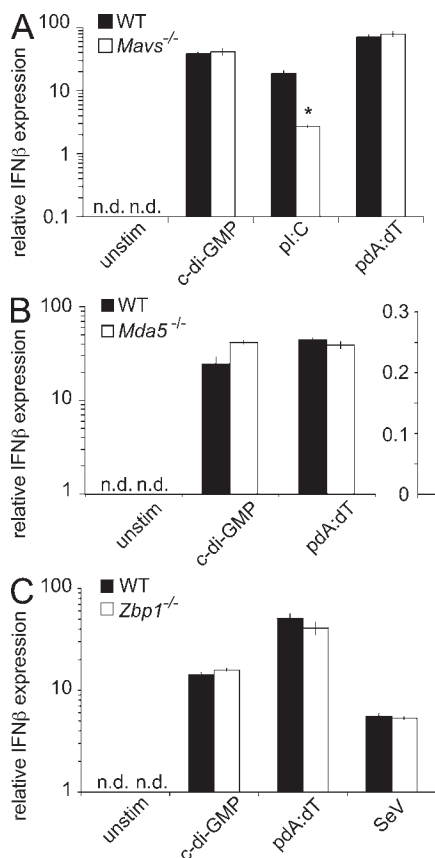
DAI, and at least one of these uncharacterized DNA sensors, or another independent sensor, may respond to c-di-GMP.

### c-di-GMP signals through IRF3 and IRF7 in vivo

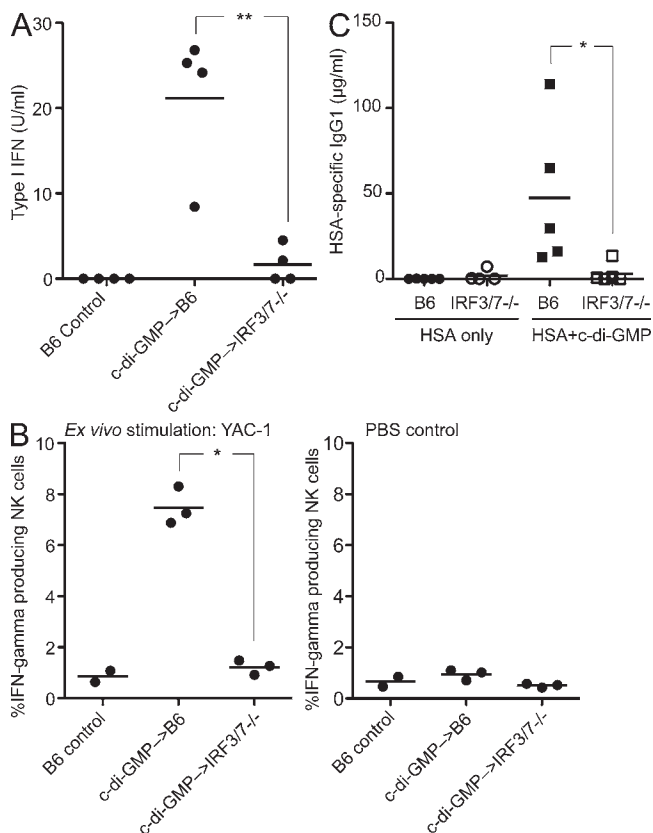
To validate our findings in vivo, we injected mice intraperitoneally with c-di-GMP (200 nmol per mouse) and measured the production of type I IFNs in the blood 18 h later. B6 mice injected with c-di-GMP produced an IFN response to c-di-GMP, and this response was abolished in *Irf3/7* double-deficient mice (Fig. 6 A). Interestingly, single-deficient *Irf3*<sup>-/-</sup> mice responded to c-di-GMP in vivo (unpublished data), implying a role for IRF7 in responses to c-di-GMP in vivo. To determine whether cytokine induction by c-di-GMP affected cellular responses in vivo, we collected splenic NK cells from mice injected with c-di-GMP

and measured their responsiveness to YAC-1 target cells ex vivo. Because NK cells respond to in vivo injection of poly I:C, a prototypical IFN inducer, we expected that c-di-GMP might also activate NK cells. Indeed, NK cells obtained from B6 mice injected with c-di-GMP responded to YAC-1 cells, as measured by intracellular cytokine staining for IFN- $\gamma$  (Fig. 6 B). However, NK cells obtained from *Irf3/7*<sup>-/-</sup> mice stimulated with c-di-GMP were nonresponsive to YAC-1 target cells. As a negative control, NK cells stimulated ex vivo with PBS did not produce IFN- $\gamma$ . These observations indicate that c-di-GMP can stimulate cytokine and NK cell responses in vivo, and these responses require the IRF3/7 transcription factors.

