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Positive Feedback Regulation of Type I IFN Production by the IFN-Inducible DNA Sensor cGAS

Feng Ma,* Bing Li,† Su-yang Liu,* Shankar S. Iyer,* Yongxin Yu,‡ Aiping Wu,* and Genhong Cheng*

Rapid and robust induction of type I IFN (IFN-I) is a critical event in host antiviral innate immune response. It has been well demonstrated that cyclic GMP-AMP (cGAMP) synthase (cGAS) plays an important role in sensing cytosolic DNA and triggering STING dependent signaling to induce IFN-I. However, it is largely unknown how cGAS itself is regulated during pathogen infection and IFN-I production. In this study, we show that pattern recognition receptor (PRR) ligands, including lipid A, LPS, poly(I:C), poly(dA:dT), and cGAMP, induce cGAS expression in an IFN-I–dependent manner in both mouse and human macrophages. Further experiments indicated that cGAS is an IFN-stimulated gene (ISG), and two adjacent IFN-sensitive response elements (ISREs) in the promoter region of cGAS mediate the induction of cGAS by IFN-I. Additionally, we show that optimal production of IFN-β triggered by poly (dA:dT) or HSV-1 requires IFNAR signaling. Knockdown of the constitutively expressed DNA sensor DDX41 attenuates poly(dA:dT)-triggered IFN-β production and cGAS induction. By analyzing the dynamic expression of poly(dA:dT)-induced IFN-β and cGAS transcripts, we have found that induction of IFN-β is earlier than cGAS. Furthermore, we have provided evidence that induction of cGAS by IFN-I mediates the subsequent positive feedback regulation of DNA-triggered IFN-I production. Thus, our study not only provides a novel mechanism of modulating cGAS expression, but also adds another layer of regulation in DNA-triggered IFN-I production by induction of cGAS. The Journal of Immunology, 2015, 194: 1545–1554.
tions of cGAS and cGAS-mediated innate immune responses have been extensively studied, the regulation of cGAS itself during pathogen infection is largely unknown. Additionally, the crosstalk between cGAS and other DNA sensors is also still unclear.

In this study, we provide data to show that cGAS is specifically induced by IFN-I through two adjacent IFN-sensitive response elements (ISREs) in the cGAS promoter. A positive feedback regulation loop is required for optimal production of DNA-triggered IFN-I production. Knockdown of the constitutively expressed DNA sensor DEAD (Asp-Glu-Ala-Asp) box polypeptide 41 (DDX41) attenuates both poly(deoxyadenylic-deoxathymidylic) acid [poly(dA:dT)]-triggered IFN-β production and cGAS induction. We further show that induction of cGAS by the first wave of IFN-I plays a role in the subsequent positive feedback regulation of DNA-triggered IFN-I production. Our study not only demonstrates that cGAS is positively regulated by IFN-I, but it also indicates that the induction of cGAS plays a role in the IFN-I positive feedback loop.

Materials and Methods

Mice and reagents

Wild-type C57BL/6 (6–8 wk of age) and age-matched Ifnar1−/−, Stat1−/−, Myd88−/−, Traf3−/−, Cardif−/−, Sting−/−, and Ifi3−/− male mice were either bred at the University of California, Los Angeles Animal Facility or purchased from The Jackson Laboratory. All mice experiments were performed in accordance with guidelines from the University of California, Los Angeles Institutional Animal Care and Use Committee. cGAM, polyinosinic-polycytidylic acid [poly(I:C)], and poly(dA:dT) were purchased from InvivoGen (San Diego, CA). Lipid A was from Enzo Life Sciences (Farmingdale, NY). LPS (Escherichia coli 011:B4), anti-α–tubulin Ab, human cGAS Ab (anti-C6ORF150), and anti-p204 Ab were from Sigma-Aldrich (St. Louis, MO). Anti-Ddx41 (H00051428) Ab was from Novus Biologicals (Littleton, CO). Anti-GAPDH (GT239) was from GeneTex (Irvine, CA). Recombinant human and mouse IFN-α was from PBL Interferon Source (Piscataway, NJ) and recombinant mouse IFN-γ was from R&D Systems (Minneapolis, MN).

