Research Article

Cytosolic d-type CpG-oligonucleotides induce a type I interferon response by activating the cGAS-STING signaling pathway

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Cytosolic DNA receptor cyclic GMP-AMP (cGAMP) synthase (cGAS) has been shown to be critically involved in the detection of cytosolic, self- and non-self-DNA, initiating a type I IFN response through the adaptor protein Stimulator of Interferon Genes (STING) and interferon regulatory factor 3 (IRF3). Current studies propose that canonical binding of dsDNA by cGAS depends on DNA length, but not on base sequence. In contrast, activation of TLR9 is sequence dependent. It requires unmethylated CpG dinucleotides in microbial DNA, which is mimicked by synthetic oligodeoxynucleotides (ODN). Here, we provide evidence that d-type ODN (D-ODN), but not K-type ODN (K-ODN), bind to human cGAS and activate downstream signaling. Transfection of D-ODN into a TLR9-deficient, human monocytic cell line (THP-1) induced phosphorylation of IRF3 and secretion of IFN. This response was absent in cells with CRISPR/Cas9-mediated cGAS- or STING-deficiency. Utilizing a protein pulldown approach, we further demonstrate direct binding of D-ODN to cGAS. Induction of a type I IFN response by D-ODN was confirmed in human primary monocytes and monocyte-derived macrophages. These results are relevant to our understanding of self–nonself-discrimination by cGAS and to the pharmacologic effects of ODN, which currently are investigated in clinical studies.

Keywords: cGAMP · cGAS · CpG-oligonucleotide · immunotherapy · innate immunity · interferons · pattern recognition receptors · STING · toll-like receptor 9 · type I interferon

Additional supporting information may be found online in the Supporting Information section at the end of the article.
Introduction

The utilization of synthetic oligonucleotides is currently being studied in a broad range of diseases, including malignancies, allergies, and infectious diseases, and oligodeoxynucleotides (ODN), have been approved as vaccine adjuvants [1, 2]. ODN contain unmethylated CpG motifs that are prevalent in bacterial and viral but not in vertebrate genomic DNA [3]. These CpG motifs are recognized by TLR9. Within human PBMC, functional TLR9 signaling in response to pathogens is restricted to B cells and plasmacytoid dendritic cells (PDC), while murine TLR9 is also expressed by monocytes and macrophages [4, 5]. Distinct classes of synthetic ODN have been described, depending on their structural and immunostimulatory properties [6–9]: K-ODN (also referred to as CpG-B) trigger early secretion of IFN-β and production of proinflammatory cytokines, including IL-6 and TNF-α, by PDC mediated by the transcription factors NF-κB and interferon regulatory factor (IRF5) [10]. K-ODN also activate B cells to proliferate and secrete immunoglobulins [11, 12]. D-ODN (also referred to as CpG-A) induce high levels of IFN-α secretion from PDC via an IRF7-dependent autocrine/paracrine feedback loop [7, 13, 14]. Structurally, K-ODN are linear, ss molecules containing multiple unmethylated CpG motifs. D-ODN form a central hairpin structure and have a poly-G tail that leads them to form G-tetrads [15].

While TLR9 binds CpG-containing DNA within endosomes, detection of cytosolic DNA is primarily mediated by the receptor cyclic GMP-AMP (cGAMP) synthase (cGAS). Upon binding of DNA, the protein cGAS induces GTP and ATP to form the second messenger cGAMP. cGAMP then binds stimulator of interferon genes (STING) [16–19], which triggers phosphorylation of IRF3 via TBK1. IRF3 acts as a transcriptional regulator of inflammatory genes and upregulates IFN-β [20]. Recent studies suggest a major role for the cGAS-STING pathway in both the immune defense against pathogens and in autoimmune diseases [21, 22]. The minimal cGAS activation motif is a matter of current scientific debate. Several studies suggest that human cGAS preferentially recognizes long dsDNA, with a minimum length requirement of 40-45 base pairs. The cGAS activation by long dsDNA is sequence independent [23–25]. This concept was challenged by Herzner et al., who observed that short stretches of base-paired DNA within ssDNA stem-loop structures of HIV-1 DNA activate cGAS in a sequence-dependent manner [26].

Although TLR9 was shown to be critical for CpG-ODN-induced immune responses, there is much less known about additional effects elicited by cytotoxic CpG-ODN. Better knowledge of potential TLR9-independent effects of CpG-ODN could lead to an improved rationale and indication of how to use various types of CpG-ODN in clinical settings.

In this study, we report that transfected D-ODN but not K-ODN initiate a type I IFN response by human monocytes and macrophages that lack functional TLR9. We further demonstrate direct binding of cGAS by D-ODN. Knockdown experiments confirmed that this immune response depends on cGAS-STING signaling and not on TLR9 sensing, suggesting that the cGAS-STING pathway plays a critical role in the cytosolic recognition of D-ODN.

Results

Cytosolic D-ODN induces IRF3 phosphorylation and type I IFN production by TLR9-deficient THP-1 cells

Primary human monocytes do not respond to CpG ODN stimulation of TLR9 [4, 27, 28] and can even suppress TLR9-induced IFN-secretion by PDC [29, 30]. Unexpectedly, we observed dose-dependent transcription and secretion of IFN-β by naive THP-1 cells transfected with D-ODN (Fig. 1A and B) at levels comparable to cytosolic herring testes DNA (htDNA, a dsDNA molecule known to activate cGAS). We also compared transfected D-ODN to the transfection reagent itself: transfected D-ODN induced significantly higher levels of IFN-β than lipofectamine alone (Supporting information Fig. S1A and B). Significant levels of IFN-β were also induced by p(I:C) and p(dA:dT) which trigger type I IFN responses through TLR3 and RIG-I-like receptors [31–33]. To examine whether transfected D-ODN induce downstream mediators of type I IFNs, we also investigated levels of CXCL10 [34]. Again, we found significant secretion of CXCL10 in response to D-ODN (Fig. 1C). Interestingly, low levels of IL-6 secretion were also induced by D-ODN transfection (Fig. 1D).