To obtain a preliminary indication as to whether adaptive immune responses could be stimulated by c-di-GMP, we immunized mice with human serum albumin (HSA) and tested whether coinjected c-di-GMP could function as an adjuvant, as previously reported (Ebensen et al., 2007; Karaolis et al.,



**Figure 5. c-di-GMP does not stimulate known cytosolic pathways for sensing nucleic acids.** (A) *Mavs*<sup>-/-</sup> and littermate wild-type macrophages were transfected with 3.3  $\mu$ g/ml c-di-GMP, poly dA:dT (pdA:dT, DNA), or poly I:C (pl:C, RNA), and transcription of the IFN- $\beta$  gene (*Ifnb*) was assessed by quantitative RT-PCR, with normalization to ribosomal protein *rps17* message. n.d., not detectable. (B) *Mda5*<sup>-/-</sup> and wild-type control macrophages were stimulated and analyzed as in A. (C) *Zbp1*<sup>-/-</sup> (DAI knockout) and wild-type control macrophages were stimulated and analyzed as in A. SeV, Sendai virus. Results are representative of at least three independent experiments. Data are means  $\pm$  SD ( $n = 3$ ). \*,  $P < 0.01$ ; and \*\*,  $P < 0.001$  as compared with wild-type bone marrow macrophages (Student's *t* test).



### Figure 6. c-di-GMP activates IRF3/7-dependent responses in vivo.

(A and B) B6 and *Irf3/7*<sup>-/-</sup> mice were injected intraperitoneally with 200 nmol c-di-GMP, and (A) serum IFN was measured by bioassay 18 h later, or (B) 36 h after injection, splenic NK responsiveness to YAC-1 target cells (or PBS control) was measured ex vivo by intracellular staining for IFN- $\gamma$ . (C) B6 and *Irf3/7*<sup>-/-</sup> mice were injected intraperitoneally with HSA  $\pm$  200 nmol c-di-GMP, and 2 wk later serum IgG1 specific for HSA was determined by ELISA. The experiments in A and B were repeated twice, and the experiment in C was performed once. Horizontal bars represent means. \*\*,  $P < 0.01$ ; and \*,  $P < 0.05$  (Student's *t* test).

2007a). Serum was collected from immunized mice 2 wk after a single intraperitoneal injection. Mice immunized with HSA alone did not mount a significant antibody response to HSA. In contrast, mice injected with HSA plus c-di-GMP produced variable but significant titers of HSA-specific IgG1 antibodies, confirming that c-di-GMP can function as an adjuvant *in vivo*. Importantly, our preliminary data indicated that the adjuvant effect of c-di-GMP depended on IRF3/7, as antibody responses in *Ifi3/7<sup>-/-</sup>* mice immunized with HSA and c-di-GMP were significantly ( $P < 0.05$ ) reduced as compared with immunized B6 mice (Fig. 6 C). Further studies will be required to establish the mechanism by which c-di-GMP functions as an adjuvant, but our results are consistent with a recent report indicating that the IRF3/7 kinase, TBK1, is required for adaptive immune responses in a DNA-vaccine model (Ishii et al., 2008).

## DISCUSSION

It has been well established that innate immune responses are initiated in response to certain microbial ligands that are evolutionarily conserved and that can be distinguished from self-ligands. Nucleic acids appear to be a favored target of immune recognition, and are sensed by a variety of endosomal and cytosolic sensor proteins. The cyclic dinucleotide c-di-GMP is a bacterial second messenger that exhibits several characteristics that are desirable in an immunostimulatory ligand: it is produced by numerous species of bacteria, it is a critical regulator of bacterial physiology, and it is not similar to host molecules. Indeed, several previous reports have demonstrated that c-di-GMP can provoke potent immune responses when injected *in vivo* into mice (Karaolis et al., 2005a; Karaolis et al., 2005b; Karaolis et al., 2007a; Karaolis et al., 2007b). However, the mechanism by which c-di-GMP stimulates immune responses remained unclear. Our data provide evidence that c-di-GMP is sensed by a novel cytosolic immunosurveillance pathway. Responsiveness to c-di-GMP is independent of TLRs that monitor the extracellular/endosomal compartments and is strongly potentiated by transfection of c-di-GMP into the host cell cytosol. Moreover, c-di-GMP provokes a transcriptional response highly reminiscent of that triggered by the cytosolic presence of DNA or RNA, and involves activation of TBK-1, MAP kinases, and the IRF-3 and NF- $\kappa$ B transcription factors. Thus, we propose that c-di-GMP is sensed in the cytosol.