Cell culture and activation

HEK293T, RAW264.7, and THP-1 cell lines were obtained from American Type Culture Collection (Manassas, VA). HEK293T and RAW264.7 cells were maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin. THP-1 cells were cultured in RPMI 1640 supplemented with 5% FBS and 1% penicillin/streptomycin. For bone marrow–derived macrophage (BMM) differentiation, bone marrow cells were harvested from wild-type (WT) or indicated gene-deficient C57BL/6 mice and differentiated in DMEM plus 10% FBS for 7 d with 10 ng/ml M-CSF. The cell culture medium was replaced on days 3 and 6, and on day 7 the cells were differentiated in DMEM plus 10% FBS for 7 d with 10 ng/ml M-CSF. For J2 virus–immortalized macrophages (J2-BMMs), a cell line (called GG2EE) transformed by retrovirus expressing v-raf and c-myc was established and grown in RPMI 1640 (10 mM HEPES [pH 7.8], 10% FBS, 1% penicillin/streptomycin). Supernatant containing J2 viruses was harvested and filtered through a 0.22-μm filter. Bone marrow cells were infected with the J2 virus and immortalized as described previously (26, 27). Fenmur and tibia from Ifi3−/− mice (8 wk old, male, C57BL6 background) were shipped overnight from Michael S. Diamond’s laboratory (Washington University). Ifi7−/− bone marrow cells were differentiated into BMMs and immortalized as Ifi7−/− J2-BMMs. To activate BMMs or J2-BMMs, 100 ng/ml LPS was added into culture medium, or the indicated amount of cGAMP, poly(I:C), or poly(dA:dT) was transfected into cells by Lipofectamine 2000 (Life Technologies). The ratio of transfection reagent to ligands was 2.5 (μl/μg). A detailed Lipofectamine 2000 transfection protocol was followed as described in a previous study and in the manufacturer’s instructions (28). Prior to being activated by stimulation with IFN-α or transfection with poly(I:C) or poly(dA:dT), THP-1 cells were differentiated into macrophages by incubating with 50 nM PMA (Sigma-Aldrich) for 16 h and further cultured for an additional 48 h without PMA.

RNA isolation and quantitative PCR

Total RNA was extracted with TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. One microgram RNA from each sample was reverse transcribed by using an iScript one-step RT-PCR kit with SYBR Green dye (Bio-Rad). Real-time quantitative RT-PCR analysis was performed by using a SensiFAST SYBR and fluoroscence kit (Bioline) and a CFX96 Touch real-time PCR detection system (Bio-Rad). Relative mRNA expression level of genes was normalized to the internal control ribosomal protein gene Rpl32 by using the 2−ΔΔCt cycle threshold method (29). Primer sequences for quantitative PCR (qPCR) were obtained from PrimerBank and are available upon request (30).

Microarray and RNA sequencing

Microarrays were performed on an Affymetrix mouse genome 430.2 array at the University of California, Los Angeles Genotyping and Sequencing Center. The data were deposited in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35825 under accession no. GSE35825). Briefly, WT BMMs were stimulated with 62.5 U/ml IFN-α or 1 U/ml IFN-γ for 2.5 h. Total RNA was extracted for the microarray experiment. In this study, we further analyzed our published microarray data and focused on the regulation of cGAS by IFN. For the RNA sequencing (RNA-Seq) experiment, day 7 BMMs differentiated from wild-type or Ifnar1−/− mice were stimulated with 100 ng/ml lipid A for 4 or 12 h. Total RNA was extracted and then cDNA libraries were constructed by using TrueSeq SBS kit v3 (FC-401-3001; Illumina, San Diego, CA) according to the manufacturer’s guidelines. Next-generation sequencing was performed by using an Illumina HiSeq 2000 with 100-bp single end reads at the High Throughput Sequencing Core of the University of California, Los Angeles Broad Stem Cell Research Center. Details of RNA-seq data analysis were described in a previous study (27).

ELISA and immunoblot

IFN-α and IFN-β in culture supernatant were quantified with a VeriKine mouse IFN-α and IFN-β ELISA kits (PBL, Interferon Source) according to the manufacturer’s instructions. For immunoblot analysis, cells were collected in Triton lysis buffer (50 mM Tris–Cl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 5% glycerol) containing complete protease inhibitors (Roche). Protein concentrations of the extracts were measured with a bicinchoninic acid assay (Thermo Scientific) and equalized with the lysis buffer. Equal amounts of the extracts were loaded and subjected to SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Millipore), and then blotted with ECL (Pierce) or Odyssey imaging systems (LI-COR Biosciences).