Since naive THP-1 cells are monocyteic they should not respond to TLR9 stimulation [4, 27, 28], an expectation confirmed by their lack of TLR9 expression determined by immunoblot (Fig. 1E). By comparison, TLR9 protein was detected in TLR9-transfected HEK293 cells and in CAL-1 cells, a PDC-like cell line known to express that receptor [7, 35] (Fig. 1E). TLR9 expression was not inducible by transfection of D-ODN, htDNA, or cGAMP (Supporting information Fig. S1C).

TLR9-dependent induction of type I IFN by CpG-ODN depends on the activation of IRF5 and IRF7 [3], whereas IRF3 mediates the IFN response to cytosolic dsDNA [20, 36, 37]. We, therefore, evaluated whether D-ODN activated IRF3 in THP-1 cells. Results showed that cells transfected with D-ODN phosphorylated IRF3 as did the positive controls dsDNA and p(dA:dT) [20, 32, 38, 39] (Fig. 1F, quantification in Supporting information Fig. S1D). In summary, these findings suggest the involvement of cytosolic receptors rather than endosomal TLR9 in the induction of type I IFN by THP-1 transfected with D-ODN.

Transfection of D-ODN induces STING oligomerization

To further define the signaling pathway triggered by cytosolic D-ODN upstream of IRF3, we evaluated the activation of the cGAS-STING pathway. Cytosolic DNA induces synthesis of cGAMP by cGAS, which acts as second messenger, promoting STING activation and oligomerization into a supramolecular complex [40]. HEK cells stably transduced with an mCherry-tagged STING construct (henceforth “HEK Sting”) do not express cGAS. Sting oligomerization within HEK Sting cells can be stimulated if cocultured with cGAMP producing cells (cGAMP is transferred between cells through gap junctions) [40–42]. HEK cells stably expressing
Figure 1. Cytosolic D-ODN induces type I IFN and pIRF3 in TLR9-deficient cells. Different doses (as indicated by color) of D-ODN, htDNA, p(dA:dT), or p(I:C) were transfected into THP-1 cells using lipofectamine. (A) Cells were harvested for RNA isolation after 6 h and relative levels of IFN-β mRNA were quantified in relation to naïve controls (qRT-PCR). Results show means ± SEMs from five independent experiments with “n”/condition: n = 5 for naïve control, D-ODN 1 μg/mL and htDNA; n = 4 for D-ODN 0.1 μg/mL and 0.3 μg/mL; n = 3 for D-ODN: 0.03 μg/mL, p(I:C) and p(dA:dT). Samples from independent experiments were performed in triplicates. (B-D) The concentration of the indicated cytokines was analyzed by ELISA 24 h after addition of ligands. Results show means ± SEMs. “n”/condition (from independent experiments): (B) n = 8 for naïve control and D-ODN 1 μg/mL; n = 7 for D-ODN 0.1 μg/mL and 0.3 μg/mL; n = 5 for htDNA 1 μg/mL; n = 4 for D-ODN 0.03 μg/mL, p(I:C) and p(dA:dT); n = 3 for D-ODN 3 μg/mL and ht-DNA 3 μg/mL. (C) n = 9 for naïve control and D-ODN 1 μg/mL; n = 8 for D-ODN 0.1 μg/mL and 0.3 μg/mL; n = 6 for htDNA 1 μg/mL and p(dA:dT);
high levels of cGAS (henceforth “HEK cGAS<sup>low</sup>”) activated HEK Sting cells in the same culture [41, 42]. STING oligomerization by HEK cells with low cGAS expression (henceforth “HEK cGAS<sup>low</sup>”) was significantly increased by D-ODN transfection (Fig. 2A, quantification in Fig. 2B), but not by K-ODN transfection (Supporting information Fig. S2). Together, these data indicate that cytosolic D-ODN induce synthesis of cGAMP and activate STING, consistent with D-ODN directly activating cGAS.

**IFN-β-induction by D-ODN requires cGAS and STING**

Since HEK cGAS<sup>low</sup> cells transfected with D-ODN triggered STING oligomerization in bystander cells, we examined whether cGAS itself was required for sensing transfected D-ODN. WT, cGAS KO, STING KO, and cGAS/STING double-knockout (DKO) THP-1 cells were transfected with D-ODN. Consistent with previous results (Fig. 1), D-ODN induced secretion of IFN-β (Fig. 3A, left panel) and CXCL10 (Fig. 3A, middle panel) by WT THP-1 cells. D-ODN also upregulated IFN-β gene expression in these cells (Supporting information Fig. S3A). This cytokine response was not detected in cGAS KO, STING KO, and DKO cells (Fig. 3B to D and Supporting information Fig. S3B to D).

A comprehensive set of control ligands established the validity of the experimental setting using KO cells. cGAMP induced expression and secretion of IFN-β and CXCL10 by cGAS KO cells (Fig. 3D) but not DKO or STING KO cells (Fig. 3B and C). In response to transfection with p(I:C) and p(dA:dT), which signal independently of cGAS or STING, secretion of IFN-β and CXCL10 was detected in WT cells and all KO cell lines (Fig. 3A to D). Similarly, LPS (signaling through TLR4) triggered IL-6 secretion by all cell lines (Fig. 3A to D) concluding that all KO effects were specific and the cells functionally intact. These findings demonstrate that both cGAS and STING are required for the type 1 IFN response elicited by cytosolic D-ODN.