How many different cytosolic sensors exist for nucleic acids? Our data suggest that sensing of c-di-GMP occurs via a novel cytosolic immunosurveillance pathway. Known nucleic acid sensors include Mda5 and RIG-I, which sense viral RNA and signal via MAVS, as well as DAI, a putative sensor of DNA that signals independently of MAVS. Based on the finding that most cell types, including MEFs, can still respond to DNA in the absence of DAI (Ishii et al., 2008), it has been proposed that an additional DNA sensor must exist and that MEFs express both of these sensors (Wang et al., 2008). Our results now suggest that there is at least one additional nucleic acid sensor in the cytosol, because we find that MEFs do not

respond well to c-di-GMP. Given the marked chemical dissimilarities among DNA, RNA, and c-di-GMP, it is perhaps not surprising that these nucleic acids appear to be recognized by different sensors. Indeed, we favor the idea that there are multiple cytosolic sensors for nucleic acids leading to induction of type I IFNs. The specificities of these various sensors will require further dissection. For example, although DAI knockout cells still responded to c-di-GMP, it remains formally possible that DAI is just one of several redundant sensors for c-di-GMP. These possibilities can be addressed once additional cytosolic nucleic acid sensors are identified.

Our results suggest that cytosolic sensing of c-di-GMP is relatively specific. Other related small nucleic acid compounds such as GTP, cGMP, ppGpp, and pGpG did not trigger transcriptional induction of type I IFNs (Fig. 1). In addition, c-di-GMP that was hydrolyzed by snake venom phosphodiesterase did not induce type I IFN (Fig. 1). However, our results cannot eliminate the possibility that a metabolite of c-di-GMP, rather than c-di-GMP itself, is the true molecular moiety that is sensed in the cytosol. Elimination of this possibility awaits identification of the c-di-GMP sensor and biophysical or crystallographic characterization of its binding to c-di-GMP. Our results also cannot eliminate the possibility that c-di-GMP triggers type I IFN expression indirectly, for example, by stimulating the synthesis of a host ligand that functions as the “true” proximal trigger of type I IFN gene expression. It is also possible that c-di-GMP acts “pharmacologically” by disrupting host physiology in a way that results in type I IFN expression. Moreover, because the signaling triggered by c-di-GMP and cytosolic DNA are very similar, it is possible that the c-di-GMP pathway is a branch off of the DNA-sensing pathway rather than a fully independent pathway. However, even if these alternative models are correct, our data indicate that the pathway downstream of c-di-GMP contains at least some novel components and is at least partially independent of known cytosolic or TLR signaling pathways.

Several bacterial pathogens, including *L. monocytogenes*, *L. pneumophila*, *F. tularensis*, *M. tuberculosis*, and group B *Streptococcus*, have been reported to induce type I IFNs (Coers et al., 2000; O’Riordan et al., 2002; Opitz et al., 2006; Stetson and Medzhitov, 2006a; Henry et al., 2007; Roux et al., 2007; Stanley et al., 2007; Charrel-Dennis et al., 2008). The mechanism by which these pathogens induce type I IFN resembles that of c-di-GMP: in all cases, type I IFN induction is independent of MyD88/Trif and TLRs, requires TBK1 and IRF3, and (with one exception; unpublished data; Opitz et al., 2006) is independent of the cytosolic RNA-sensing pathway. It is widely assumed that bacterial DNA, perhaps released after bacterial cell lysis, is the relevant ligand that triggers type I IFNs in response to pathogens. There are no data that eliminate or confirm this possibility, as sensors required for IFN induction in response to cytosolic bacteria have not yet been reported. However, it is noteworthy that in all cases, induction of type I IFNs by cytosolic bacteria requires expression of an auxiliary secretion system, namely the ESX-1 system of *M. tuberculosis*, the *Francisella* pathogenicity island-encoded