cGAS promoter reporter and dual-luciferase reporter assay

The potential transcription factor (TF) binding sites in the mouse cGAS gene promoter region were predicted by MatInspector (Genomatix, Ann Arbor, MI) (31). Conservation analysis of the TF binding sites among the mammalian species was analyzed and viewed by the University of California, Santa Cruz genome browser (http://genome.ucsc.edu/). Different lengths of cGAS promoters were amplified from C57BL/6 genome DNA and subcloned into the pGL4.20 (luc2/Puro) vector (Promega, Madison, WI) to generate WT-luc and ΔJ2-luc reporter constructs. The IRES2#2, IRES1#1, and Stat1 binding site of ΔJ2–luc reporter were mutated to generate ΔJ3#1–luc/mut1–luc, ΔJ3#2–luc/mut1–luc, and ΔJ3#2–luc/mut1–Stat1–luc reporter constructs, respectively, via a QuickChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The indicated cGAS promoter reporter construct was cotransfected with Renilla luciferase reporter into RAW264.7 cells by an Amaxa Cell Line Nucleofector kit V (Lonza). At 12 h after transfection, the cell culture medium was replaced and stimulated with 100 ng/ml LPS for another 12 h. The cells were lysed by passive lysis buffer, and the firefly luciferase activity of the cGAS reporters was measured and normalized by Renilla luciferase activity according to the manufacturer’s instructions (Dual-Luciferase reporter assay system from Promega). The transfection of cGAS reporters constructs in HEK293T cells was recorded according to the manufacturer’s instructions of jetPEI (Polyplus Transfection). At 24 h after transfection, the cells were lysed and the relative luciferase activity was measured as in RAW264.7 cells.

Chromatin immunoprecipitation sequencing data analysis

Stat1 chromatin immunoprecipitation sequencing (ChIP-Seq) raw data from BMMs were downloaded from the Gene Expression Omnibus (accession no. GSE33913). BMM differentiation and activation were described previously (32). Briefly, BMMs were differentiated with M-CSF and treated with IFN-β or IFN-γ for 6 h before crosslinking for chromatin isolation. ChIP reactions were performed with anti-STAT1a Ab from Santa Cruz Biotechnology, and libraries were generated by standard Illumina protocols. Sequenced reads were aligned to mouse genome (mm9) allowing up to two mismatches using Bowtie (33). The data were processed as previously described (34). For peak calling, mouse genome was divided into
100-bp windows. A p value for Poisson distribution of enriched chromatin immunoprecipitated DNA over input DNA for each window was calculated. Significant peaks were defined as the windows with a significant p value of \(<10^{-5}\) and with two neighboring windows at the same significance.

**DDx41 and p204 small interfering RNA knockdown**

BMMs (2 × 10^6) were differentiated for 7 d in a 12-well plate. On day 7, cell culture medium was replaced and the cells were transfected with 20 nM nontargeting control, Ddx41-specific small interfering RNA (siRNA; Dharmacon RNA interference and gene expression, SMARTpool: siGENOME Ddx41 siRNA), or p204-specific siRNA (Dharmacon RNA interference and gene expression, SMARTpool: siGENOME p204 siRNA) by using INTERFERin transfection reagent (Polyplus Transfection) according to the manufacturer’s instructions. At 36 h after transfection, the knockdown efficiency was measured by Western blot.

**Lentivirus packaging and lentiviral transduction**

Full lengths of mouse cGAS were cloned into the lentivector pCDFl-CMV-MCS2-EF1-copGFP (CD111B-1; System Biosciences) to make the expression constructs LV-cGAS. LV-Ctrl or LV-cGAS vector was cotransfected into HEK293T cells with the pPACKF1 packaging plasmids mix (LV100A-1; System Biosciences). Control or cGAS-overexpressing lentiviruses were produced, and WT or Ifnar1^−/−^ BMMs were transfected by these lentiviruses according to the user’s manual (System Biosciences) and a previous study (35).

**Software and graphing**

Microarray analysis was performed by using the Bioconductor affy package (http://www.bioconductor.org/). RNA-Seq data were analyzed on University of California, Los Angeles Galaxy server (http://galaxy.hoffman2.idre.ucla.edu/root). All graphs were generated with GraphPad Prism and Photoshop.