**D-ODN but not K-ODN binds to and activates cGAS**

The ability of nontransfected D-ODN to induce type I IFN production is sequence dependent. The critical hexameric CpG motif lies at the apex of a stem-loop structure composed of three self-complimentary phosphodiester base pairs [6]. This class of ODN also carries a 3′-prime poly-G-tail composed of phosphorothioate (PO) nucleotides. In contrast, K-ODN are ss, linear 12–30 mers with up to three CpG-motifs composed completely of phosphodiester (PS) nucleotides and lacking either a palindromic motif or poly-G tail [7].

We set out to determine whether cGAS/STING activation depends on the class of the transfected CpG ODN. In contrast to htDNA and D-ODN, transfection with K-ODN did not induce significant IFN-β mRNA expression by THP-1 cells (Fig. 4A, left panel). K-ODN similarly failed to induce secretion of IFN-β, CXCL10 (Fig. 4A, middle and right panel) or IL-6 (data not shown). These findings verify that THP-1 cells do not respond to TLR9 agonists [7].

G10 is a D-ODN used in clinical studies targeting allergic diseases and solid tumors [43–46]. G10 is composed of three central CpG motifs in a 30 mer and contains both a 3′ and 5′ poly-G-tails on a PO backbone. To investigate whether D-ODN lacking a PS backbone can activate cGAS, we transfected WT and KO cells with G10 and compared the effect to D-ODNs with backbones including PS nucleotides. Significant secretion of IFN-β and CXCL10 was elicited by all of these constructs in the WT but not STING KO or cGAS KO cells (Fig. 4). Stimulation with p(dA:dT) or LPS induced CXCL10 and IL-6 secretion (respectively) in all cell lines, confirming their viability and responsiveness (Fig. 4B to D). Following transfection of cGAMP, we detected significant secretion of IFN-β and CXCL10 in WT and cGAS KO but not in STING KO cells.

To further corroborate these results, we also studied IRF3 phosphorylation upstream of IFN secretion. Phosphorylation of IRF3 was observed after transfection of both D-ODN and G10 but not K-ODN (Fig. 4E). cGAMP and htDNA were used as positive controls.

Utilizing a protein pulldown approach, we investigated whether these ODN interacted directly with cGAS and whether this interaction was ODN class dependent. THP-1 lysates were exposed to biotinylated DNA constructs which were then captured by streptavidin beads. Biotinylated htDNA, D-ODN, and G10 all pulled down cGAS protein while K-ODN did not (Fig. 4F and Supporting information Fig. S4). As expected, biotinylated D-ODN and htDNA did not bind cGAS in cGAS-KO cells. This set of experiments shows that cytosolic D- but not K-class ODN binds to and activate the canonical cGAS/STING signaling pathway.

**Transfected D-ODN induces type I IFN in primary human monocytes and macrophages**

THP-1 is a monocyte-like cell line originally derived from a patient with acute monocytic leukemia. To determine whether primary monocytes from healthy humans are capable of sensing cytosolic D-ODN, we isolated CD14<sup>+</sup> cells from healthy volunteers. When transfected with D- but not K-ODN, these cells responded with IFN-β and IL-6 secretion (Fig. 5A). In monocytes,
Figure 2. Transfection of D-ODN induces STING oligomerization. STING aggregate formation (mCherry clustering) was examined by fluorescence microscopy in HEK Sting cells (see Materials & Methods) cocultured for 24 h with either HEK cGAS low or HEK cGAS high cells transfected with the indicated DNA ligands (3 μg/mL). (A) Representative images from three independent experiments are shown (20× magnification, scale bar 50 μm). (B) STING aggregates were automatically quantified, means ± SEMs from three representative visual fields from independent experiments are shown. Statistical significance was determined by unpaired student’s t-test. *p < 0.05 versus negative control (HEK cGAS low + HEK Sting).

CXCL10 secretion was significantly higher after transfection of D-ODN compared to K-ODN. cGAMP and htDNA served as positive controls in these experiments. Similarly, IFN-β, CXCL10, and IL-6 secretion was significantly detected in human macrophages derived from CD14+ selected monocytes transfected with D-ODN. (Fig. 5B). Of note, transfection reagent alone or nontransfected D-ODN had no effects on cytokine production. Transfection of all DNA-constructs exerted some cytotoxic effects (Supporting information Fig. S5), as measured by activity of lactate dehydrogenase (LDH) in the supernatant. These effects, however, did not correlate with the capability of DNA-constructs to induce IFN-β.

Consistent with these findings, we observed expression of cGAS, but not TLR9 in human monocyte-derived macrophages by using immunoblot (Fig. 5C and D).

We conclude that the ability to sense cytosolic D-ODN is not confined to immortalized cell lines (HEK293, THP-1).

Discussion

Previous studies showed that D-ODN-driven IFN production was TLR9 dependent (cells lacking TLR9 did not respond). This manuscript suggests that D-ODN can signal via two independent mechanisms of action. One is TLR9 dependent and the other is cGAS/STING dependent.

This work describes a novel signaling pathway through which D-class ODN induces type I IFN secretion. TLR9-negative THP-1 cells transfected with D-ODN were stimulated to initiate robust IFN-β and CXCL10 secretion. Importantly, K-ODN did not activate these cells suggesting that the CpG sequence alone is insufficient to elicit this response. We further demonstrated STING oligomerization in response to transfected D-ODN in TLR9-deficient HEK293 cells. Utilizing cGAS/STING-KO cells, we proved that the cGAS/STING-pathway is required to detect cytosolic D-ODN. Finally, we demonstrate that D- but not K-ODN directly binds to cGAS. These findings were validated in primary human monocytes, increasing their clinical relevance, and represent the first description of CpG-ODN-mediated activation of the cGAS/STING-signaling pathway.