secretion system of *F. tularensis*, the Dot/Icm system of *L. pneumophila*, or the MdrM multidrug efflux pump of *L. monocytogenes*. It is tempting to speculate that a small, bacterially derived molecule such as c-di-GMP could be transported or leak through these secretion systems. It has not yet been possible to test this idea directly because all of these bacterial species encode numerous c-di-GMP synthases, and a strain lacking all c-di-GMP synthesis has not been reported. In any case, interpretation of these experiments would be complicated by the fact that c-di-GMP plays important regulatory roles in bacterial physiology and pathogenesis. Nevertheless, our results with synthetic purified c-di-GMP suggest that in addition to DNA, c-di-GMP is a candidate for a conserved molecule unique to bacteria that is responsible for triggering transcription of type I IFN genes. In light of a recent report that bacteria appear to be able to synthesize c-di-AMP (Witte et al., 2008), it is interesting to consider whether additional nucleic acids produced specifically by bacteria might also trigger host immunosurveillance pathways. Non-nucleic acid small molecules, such as the drug DMXAA, also appear to be able to stimulate the TBK1-IRF3 axis (Roberts et al., 2007). Indeed, there may be multiple redundant pathways for cytosolic sensing of bacteria. Collectively, the available evidence suggests that host cells may sense a wider array of bacterial ligands than was previously appreciated.

Our results may have important implications for the design of new adjuvants and vaccines. DNA vaccines have attracted considerable enthusiasm as an approach for protecting against a variety of infectious diseases (Wang et al., 2001; Yang et al., 2004), but there are safety concerns about the insertional mutagenic potential of DNA vaccines and/or their potential to trigger pathogenic anti-DNA autoimmune antibody responses (Schalk et al., 2006). The potency of DNA vaccines appears to derive from their ability to stimulate the TBK1 and innate cytosolic DNA-sensing pathways (Ishii et al., 2006). Thus, our demonstration that a synthetic nonself non-DNA molecule such as c-di-GMP can stimulate an in vitro and in vivo innate and adaptive immune response (Fig. 6) similar to that induced by DNA, without similar autoimmune or mutagenic risks, suggests that c-di-GMP might have valuable application as a small-molecule adjuvant. Understanding the molecular basis of c-di-GMP signaling in mammalian cells will be a crucial step toward achieving this aim.

## MATERIALS AND METHODS

**Mice and cell lines.** Bone marrow macrophages were derived from various mouse strains, including *Mavs*<sup>-/-</sup> (Sun et al., 2006), *Mda5*<sup>-/-</sup> (Gitlin et al., 2006), and *Zbp1*<sup>-/-</sup> (Ishii et al., 2008). Wild-type C57BL/6 and *Tnfr1*<sup>-/-</sup> mice were from the Jackson Laboratory. *Myd88*<sup>-/-</sup>/*Trif*<sup>-/-</sup> mice were obtained from G. Barton (University of California, Berkeley, CA). *Irf1*<sup>-/-</sup>, *Irf3*<sup>-/-</sup>, *Irf5*<sup>-/-</sup>, and *Irf7*<sup>-/-</sup> mice were obtained from T. Taniguchi (University of Tokyo, Tokyo, Japan). *Tbk1*<sup>-/-</sup>/*Tnfr1*<sup>-/-</sup> mice were generated by crossing *Tbk1*<sup>-/-</sup> mice (provided by W.-C. Yeh, University of Toronto, Toronto, Canada) with *Tnfr1*<sup>-/-</sup> mice. *Rip2*<sup>-/-</sup> mice were obtained from R. Medzhitov (Yale University, New Haven, CT). *Nod1*<sup>-/-</sup>/*Nod2*<sup>-/-</sup> mice were obtained from D. Portnoy (University of California, Berkeley, CA). *Nalp3*<sup>-/-</sup> mice were obtained from V. Dixit (Genentech, South San Francisco, CA). ISRE-L929 IFN reporter cells were obtained from

B. Beutler (The Scripps Research Institute, La Jolla, CA), and RAW-κB cells were obtained from G. Barton. Animal protocols were approved by the University of California, Berkeley Animal Care and Use Committee.

**Reagents.** c-di-GMP was synthesized as previously described (Kawai et al., 2003), poly I:C was obtained from GE Biosciences, pA:dT (poly(dA-dT):poly(dA-dT)) was purchased from Sigma-Aldrich, ppGpp (guanosine-3',5'-bisphosphate) was obtained from TriLink Biotechnologies, pGpG (di-guanosine) was purchased from IBA GmbH, GTP and cGMP were obtained from Sigma-Aldrich, purified LPS was purchased from InvivoGen, and Sendai virus was obtained from Charles River Laboratories. Theiler's virus was the gift of M. Brahic (Stanford University, Stanford, CA).