**Results**

cGAS is IFN-I-inducible whereas DDX41 is constitutively expressed in BMMs

By analyzing the gene expression profile of the IFN-I- and type II IFN (IFN-II)-stimulated BMMs (5), we found that cGAS mRNA expression was significantly upregulated in IFN-α-treated BMMs (Fig. 1A). We compared the cGAS mRNA level in WT and Ifnar1^−/−^ BMMs activated by TLR4 ligand lipid A. Our RNA-Seq data indicated that cGAS was significantly induced by lipid A in WT BMMs but not in Ifnar1^−/−^ BMMs (Fig. 1B). A higher cGAS mRNA level was detected in WT BMMs than in Ifnar1^−/−^ BMMs when the cells were activated by lipid A (Fig. 1B). However, the expression of another DNA sensor, DDX41, was not affected by IFN-α treatment in BMMs (Fig. 1C). No significant difference of DDX41 mRNA level was detected between WT and Ifnar1^−/−^ BMMs, neither in the resting condition nor in the lipid A–activated condition (Fig. 1D). Furthermore, both poly(I:C) and poly(dA:dT) significantly induced cGAS expression in WT BMMs; however, the induction of cGAS was completely abolished in Ifnar1^−/−^ BMMs (Fig. 1E). These data suggest that cGAS is an ISG and DDX41 is constitutively expressed in BMMs.

cGAS is specifically induced by IFN-I

To determine the specificity of cGAS induction by IFN-I, we treated the WT and several gene-deficient BMMs with different PRR ligands. It is well known that LPS activates NF-κB and MAPK signaling through Myd88-dependent pathway, and it triggers IFN-I production through the TRIF-dependent pathway (6). We found that LPS stimulation significantly induced cGAS expression in WT and Myd88^−/−^ BMMs, but not in Trif^−/−^ BMMs, which indicated that LPS could induce cGAS expression through the TRIF-dependent pathway (Fig. 2A). Transfection of poly(I:C) triggers IFN-I production mainly through the RIG-I–CARDIF-dependent pathway whereas transfection of poly(dA:dT) triggers IFN-I production through a STING-dependent pathway (2, 6, 20).

We found that both poly(I:C) and poly(dA:dT) induced cGAS expression in WT BMMs. However, the induction of cGAS was significantly impaired in Cardif^−/−^ BMMs activated by poly(I:C) transfection but not in Cardif^−/−^ BMMs activated by poly(dA:dT).
transfection. Attenuated poly(dA:dT)-triggered IFN-I production was observed in *Sting<sup>−/−</sup>* macrophages, which fail to produce detectable STING protein (36, 37). We found that the induction of cGAS was significantly impaired in *Sting<sup>−/−</sup>* BMMs activated by transfection of poly(dA:dT) but not in *Sting<sup>−/−</sup>* BMMs activated by transfection of poly(I:C) (Fig. 2B). It has been shown that cGAS converts DNA to cGAMP to trigger the STING-dependent IFN-I production (12). Interestingly, our results indicated that cGAMP, in turn, could induce cGAS mRNA in a dose-dependent manner in WT BMMs but not in *Ifnar1<sup>−/−</sup>* BMMs (Fig. 2C).

Taken together, these data suggest that multiple PRR ligands could induce cGAS expression by triggering IFN-I production and activating the IFNAR signaling, whereas activating other signaling such as the MyD88-dependent pathways does not seem to affect cGAS expression. To test whether cGAS is also induced by IFN-I in human cells, we treated THP-1 cells with IFN-α and different PRR ligands. As shown in Fig. 2D, cGAS was significantly induced by IFN-α in THP-1 cells at both time points we examined. IFN-α triggered cGAS expression in a dose-dependent manner (Fig. 2E). Similar to the data from mouse BMMs, both poly(I:C) and poly(dA:dT) induced cGAS in THP-1 cells (Fig. 2F). Additionally, by using a commercial Ab specifically against human cGAS, we found that IFN-α significantly induced cGAS protein expression in THP-1 cells (Fig. 2G). Therefore, our data indicate that cGAS could be induced by IFN-I specifically in both mouse and human macrophages.

**FIGURE 2.** cGAS is specifically induced by IFN-I in mouse and human macrophages. (A) WT, *Myd88<sup>−/−</sup>*, and *Trif<sup>−/−</sup>* BMMs were stimulated with 100 ng/ml LPS for indicated time points, and cGAS mRNA level in these cells was detected by qPCR and normalized to Rpl32. (B) WT, *Cardif<sup>−/−</sup>*, and *Sting<sup>gt/gt</sup>* BMMs were transfected with 1 μg/ml poly(I:C) or poly(dA:dT) for 4 h, and cGAS mRNA level in these cells was detected by qPCR and normalized to Rpl32. (C) WT and *Ifnar1<sup>−/−</sup>* BMMs were transfected with the indicated amount of cGAMP for 4 h, and cGAS mRNA level in these cells was detected by qPCR and normalized to Rpl32. (D) THP-1–differentiated macrophages were treated with 500 U/ml human IFN-α for indicated time points, RNA was extracted from these cells, and cGAS mRNA level was detected by qPCR and normalized to *RPL32*. (E) THP-1 cells were treated with the indicated amount of human IFN-α (10–1000 U/ml) for 4 h, RNA was extracted from these cells, and cGAS mRNA level was detected by qPCR and normalized to *RPL32*. (F) THP-1 cells were transfected with 1 μg/ml poly(I:C) or poly(dA:dT) for 4 h, RNA was extracted from these cells, and cGAS mRNA level was detected by qPCR and normalized to *RPL32*. (G) THP-1 cells were treated with 500 U/ml human IFN-α for indicated time points, and cGAS protein level was detected by Western blot. *α-Tubulin is shown as a loading control. *p < 0.05, **p < 0.01 (Student t test). Data in (A)–(F) are from three independent experiments (mean ± SEM). Data in (G) are from one representative of three independent experiments.