Current research shows that cGAS binds to the sugar phosphate backbone of dsDNA [47, 48]. Indeed, we previously demonstrated that inhibition of cGAS activity by binding the 24-bp ODN A151 is partially mediated by its PS backbone [42]. Consistent with this, we could not detect cGAS activation by K-ODN on a PS backbone (Fig. 4). By comparison, the D-ODNs that activated cGAS both contained stretches of PO nucleotide DNA (Fig. 3 and 4).

Recent studies demonstrated that the backbone structure and length but not sequence of dsDNA determine cGAS:dsDNA interaction and downstream signaling [21, 23–25, 47–50]. Jakobsen et al. showed that cytosolic HIV-1 derived ssDNA shorter than 40-bp is also capable of inducing type I IFN [51]. This stimulatory capacity depended on stretches of dsDNA within the stem-loop structure and was mediated by IFI16 [51, 52]. Similarly, Herzner et al. demonstrated cGAS-signaling in response to HIV-1-derived short ssDNA [26]. The stimulatory capacity of such short ssDNA was found to be sequence dependent: the minimal cGAS recognition motif (termed “G3-YSD”) forms a stem-loop structure due to its palindromic DNA sequence, with a base-paired stem of...
Figure 3. IFN-β induction by D-ODN requires cGAS and STING. (A) Naïve THP-1 cells (WT) were transfected with the indicated ligands or treated with LPS (all ligands at 1 μg/ml). After 24 h, supernatants were analyzed for IFN-β, CXCL10, and IL-6. Results show means ± SEMs from four (left panel, IFN-β), seven (middle panel, CXCL10), and five (right panel, IL-6) samples from independent experiments. (B) THP-1 cells with combined KO of cGAS and STING were treated and analyzed as in (A). Results show means ± SEMs from four (left panel, IFN-β), six (middle panel, CXCL10), and four (right panel, IL-6) samples from independent experiments. (C) THP-1 cells with exclusive KO of STING were treated and analyzed as in (A). Results show means ± SEMs from four (left panel, IFN-β), seven (middle panel, CXCL10), and five (right panel, IL-6) samples from independent experiments. (D) THP-1 cells with exclusive KO of cGAS were treated and analyzed as in (A). Results show means ± SEMs from four (left panel, IFN-β), seven (middle panel, CXCL10), and five (right panel, IL-6) samples from independent experiments. Statistical significance was determined by unpaired student’s t-test. *p < 0.05 versus negative control. #p < 0.05 versus D-ODN.
Figure 4. D-ODN but not K-ODN binds and activates cGAS. (A) Naïve THP-1 cells (WT) were exposed to LPS or transfected with the indicated ligands (all ligands: 1 μg/mL). After 6 h, cells were harvested and IFN-β mRNA was quantified by qRT-PCR (left panels) or concentrations of the indicated cytokines were determined 24 h after transfection in cell supernatants by ELISA (middle and right panels). Results show means + SEMs from six independent experiments performed in triplicates (left panel, qRT-PCR). "n/condition (from independent experiments) for IFN-β protein: n = 8 for naïve control and D-ODN; n = 5 for htDNA and K-ODN. *”/condition (from independent experiments) for CXCL10 protein: n = 8 for naïve control and D-ODN; n = 6 for htDNA; n = 5 for K-ODN. (B) Naïve THP-1 cells (WT) were transfected with the indicated ligands. Concentrations of indicated cytokines were determined as in (A). Results show means + SEMs from three samples from independent experiments. (C) THP-1 cells with KO of STING were treated and analyzed as in (A). Results show means + SEMs from samples from three (IFN-β, left panel), six (CXCL10, middle panel), or five (IL6, right panel) independent experiments. (D) THP-1 cells with KO of cGAS were treated and analyzed as in (A). Results show means + SEMs from samples from three (IFN-β, left panel), six (CXCL10, middle panel), or five (IL6, right panel) independent experiments. (E) Left panel: phosphorylation of IRF3 was determined by western blot 24 h after transfection of indicated ligands into WT THP-1 cells. "—": Untreated cells. Right panel: quantification of relative protein (pIRF3/IRF3 in %) as determined by western blot from three independent experiments. (E, F) One representative blot of three independent experiments is shown. Statistical significance was determined by paired (qRT-PCR) and unpaired (ELISA) student’s t-test (B through D: one-sided). * p < 0.05 versus negative control.

12-20 bp of dsDNA flanked by two or three unpaired Gs at either side. Of note, Herzner et al. concluded that intermolecular quadruplex interactions—which are facilitated by longer G-rich sequences—were not involved in G3-YSD recognition. In our study, we used predefined ODN which had been designed for optimal stimulation of TLR9 in primary human PBMC [7, 10, 53]. Interestingly, all D-ODN contained a palindromic sequence, forming stem-loop structures with a stem of 3-4 bp of dsDNA and 2-9 Gs at either end. In principle, this is consistent with the minimal cGAS activation motif described by Herzner et al., extending
their finding that stretches of 12-bp dsDNA are sufficient for cGAS activation to even shorter stretches of dsDNA [26].

In summary, we demonstrate a completely novel mechanism of ODN-sensing by the innate immune system in the cytosol. Our work is the first to establish that cytosolic D-ODN, but not K-ODN, are capable of inducing a robust cGAS/STING-dependent IFN response. Our study also confirms that cGAS is capable of sensing short stretches of dsDNA within ssDNA stem-loop structures. Future studies should be directed toward dissecting the precise requirements for D-ODN sensing by cGAS. Based on our findings, we suggest that activation of cGAS by cytosolic D-ODN depends on a central palindromic sequence, resulting in a stem-loop structure with stretches of dsDNA, flanked by at least two Gs at either side. We cannot rule out the possibility that the ODN backbone also contributes to cGAS activity. Future studies need to investigate whether cGAS-stimulation depends on higher, quaternary structures mediated by longer poly-G tails and Hoge-steen effects and whether D-ODN on PS backbones are capable of cGAS signaling.