**Cell culture.** L929, RAW 264.7, and MEF cell lines were cultured in DMEM containing 10% FBS, glutamine, and penicillin-streptomycin. For bone marrow-derived macrophages, bone marrow cells from femurs and tibias were cultured for 7 d in RPMI 1640 media containing 10% FBS, glutamine, penicillin-streptomycin, and 10% CSF from 3T3 cells, with feeding on the fourth day of growth. For conventional dendritic cells (GM-CSF-dendritic cells), bone marrow cells were cultured for 5 d with RPMI 1640 containing 10% FBS, glutamine, penicillin-streptomycin, β-mercaptoethanol, and GM-CSF, with fresh media added on the second and fourth days of growth. Peritoneal macrophages were elicited by injection of 2 ml 4% thioglycollate (Fluid Thioglycollate Medium; BD), and were obtained 4 d later by lavage of the peritoneal cavity with RPMI 1640.

**Cell stimulations (transfections).** Cells were transfected using Lipofectamine 2000 (LF2000; Invitrogen) according to the manufacturer's protocol. All nucleic acid stimulants were mixed with LF2000 at a ratio of 1 μl LF2000/1 μg nucleic acid, incubated at room temperature for 20–30 min, and added to cells at a final concentration of 3.3 μg/ml (96-well plates) or 4 μg/ml (6-well plates). For pI:C, 2 mg/ml of the stock solution was heated at 50°C for 10 min and cooled to room temperature before mixing with LF2000. Transfection experiments were done for 6 h, unless otherwise stated in the figures. For phosphodiesterase treatment of c-di-GMP, 0.5 μg/μl c-di-GMP was incubated for 2 h at room temperature in OptiMem buffer (Invitrogen), with 15 mM MgCl<sub>2</sub> and 1 U Phosphodiesterase I (GE Healthcare).

**Quantitative PCR.** Stimulated cells were overlaid with RNAlater (Applied Biosystems) and stored. RNA was isolated using the RNeasy kit (QIAGEN) according to the manufacturer's protocol, and was treated with RQ1 RNase-free DNase (Promega). 0.5 μg RNA was reverse transcribed with Superscript III (Invitrogen). Platinum Taq DNA polymerase (Invitrogen) and EvaGreen dye (Biotium) were used for quantitative PCR assays and analyzed with a real-time PCR system (StepOnePlus; Applied Biosystems). All gene expression values were normalized to *Rps17* (mouse) or *SP9* (human) levels for each sample. The following primer sequences were used: mouse *Irf1b*, (forward) 5'-ATAAGCAGCTCCAGCTCCAA-3' and (reverse) 5'-CTGTCTGCTGGTGGAGTTCA-3'; mouse *Irfα5*, (forward) 5'-TGACCTCAAAGCCTGTGTGATG-3' and (reverse) 5'-AAGTATTTCTCCACAGCCAGCAG-3'; mouse *Rps17*, (forward) 5'-CGCCATTATCCCCAGCAAG-3' and (reverse) 5'-TGTCGGGATCCACCTCAATG-3'; human *IFNβ*, (forward) 5'-AAACTCATGAGCAGCTTGCA-3' and (reverse) 5'-AGGAGATCTTCAGTTTCGGAGG-3'; and human *SP9*, (forward) 5'-ATCCGCCAGCGCCATA-3' and (reverse) 5'-TCAATGTGCTTCTGGGAATCC-3'.

**Type I IFN bioassay and luciferase reporter assay.** Cell-culture supernatants from stimulated cells were overlaid on top of ISRE-L929 IFN reporter cells (Jiang et al., 2005) and incubated for 4–6 h (96-well plate). The reporter cells were lysed in Passive Lysis Buffer (Promega) for 5 min at room temperature, mixed with firefly luciferin substrate (Biosynth), and measured on a luminometer (LmaxII<sup>384</sup>; MDS Analytical Technologies). Levels of type I IFN were calculated from a standard curve using recombinant mouse IFN-β (R&D Systems).



**ELISA.** Levels of Rantes protein in supernatants from bone marrow macrophages were measured using the Rantes DuoSet Elisa kit (R&D Systems) according to the manufacturer's protocol.