ISREs in cGAS promoter are critical for IFN-I–triggered cGAS expression

To determine how cGAS is induced by IFN-I, we analyzed the potential TF binding sites in the cGAS 5′ untranslated region. Mouse *cGas* locates on chromosome 9 and is encoded by the negative strand of DNA (Fig. 3A). Among all the predicted TF binding sites around the *cGas* 5′ untranslated regions (Fig. 3B). Given that ISRE is the motif bound by ISG factor 3, a tripartite complex of tyrosine-phosphorylated STAT1/STAT2 and IRF9 (38), we analyzed the STAT1 ChIP-Seq data from BMMs to determine whether STAT1 could bind to these predicted ISREs and the Stat1 binding site. According to the STAT1 ChIP-Seq data from Maniatis and colleagues (32), we noticed a significant STAT1 binding peak in the promoter region of *cGas* in BMMs treated with IFN-β or IFN-γ. Both ISRE#1 and ISRE#2 were located in the middle region of the peak whereas ISRE#3 was not in the peak region. Although the predicted Stat1 binding site was within the peak region, far fewer STAT1 chromatin-immunoprecipitated reads were aligned in the predicted Stat1 binding site than in ISRE#1 and ISRE#2 (Fig. 3C). To verify the potential functions of these TF binding sites, several reporter constructs were made and luciferase reporter assays were performed (Fig. 3D). As shown in Fig. 3E, the IFN-β–luc reporter constructs were activated by IFN-β but not IFN-α. We also examined the potential TF binding sites in the cGAS transcription start site, there are three ISREs and one STAT1 binding site that are potentially responsible for the induction of cGAS by IFN-I. The sequence of ISRE#2 is very conserved in multiple mammalian *cGas* 5′ untranslated regions (Fig. 3B).
FIGURE 3. The ISREs in the cGAS promoter mediate the induction of cGAS by IFN-I. (A) Chromosome locations of mouse cGas and its nearby genes. The diagram was modified from National Center for Biotechnology Information gene ID no. 214763. (B) Potential ISREs and STAT1 binding site in the promoter of cGas. TF binding site prediction was performed by MatInspector. The location of the ISREs and STAT1 binding site and the conservation score are shown. The conservation comparison of ISRE#2 between human, rat, chimpanzee, rabbit, tree shrew, dog, and elephant was according to the sequence from University of California, Santa Cruz Genome Browser. CDS, coding DNA sequence; TSS, transcription start site. (C) BMMs were treated with 100 U/ml IFN-β and IFN-γ for 6 h, STAT1 ChIP-Seq data were analyzed, and the Stat1 binding region in mouse cGas promoter is shown. The Stat1 ChIP-Seq raw data were downloaded from Gene Expression Omnibus (accession no. GSE33913). (D) Sequence of the mutated ISRE#2, mutated ISRE#1, mutated Stat1, and the schematic diagram of the cGAS promoter reporter plasmids. (E) Indicated cGAS promoter reporter constructs or IFN-β luciferase reporter (IFN-β-luc), which expressing firefly luciferase, was transfected into RAW264.7 cells by a nucleofection system. pRL-TK-luc vector expressing Renilla luciferase was cotransfected as a control for transfection efficiency. Data are shown as the relative luciferase activity. (F) Flag, TBK1, or IRF1 was cotransfected with indicated promoter reporter constructs and pRL-TK-luc vector. Data are shown as the relative luciferase activity. (G) BMMs (Figure legend continues)
was significantly activated by LPS in RAW264.7 cells. Using the similar experimental system, we found that LPS activated WT, Δ#3, and Δ#3-mutStat1 luciferase reporters, but not the Δ#3-mut#1 and Δ#3-mut#2 reporters in RAW264.7 cells (Fig. 3E), which suggested that ISRE#1 and ISRE#2 played a major role for regulating cGAS expression by IFN-I. It has been known that TNK1 and IRF1 trigger IFN-I production in HEK293T cells (39, 40). Consistent with the results from RAW264.7 cells, both TNK1 and IRF1 activated WT, Δ#3, and Δ#3-mutStat1 luciferase reporters, but not the Δ#3-mut#1 and Δ#3-mut#2 reporters in HEK293T cells (Fig. 3F). Considering that multiple common ISGs could be induced by both IFN-I and IFN-II (5), we checked the cGAS mRNA level in IFN-γ-stimulated BMMs. Comparing the induction of cGAS by IFN-I (Figs. 1A, 3G, 3H), IFN-γ stimulation only modestly upregulated cGAS mRNA in BMMs and THP-1 cells (Fig. 3G, 3H), which was consistent with less STAT1 binding in cGAS promoter during IFN-γ treatment than IFN-β treatment based on the analysis of STAT1 ChIP-Seq data (Fig. 3C). These data further suggested that induction of cGAS is mainly mediated by ISREs rather than the IFN-γ-activated sites (GAS).