Some earlier studies also documented TLR9-independent immunological effects of ODN [13, 54–57]. Based on our data, experimental results obtained with transfected CpG-ODNs [13, 56, 57] must be interpreted carefully, meticulously distinguishing between TLR9- and cGAS-dependent effects. Future studies need to clarify whether cGAS-stimulation by ODN can be exploited clinically such as for vaccination or cancer therapy. Carefully selected and transfected ODN could potentially combine the activation of B cells, PDC, and macrophages. In the case of tolerogenic ODN, it needs to be elucidated whether cGAS stimulation needs to be avoided. In addition, future studies need to investigate whether highly TLR9- or cGAS-selective ODN can be synthesized, depending on optimal sequence and structure.

Materials and methods

All cell culture and transfection reagents were tested negative for endotoxin (<0.1 EU/mL) by LAL assay.

Transfection of cGAMP, htDNA, and ODN

Endotoxin-free (lack of response in HEK-TLR4 cells) cGAMP (cyclic [G(2',5')pA(3',5')p]) was purchased from Invivogen (San Diego, CA, USA). dsDNA isolated from herring testis (htDNA) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). For some studies, an equimolar mixture of three ODN sequences was used (“D-ODN” and “K-ODN”), as previous studies demonstrated that such mixtures more consistently stimulated cells from multiple donors than did individual ODN. Sequences and backbone structure can be found in Table 1. Endotoxin-free (<0.1 EU/mL at 1 mg/mL) D-ODN (equimolar mixture of D19, D29, and D35) and K-ODN (equimolar mixture of K3, K23, and K123) were synthesized at the Core Facility of the Center for Biologics Evaluation and Research facility, Food and Drug Administration (Bethesda, MD, USA) and kindly provided by D. M. Klinman [6]. A. M. Krieg kindly provided G10.

Lipofectamine 2000 was purchased from Thermo Fisher Scientifics (Walhach, MA, USA). All stimulation experiments were performed according to the manufacturer’s instructions. For transfection experiments, cells were seeded at 1 × 10⁶/well (24-well format) in 1 mL of culture medium. Primary cells were seeded at lower densities (3 × 10⁵–8 × 10⁵/well) and reagents were downscaled accordingly.
Figure 5. Transfected D-ODN induces type I IFN in primary human monocytes and macrophages. (A) Primary human monocytes isolated by CD14-selective MACS were transfected with the indicated ligands (1 μg/mL). After 24 h, concentrations of IFN-β (left panel), CXCL10 (middle panel), and IL-6 (right panel) in the supernatants were determined by ELISA. Results show means ± SEMs from three independent experiments with a total of six donors. (B) Concentrations of IFN-β (left panel), CXCL10 (middle panel), and IL-6 (right panel) in the supernatants of human monocyte-derived macrophages were determined as in (A). Results show means ± SEMs from four independent experiments with a total of six individual donors. (C) cGAS-protein and (D) TLR9-protein were determined in cell lysates from human monocyte-derived macrophages after stimulation with the indicated ligands for 24 h (western blot). HEK293 cells with TLR9 served as control. “—”: Untreated cells. In (C) and (D), two independent experiments with two individual donors are shown. Statistical significance was determined by unpaired student’s t-test. *p < 0.05 versus negative control. **p < 0.05 versus K-ODN.

Cell culture

The CAL-1 pDC line was grown in complete RPMI 1640 medium (Lonza, Walkersville, MD, USA), supplemented with heat-inactivated 10% FCS (Biochrome, Berlin, Germany). HEK293T cells were maintained in DMEM supplemented with heat-inactivated 10% FCS and 1% sodium-pyruvate (Thermo Fisher Scientific). THP-1 cells with targeted mutations were kindly provided by T. Zillinger. To generate targeted mutations, THP-1 cells were coelectroporated with a gRNA- and a Cas9-expression plasmid for cGAS KO and for STING KO as described earlier [42]. The gRNA target sequences used were GCGCGCCGTC-CGCGCAACT(GGG) for cGAS KO and CTAGCCCCAAAGGGTG- CACC(AGG) for STING KO (PAM sequence in parenthesis). Sequential KO procedures were performed to create DKO cells.

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Table 1. Classification, names, and sequences of all ODN used in this study

| ODN-type | Name  | Sequence                  |
|----------|-------|---------------------------|
| D        | D19   | 5’-GGtgcatcgatgcagGGGGG-3’|
| D        | D29   | 5’-GGtgccaggcgcagGGGGG-3’|
| D        | D35   | 5’-GgtgcatcgatgcaggggGG-3’|
| D        | G10   | 5’-gggggggggggacgatcgtcgggggggggg-3’|
| K        | K3    | 5’-ATCGACTCTCGAGCGTTCTC-3’|
| K        | K23   | 5’-TCGAGGTTCTCTC-3’       |
| K        | K123  | 5’-TCGTTCTGTTCTC-3’       |

Capital letters depict bases with phosphorothioate backbone, lowercase letters depict a phosphodiester backbone.

All studies using primary cells were performed after written approval from the ethics committee of the medical faculty of the university of Bonn (“Ethik-Kommission der Medizinischen Fakultät,” “Rheinische Friedrich-Wilhelms Universität Bonn”) and after obtaining written informed consent from the donors. Investigations were conducted according to the principles expressed in the Declaration of Helsinki. Primary human monocytes were isolated from peripheral blood by CD14+ selection, following the manufacturer’s protocol (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in supplemented RPMI 1640 (including L-glutamine, 10% heat-inactivated FCS, and 1% sodium pyruvate).