**Microarray analysis.** Macrophage RNA from  $10^6$  cells (6-well dishes) was isolated using the Ambion RNAqueous kit (Applied Biosystems) and amplified with the Ambion Amino Allyl MessageAmp II aRNA Amplification kit (Applied Biosystems) according to the manufacturer's protocol. Microarrays were performed as previously described (Leber et al., 2008). In brief, spotted microarrays using the MEEBO 70-mer oligonucleotide set (Illumina) were printed at the University of California, San Francisco Center for Advanced Technology. Microarray probes were generated by coupling amplified RNA to Cy dyes. After hybridization, arrays were washed, scanned on a GenePix 4000B Scanner (MDS Analytical Technologies), and gridded using SpotReader software (Niles Scientific). Analysis was performed using the GenePix Pro 6 and Acuity 4 software packages (MDS Analytical Technologies). Two independent experiments were performed. Microarray data have been deposited in the Gene Expression Omnibus under accession no. GSE16943.

**Cell fractionation, immunoblotting, and gel shift assays.** For analysis of MAP kinase activation, whole-cell extracts were prepared from  $2 \times 10^6$  bone marrow macrophages stimulated for the times indicated in the figures and lysed in RIPA buffer supplemented with 50 mM NaF, 2 mM  $\text{NaVO}_3$ , 25 mM  $\beta$ -glycerol phosphate, 1 mM PMSF, and 1 $\times$  Complete Protease Inhibitor Cocktail (Roche). For analysis of IRF3 nuclear translocation, nuclear extracts were prepared from  $2 \times 10^6$  bone marrow macrophages stimulated for the times indicated in the figures using NE-PER nuclear extraction reagent (Pierce), supplemented with 1 $\times$  Complete Protease Inhibitor Cocktail, using the manufacturer's protocol. Protein levels were normalized using the micro-BCA kit (Thermo Fisher Scientific) and separated on 10% NuPAGE bis-tris gels (Invitrogen). Proteins were transferred to polyvinylidene fluoride membranes and immunoblotted with various primary antibodies. Whole-cell extracts were probed with anti-p38 MAP kinase, anti-phospho-p38 MAP kinase (Thr180/Tyr182), anti-ERK1/2, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-SAPK/JNK, and anti-phospho-SAPK/JNK (Thr183/Tyr185) antibodies from Cell Signaling Technology. Nuclear extracts were probed with anti-IRF3 and anti-lamin B (FL-425 and M-20; Santa Cruz Biotechnology, Inc.). For EMSA, nuclear extracts from stimulated bone marrow macrophages were incubated with a  $^{32}\text{P}$  end-labeled NF- $\kappa\text{B}$  probe (5'-GAT-CAGTTGAGGGGACTTTCCAGGC-3'). Protein-DNA complexes were resolved by native gel electrophoresis and visualized by autoradiography. Anti-p65 (C-20) was obtained from Santa Cruz Biotechnology, Inc.

**In vivo experiments.** C57BL/6 or *Ifi3/7*<sup>-/-</sup> mice were injected intraperitoneally with 200 nmol of chemically synthesized c-di-GMP. For analysis of innate immune responses, mice were sacrificed and analyzed 18–36 h after injection, as indicated in the figures. Serum was collected and tested for IFN as described above. Splenic NK cells were assayed ex vivo for production of IFN- $\gamma$  in response to YAC-1 target cells, as described previously (Joncker et al., 2009). For antibody responses, mice were injected with 25  $\mu\text{g}$  of endotoxin-free HSA (Sigma-Aldrich) mixed with 200 nmol c-di-GMP or saline. 2 wk after injection, mice were bled and serum IgG1 specific for HSA was measured by ELISA.

**Online supplemental material.** Fig. S1 shows production of Rantes in response to c-di-GMP in wild-type, *Myd88*<sup>-/-</sup>, *Trif*<sup>-/-</sup>, *Tnfr1*<sup>-/-</sup>, *Tbk1*<sup>-/-</sup>, *Tnfr1*<sup>-/-</sup>, *Mavs*<sup>-/-</sup>, *Ifi1*<sup>-/-</sup>, *Ifi3*<sup>-/-</sup>, *Ifi5*<sup>-/-</sup>, *Ifi7*<sup>-/-</sup>, and *Ifnar*<sup>-/-</sup> bone marrow-derived macrophages. Table S1 contains whole-transcriptome microarray gene expression data for bone marrow-derived macrophages transfected with c-di-GMP and pA:dT. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20082874/DC1>.

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