**Optimal production of viral DNA–triggered IFN-I requires IFNAR signaling**

In response to viral DNA, DDX41 and cGAS recognize viral DNAs and activate the STING/TBK1/IRF3 signaling axis by directly binding to STING or producing the endogenous cyclic di-nucleotide, cGAMP (12, 41). Host cells can produce a large amount of IFN-I to defend the DNA viral infections upon the activation of the STING/DBK1/IRF3-dependent pathway. Interestingly, less production of IFN-β transcript and protein could be detected in poly(dA:dT)-transfected or HSV-1–infected WT J2-BMMs than in Ifnar1−/− and Stat1−/− J2-BMMs (Fig. 4A, 4B), which suggested that optimal production of viral DNA–triggered IFN-I requires IFNAR signaling. IFN-I–inducible TF IRF7 is a well-known ISG that mediates the IFN-I positive feedback loop through the IRF3/IFN-β/IRF7/IFN-α-β axis during viral infection (42–44). To test whether IRF7 is also required for viral DNA–triggered IFN-I induction in macrophages, we compared the IFN-I transcripts and supernatant IFN-I protein from poly(dA:dT)-transfected WT, Ifn3−/−, and Ifn7−/− J2-BMMs. Significantly attenuated induction of IFN-β mRNA was observed in Ifn3−/− but not in Ifn7−/− J2-BMMs activated by transfection of poly(dA:dT) for 4 h, whereas IFN-α mRNA induction was impaired in both Ifn3−/− and Ifn7−/− J2-BMMs at this time point (Fig. 4C, 4D). Significant less supernatant IFN-β and IFN-α were detected in Ifn3−/− and Ifn7−/− J2-BMMs activated by transfection of poly(dA:dT) for 12 h, although the downregulation of IFN-β was not as dramatic as IFN-α in Ifn7−/− J2-BMMs (Fig. 4E, 4F). Consistent with the IFN-I production results, induction of cGAS was impaired in Ifn3−/− J2-BMMs activated by transfection of poly(dA:dT) for 12 h, although the downregulation of IFN-β was not as dramatic as IFN-α in Ifn7−/− J2-BMMs (Fig. 4E, 4F).
poly(dA:dT) for 4 and 12 h, whereas modest but significant down-regulation of cGAS induction in Ifn7−/− J2-BMMs was activated by transfection of poly(dA:dT) for 12 h but not 4 h (Fig. 4G). These data indicate that IRF7 is critical for viral DNA–triggered IFN-α production and modestly regulates IFN-β production at the later stage. However, IRF7 is dispensable for the early stage of IFN-β induction during viral DNA activation. Compared to WT cells, Ifn7−/− J2-BMMs could produce as much as 50% IFN-β whereas Ifnar1−/− and Stat1−/− J2-BMMs only produced 25% IFN-β (Fig. 4A, 4E), which suggested that a defect of IRF7 induction in Ifnar1−/− and Stat1−/− J2-BMMs could not account for less viral DNA–triggered IFN-1 in these cells. Given that cGAS induction is IFNAR-dependent but IRF7-independent at the early stage of viral DNA activation, no induction of DNA sensors such as cGAS in Ifnar1−/− and Stat1−/− cells is an alternative explanation of attenuated viral DNA–triggered IFN-1 in these cells.