Detection of cytokines by ELISA

Commercially available ELISA kits for IFN-β, IL6, and CXCL10 were purchased from R&D Systems (Minneapolis, MN, USA). All ELISA assays were performed according to the manufacturer’s instructions.

qRT-PCR

Total RNA was extracted from cells using TRIzol reagent (Thermo Fisher Scientific), as specified by the manufacturer; cDNA was synthesized with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer’s instructions. Gene expression levels (normalized to 18s) were analyzed using the ViiA 7 Real-Time PCR system (Applied Biosystems). All reagents and probes used in these studies were purchased from Applied Biosystems. The following TaqMan assays were used: IFN-B (Hs00985639 m1), 18s (Hs0287368 g1).

Western Blots

Cells were lysed in Laemmlı buffer and denatured at 95°C for 5min. Cell lysates were separated by 4-15% Mini-PROTEAN TGX gel (Bio-Rad Laboratories, Munich, Germany) and transferred onto nitrocellulose membranes. Blots were incubated with antiphospho-IRF3 (4D4G) or anti-IRF3 (D83B9) primary antibodies and anti-rabbit-IgG-HRP as secondary antibody. TLR9 blots were performed accordingly, with anti-TLR9 (D9M9H) as primary and anti-rabbit-IgG-HRP as secondary antibody. All antibodies were from Cell Signaling Technology (Cambridge, UK).

Relative amount protein was quantified using ImageJ software (NIH, USA). The amount of protein was measured as the grey mean value within a specified region of interest. Background measurements were subtracted. Finally, the amount of protein was expressed in relation to the corresponding loading control, corrected for background.

Pulldown assay

DNA (either D-ODN, K-ODN, G10, or htDNA) was biotinylated using the biotin-high prime kit (#11585649910; Roche Diagnostics, Mannheim, Germany). For pulldown of endogenous cGAS, 2 × 10^6 THP-1 cells were lysed in RIPA lysis buffer (#9806S, Cell Signaling Technology). Cell debris was removed by centrifugation. Total cell lysate was incubated with 6 μg 3’-biotinylated DNA (either D-ODN, K-ODN, G10, or htDNA) for 2 h at 4°C followed by prewashed streptavidin-agarose beads (50% w/v) for 2 h at 4°C. bead pellets were washed, boiled in Laemmlı buffer, and run on a 10% SDS-polyacrylamide gel. Blots were probed with anti-cGAS (D1D3G, Cell Signaling Technology).

Fluorescence microscopy

Coculture experiments of cGASHigh and cGASlow HEK293T cells with STING-expressing HEK293T cells were performed as previously published [42]. Briefly, 1.5 × 10^4 cGASHigh or cGASlow cells were cocultured with 1.5 × 10^4 STING reporter cells in a 96-well format and stimulated as described above. After 24 h, images were collected using an Olympus IX81 microscope with 20× magnification. STING complexes were automatically counted using the ZEN imaging software (Zeiss, Jena, Germany).

Cytotoxicity assay

Cytotoxic effects of DNA transfection were measured utilizing a commercially available assay (Promega, USA). The colorimetric assay indirectly quantifies the activity of LDH in the supernatant by measuring the production of formazan. Cells treated with lysis buffer were used as positive control.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Data are expressed as mean ± SEM with p < 0.05 considered statistically significant, as determined by two-sided student’s t-test and indicated by “*” within the figures, unless stated otherwise.
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References

1 Vollmer, J. and Krieg, A. M., Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. Adv. Drug Deliv. Rev. 2009. 61: 195–204.
2 Scheiermann, J. and Klinman, D. M., Clinical evaluation of CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases and cancer. Vaccine. 2014. 32: 6377–6389.
3 Kawai, T. and Akira, S., The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Immunol. 2010. 11: 373–384.
4 Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdörfer, B., Giese, T., Endres, S. et al., Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J. Immunol. 2002. 168: 4531–4537.
5 Krieg, A. M., CpG motifs in bacterial DNA and their immune effects. Annu. Rev. Immunol. 2002. 20: 709–760.
6 Verthelyi, D., Ishii, K. J., Gursel, I., Takeshita, F. and Klinman, D. M., Human peripheral blood cells differentially recognize and respond to two distinct CpG motifs. J. Immunol. 2001. 166: 2372–2377.
7 Steinhagen, F., Meyer, C., Tross, D., Gursel, M., Maeda, T., Klaschik, S. and Klinman, D. M., Activation of type I interferon-dependent genes characterizes the “core response” induced by CpG DNA. J. Leukoc. Biol. 2012. 92: 775–785.
8 Bode, C., Zhao, G., Steinhagen, F., Kinjo, T. and Klinman, D. M., CpG DNA as a vaccine adjuvant. Expert Rev. Vaccines. 2011. 10: 499–511.
9 Steinhagen, F., Kinjo, T., Bode, C. and Klinman, D. M., TLR-based immune adjuvants. Vaccine. 2011. 29: 3341–3355.
10 Steinhagen, F., McFarland, A. P., Rodriguez, L. G., Tewary, P., Jarret, A., Savan, R. and Klinman, D. M., IRF-5 and NF-κB p50 co-regulate IFN-α and IL-6 expression in TLR9-stimulated human plasmacytoid dendritic cells: innate immunity. Eur. J. Immunol. 2013. 43: 1896–1906.
11 Hartmann, G., Weaverna, R. D., Ballas, Z. K., Payette, P., Blackwell, S., Suparto, I., Rasmussen, W. L. et al., Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. J. Immunol. 2000. 164: 1617–1624.
12 Gürsel, I., Verthelyi, D., Gursel, I., Ishii, K. J. and Klinman, D. M., Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotides. J. Leukoc. Biol. 2002. 71: 813–820.
13 Honda, K., Ohba, Y., Yanai, H., Negishi, H., Mizutani, T., Takaoka, A., Taya, C. et al., Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. Nature. 2005. 434: 1035–1040.
14 Kerkmann, M., Rothenfusser, S., Hornung, V., Towarowski, A., Wagner, M., Sarris, A., Giese, T. et al., Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of type I IFN synthesis in human plasmacytoid dendritic cells. J. Immunol. 2003. 170: 4465–4474.
15 Klinman, D. M., Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat. Rev. Immunol. 2004. 4: 249–258.
16 Ablasser, A., Gerlof, M., Cavlar, T., Deimling, T., Witte, G., Röhl, I., Hopfner, K.-P. et al., CGAS produces a 2′-5′-linked cyclic dinucleotide second messenger that activates STING. Nature. 2013. 498: 380–384.
17 Diner, E. J., Burdette, D. L., Wilson, S. C., Monroe, K. M., Kellenberger, C. A., Hyodo, M., Hayakawa, Y. et al., The innate immune DNA sensor cGAS produces a noncanonical cyclic dinucleotide that activates human STING. Cell Reports. 2013. 1: 1355–1361.
18 Du, M. and Chen, Z. J., DNA-induced liquid phase condensation of cGAS activates innate immune signaling. Science. 2018. 361: 704–709.
19 Burdette, D. L., Monroe, K. M., Sotelo-Troha, K., Iwig, J. S., Eckert, B., Hyodo, M., Hayakawa, Y. et al., STING is a direct innate immune sensor of cyclic di-GMP. Nature. 2011. 478: 515–518.
20 Liu, S., Cai, X., Wu, J., Cong, Q., Chen, X., Li, T., Du, F. et al., Phosphorylation of innate immune adaptor proteins MAVS, STING, and TRIF induces IRF3 activation. Science. 2015. 347: aaa2630–aaa2630.
21 Ablasser, A. and Chen, Z. J., CGAS in action: expanding roles in immunity and inflammation. Science. 2019. 363: eaat8657.
22 Cai, X., Chiu, Y.-H. and Chen, Z. J., The cGAS-cGAMP-STING pathway of cytosolic DNA sensing and signaling. Molecular Cell. 2014. 54: 289–296.
23 Wu, J., Sun, L., Chen, X., Du, F., Shi, H., Chen, C. and Chen, Z. J., Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cyclic di-GMP. Science. 2013. 339: 826–830.
24 Andreeva, L., Hiller, B., Kostrewa, D., Lässig, C., de Oliveira Mann, C. C., Jan Drexl, D.,aiser, M. et al., cGAS senses long and HMGB/TFAM-bound U-turn DNA by forming protein-DNA ladders. Nature. 2017. 549: 394–398.
25 Zhou, W., Whiteley, A. T., de Oliveira Mann, C. C., Morehouse, B. R., Nowak, R. P., Fischer, E. S., Gray, N. S. et al., Structure of the human cGAS–DNA complex reveals enhanced control of immune surveillance. Cell. 2018. 174: 300–311.e11.
26 Herzner, A.-M., Hagmann, C. A., Goldeck, M., Wolter, S., Kübler, K., Wittmann, S., Gramberg, T. et al., Sequence-specific activation of the DNA sensor CGAS by Y-form DNA structures as found in primary HIV-1 cDNA. Nat Immunol. 2015. 16: 1025–1033.
27 Kadewski, N., Ho, S., Antonenko, S., Malefyt, R. W., Kastelein, R. A., Bazan, F. and Liu, Y. J., Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J. Exp. Med. 2001. 194: 863–869.
28 Iwasaki, A. and Medzhitov, R., Toll-like receptor control of the adaptive immune responses. Nat Immunol. 2004. 5: 987–995.
clear cells. TLR9-dependent IFNA1 secretion in human peripheral blood mononuclear cells. PloS ONE. 2013: e65024.

Alexopoulou, L., Takaoka, A., Bongartz, T., Speiser, D. E., Ablasser, A., Latz, E., Katsouris, P., Klune, B., Bode, C., Hoeft, A. and Poth, J. M., Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. 2001: 413: 732–738.

Ablasser, A., Baurainfeird, F., Hartmann, G., Latz, E., Fitzgerald, K. A. and Honung, V., RIG-I-dependent sensing of poly(AD:DT) through the induction of an RNA polymerase III-transcribed RNA intermediate. Nat. Immunol. 2009: 10: 1065–1072.

Kawai, T. and Akira, S., Toll-like receptor and RIG-I-like receptor signaling. Ann. N. Y. Acad. Sci. 2008: 1143: 1–20.

Holm, C. K., Jensen, S. B., Jomhorsen, N., Horan, K. A., Moeller, H. B., Gonzalez-Dosal, R. et al., Virus-cell fusion as a trigger of innate immunity dependent on the adaptor STING. Nat. Immunol. 2012: 13: 737–743.

Hiltbert, T., Steinhagen, F., Weisheit, C., Baumgarten, G., Hoef, A. and Poissant, J. M., Synergistic stimulation with different TLR7 ligands modulates gene expression patterns in the human plasmacytoid dendritic cell line CAL-1. Mediators Inflamm. 2015: 2015: 948540.

Honung, V. SnapShot: nucleic acid immune sensors, Part 1. Immunity. 2014: 41: 868–888.

Honung, V. SnapShot: nucleic acid immune sensors, Part 2. Immunity. 2014: 41: 1066–1066.

Cheng, G., Zhong, J., Chung, J. and Chisari, F. V., Double-stranded DNA and double-stranded RNA induce a common antiviral signaling pathway in human cells. Proc. Natl. Acad. Sci. U.S.A. 2007: 104: 9035–9040.

Takaoaka, A., Wang, Z., Choi, M. K., Yanai, H., Negishi, H., Ban, T., Li, Y. et al., DAI (DLM-1/2BP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature. 2007: 448: 501–505.