Knockdown of DDX41 attenuated poly(dA:dT)-triggered IFN-1 production and subsequent cGAS induction

Given that DDX41 is constitutively expressed and cGAS is inducible by IFN-1 in BMMs, we hypothesized that the first wave of IFN-1 production was triggered by DDX41-dependent signaling and that induction of cGAS by IFN-1 mediated the subsequent robust IFN-1 production in viral DNA–activated BMMs. Consistent with our hypothesis, we found that knockdown of DDX41 significantly reduced induction of IFN-1 triggered by poly(dA:dT) and cGAMP in BMMs (Fig. 5A, 5B). Meanwhile, less cGAS mRNA was induced by poly(dA:dT) but not by cGAMP in si-Ddx41–transfected BMMs (Fig. 5C). P204 is another IFN-inducible DNA sensor reported to sense poly(dA:dT) and trigger IFN-1 production (7). Knockdown of p204 significantly reduced induction of IFN-1 triggered by poly(dA:dT) but did not affect cGAS induction (Fig. 5D–F). As a control, knockdown of DDX41 or p204 did not affect the induction of IFN-β and cGAS in BMMs activated by poly(I:C) (Fig. 5A–F). Taken together, these data suggest that DDX41 not only regulates the production of IFN-1 by DNA, but it also affects the induction of the IFN-inducible sensor cGAS. Furthermore, we found that the induction of IFN-β was earlier than generation of cGAS in poly(dA:dT)-activated BMMs, which implied that the constitutively expressed DDX41 and/or basal level of cGAS mediated the first wave of IFN-1 production before induction of cGAS expression (Fig. 5G).

Overexpression of cGAS reduced the difference of poly(dA:dT)-triggered IFN-1 production between WT and Ifnar1−/− macrophages

To determine whether the induction of cGAS by the first wave production of IFN-1 plays a role in the positive feedback loop of DNA-triggered IFN-1 production, we overexpressed mouse cGAS in both WT and Ifnar1−/− J2-BMMs by lentiviral gene transduction to get a similar level of cGAS expression during poly(dA:dT) activation. cGAS mRNA was elevated dramatically after trans-

**FIGURE 5.** Knockdown of DDX41 attenuated poly(dA:dT)-triggered IFN-1 production and subsequent cGAS induction. (A–C) BMMs were transfected with 20 nM control siRNA (si-Ctrl) or si-Ddx41 for 36 h, and then cells were activated by transfection with 1 μg/ml poly(dA:dT), 3 μg/ml cGAMP, or 1 μg/ml poly(I:C) for another 12 h. The Ddx41 protein level was measured by Western blot (A), the supernatant IFN-β from these activated cells was detected by ELISA (B), and the cGas mRNA in these cells was detected by qPCR and normalized to Rp32 (C). (D–F) BMMs were transfected with 20 nM si-Ctrl or si-p204 for 36 h, and then cells were activated by transfection with 1 μg/ml poly(dA:dT) or poly(I:C) for another 12 h. The p204 protein level was measured by Western blot (D), the supernatant IFN-β from these activated cells was detected by ELISA (E), and the cGas mRNA in these cells was detected by qPCR and normalized to Rp32 (F). (G) BMMs were transfected with 1 μg/ml poly(dA:dT) for indicated time points, and Hbb and cGas mRNA was measured by qPCR and normalized to Rp32. Data in (A) and (D) are representative of three independent experiments. Data in (B), (C), and (E–G) are from three independent experiments (mean ± SEM). *p < 0.05, **p < 0.01 (Student t test).
ducing with cGAS-overexpressing lentiviruses, and the mRNA expression levels of cGAS were comparable between cGAS-overexpressed WT and Ifnar1<sup>−/−</sup> J2-BMMs (Fig. 6A). Considering that cGAS expression is almost saturated in cGAS-overexpressed cells, DNA-triggered IFN-I may not be able to further upregulate cGAS expression in these cells. We found that poly(dA:dT)-triggered IFN-β production has less change between cGAS-overexpressed WT and Ifnar1<sup>−/−</sup> J2-BMMs when compared with empty lentiviral-transduced WT and Ifnar1<sup>−/−</sup> J2-BMMs (Fig. 6B, 6C). These results indicated that overexpression of cGAS at least partially rescued the defect of IFN-β production in Ifnar1<sup>−/−</sup> macrophages. Reduced differential production of poly(dA:dT)-triggered IFN-β between WT and Ifnar1<sup>−/−</sup> J2-BMMs suggested that induction of cGAS by IFN-I contributed to the positive feedback loop of IFN-I production (Fig. 6D). Although we cannot exclude the possibility that other ISGs may also regulate the positive feedback of poly(dA:dT)-triggered IFN-I production, cGAS is likely to be one of the ISGs that plays a role in this positive feedback loop.