Ablasser, A., Schmid-Burgk, J. L., Hemmerling, I., Horvath, G. L., Schmidt, T., Latz, E. and Honung, V., Cell intrinsic immunity spreads to bystander cells via the intercellular transfer of cGAMP. Nature. 2013: 503: 530–534.

Bode, C., Fox, M., Tewary, P., Steinhagen, A., Ellermann, R. K., Klinman, D., Baumgarten, G. et al., Human plasmacytoid dendritic cells elicit a Type I interferon response by sensing DNA via the cGAS-STING signaling pathway: innate immunity. Eur. J. Immunol. 2016: 46: 1615–1621.

Steinhagen, F., Zillinger, T., Peukert, K., Fox, M., Thudium, M., Barchet, W., Putensen, C. et al., Suppressive oligodeoxynucleotides containing TTAGGG motifs inhibit cGAS activation in human monocytes. Eur. J. Immunol. 2018: 48: 605–611.

Sent, G., Johansen, P., Haug, S., Bull, C., Gottschall, C., Müller, P., Pfister, T. et al., Use of A-type CpG oligodeoxynucleotides as an adjuvant in allergen-specific immunotherapy in humans: a phase IIa clinical trial. Clin. Exp. Allergy. 2009: 39: 562–570.

Spieker, D. E., Schwarz, K., Baumgaertner, P., Manolova, V., Devere, E., Sterry, W., Walden, P. et al., Memory and effector CD8 T-cell responses after nanoparticle vaccination of melanoma patients. J. Immunol. 2010: 83: 848–857.

Klimek, L., Willers, J., Hammann-Haenni, A., Pfaar, O., Stocker, H., Mueller, P., Renner, W. N. et al., Assessment of clinical efficacy of CYT003-QbG10 in patients with allergic rhinoconjunctivitis: a phase IIb study. Clin. Exp. Allergy. 2011: 41: 1305–1312.

Beeh, K.-M., Knauss, F., Wagner, F., Schilder, C., Naudts, I., Hammann-Haenni, A., Willers, J. et al., The novel TLR-9 agonist QbG10 shows clinical efficacy in persistent allergic asthma. J. Allerg. Clin. Immunol. 2013: 131: 866–874.

Civil, F., Deiming, T., de Oliveira Mann, C. C., Ablasser, A., Moldt, M., Witte, G., Honung, V. et al., Structural mechanism of cytosolic DNA sensing by cGAS. Nature. 2013: 498: 332–337.

Gao, P., Ascana, M., Wu, Y., Barchet, W., Gaffney, B. L., Zillinger, T., Seganov, A. A. et al., Cyclic [G(2',5')pA(3',5')p] is the metazoan second messenger produced by DNA-activated cyclic GMP-AMP synthase. Cell. 2013: 153: 1094–1107.

Li, X., Shu, C., Yi, G., Chaton, C. T., Shelton, C. L., Diao, J., Zuo, X. et al., Cyclic GMP-AMP synthase is activated by double-stranded DNA-induced oligomerization. Immunity. 2013: 39: 1019–1031.

Zhang, X., Wu, J., Du, F., Xu, H., Sun, L., Chen, Z., Brautigam, C. A. et al., The cytosolic DNA sensor cGAS forms an oligomeric complex with DNA and undergoes switch-like conformational changes in the activation loop. Cell Reports. 2014: 6: 421–430.

Jakobsen, M. R., Bak, R. O., Andersen, A., Berg, R. K., Jensen, S. B., Jin, T., Laustsen, A. et al., PNAS Plus: from the Cover: IFI16 senses DNA forms of the lentiviral replication cycle and controls HIV-1 replication. Proc Natl Acad Sci. 2013: 110: E4571–E4580.

Unterholzer, L., Keating, S. E., Baron, M., Horan, K. A., Sharma, S., Siros, C. M. et al., IFI16 is an innate immune sensor for intracellular DNA. Nat. Immunol. 2010: 11: 997–1004.

Verethyi, D., Kenney, R. T., Seder, R. A., Gam, A. A., Friedag, B. and Klinman, D. M., CpG oligodeoxynucleotides as vaccine adjuvants in primates. J. Immunol. 2002: 168: 1659–1663.

Sanjuan, M. A., Rao, N., Lai, K.-T. A., Gu, Y., Sun, S., Fuchs, A., Fung-Leung, W.-P. et al., CpG-induced tyrosine phosphorylation occurs via a TLR9-independent mechanism and is required for cytokine secretion. J. Cell Biol. 2006: 172: 1057–1068.

Pivarsci, A., Toll-like receptor 9-independent suppression of skin inflammation by oligonucleotides. J. Invest. Dermatol. 2007: 127: 746–748.

Yasuda, K., Yu, P., Kirschning, C. J., Schlatter, B., Schmitz, F., Heit, A., Bauer, S. et al., Endosomal translocation of vertebrate DNA activates dendritic cells via TLR9-dependent and -independent pathways. J. Immunol. 2005: 174: 6129–6136.

Akkaya, M., Akkaya, B., Miozzo, P., Rawat, M., Pena, M., Sheehan, P. W., Kim, A. S. et al., B cells produce type I IFNs in response to the TLR9 agonist CpG-A conjugated to cationic lipids. J. Immunol. 2017: 199: 931–940.

Abbreviations: cGAMP: 2′-3′-cyclic [G(2′,5′)pA(3′,5′)p] (cyclic GMP-AMP); cGAS: cyclic GMP-AMP synthase; CXCL10: C-X-C motif chemokine ligand 10 (also interferon γ induced protein 10 [CXCL10]); DKO: double-knockout; htdNA: herring testes DNA; IFR: interferon regulatory factor; LDH: lactate dehydrogenase; ODN: oligodeoxynucleotide; PDC: plasmacytoid dendritic cell; PO: phosphorothioate; PS: phosphodiester; STING: stimulator of interferon genes.

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