In summary, our study has provided a novel mechanism by which cGAS is induced by IFN-I, and it suggested a role of cGAS induction in the IFN-I positive feedback regulation loop.

**Discussion**

cGAS (formerly C6orf150) was among the numerous ISGs according to the published microarray datasets from IFN-treated cells or tissues (45–52). Our previous IFN-treated mouse BMMs gene expression profile also showed that mouse cGAS (formerly E330016A19Rik) could be significantly induced by IFN-I and IFN-II (5). In this study, we took advantage of different PRR ligands and gene-deficient BMMs to test the signaling pathways that regulate cGAS expression. In TLR4 ligand–triggered BMMs, deficiency of Myd88 did not affect the induction of cGAS; however, deletion of Ifnar1 and Trif completely abolished the activation of cGAS expression. These data suggest that cGAS expression is not regulated by MyD88-dependent pathways. Poly(I:C), poly(dA:dT), and cGAMP activate both IRF3 and NF-κB by recruiting the kinases TBK1 and IKK, respectively (6, 20, 21). In Ifnar1<sup>−/−</sup> BMMs, the cGAS gene was not induced in response to stimulation of poly(I:C), poly(dA:dT), and cGAMP. Thus, in the present study we not only have verified that cGAS is an ISG, but we also demonstrate that cGAS expression is specifically regulated by IFNAR signaling. Furthermore, we also have identified two adjacent ISREs in the cGAS promoter that mediate the induction of cGAS by IFN-I. Although both IFN-I and IFN-II sig-
ificantly induce cGAS expression, IFN-I induced more cGAS transcript than did IFN-II in both mouse and human macrophages. This is consistent with ISRE-dependent regulation of cGAS. A recent study has shown that autophagy protein Beclin-1 suppresses cGAMP synthesis and halts IFN production by directly interacting with cGAS (53). Treatment with PMA dramatically downregulated cGAS protein level in THP-1 cells via an unknown mechanism (25). Although these studies identified the potential negative regulation of cGAS protein, in the present study we have provided a novel mechanism by which cGAS transcription and its downstream signaling are positively regulated by IFN-I.

DDX41, IFI16, and cGAS are among the numerous described cytosolic DNA sensors in the past several years. All of them could trigger STING-dependent signaling to induce IFN-I following poly(dA:dT) transfection or DNA virus infection (7, 12, 41). DDX41 is constitutively expressed in myeloid dendritic cells. Knockdown of DDX41 blocked the induction of IFI16 in poly(dA:dT)-transfected myeloid dendritic cells (41). A previous study indicated that DDX41 is more important than IFI16 in the initial sensing viral DNA and triggering the early burst of the IFN-I response (41). In the present study, we found that DDX41 expression is not altered in both lipid A-stimulated WT and Ifnar1−/− BMMs, which indicates that DDX41 is also constitutively expressed in BMMs. Knockdown of DDX41 attenuates the induction of cGAS by poly(dA:dT) in BMMs. The results of dynamic induction of IFN-β and cGAS transcripts by poly(dA:dT) show that induction of IFN-β is earlier than cGAS. Taken together, our data suggest that DDX41 and/or basal level of cGAS are likely to mediate the first wave of IFN-I production, and induction of cGAS by IFN-I contributes to the subsequent positive feedback loop of IFN-I. Although more evidence is required to support the model in which DNA sensors may act sequentially over time, our present study at least is very similar to the model proposed for the RNA helicase DDX3, which has been suggested to act as a “sentinel sensor” for viral RNA before RIG-I (which, similar to IFI16 and cGAS, is also an ISG) becomes the principal RNA sensor (54).

IFNAR signaling is required for the induction of cGAS. Optimal production of IFN-β triggered by poly(dA:dT) and HSV-1 also requires IFNAR signaling. These data further suggest that induction of cGAS by IFN-I plays a role in the IFN-I positive regulation loop. The IRF3/IFN-β/IRF7/cGAS-α/β signaling axis is a well-established loop for the IFN-I positive regulation loop. The IRF3/IFN-β/cGAS-cGAMP signaling in antiviral defense and immune adjuvant effects. The Journal of Immunology 1553.

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Disclosures

The authors have no financial conflicts of interest.

References


