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*J Immunol* published online 21 January 2015
http://www.jimmunol.org/content/early/2015/01/20/jimmunol.1402066

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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(1: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: 000–000.

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Received for publication August 13, 2014. Accepted for publication December 3, 2014.

This work was supported by a Tumor Immunology Training Grant (National Institutes of Health/National Cancer Institute Grant 5T32CA009120), as well as by National Institutes of Health Grants R01 AI078389 and R01 AI069120 and the Medical Scientist Training Program.

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Abbreviations used in this article: BMM, bone marrow–derived macrophage; cGAMP, cyclic GMP-AMP; cGAS, cyclic GMP-AMP synthase; ChIP, chromatin immunoprecipitation; ChIP-Seq, chromatin immunoprecipitation sequencing; DDX41, DEAD (Asp-Glu-Ala-Asp) box polypeptide 41; IFN-I, type I IFN; IFN-II, type II IFN; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; ISRE, IFN-sensitive response element; poly(dA:dT), poly(deoxyadenylic-deoxythymidylic) acid; poly(1:C), polynosinic-polycytidylic acid; PRR, pattern recognition receptor; qPCR, quantitative PCR; RNA-Seq, RNA sequencing; siRNA, small interfering RNA; TBK1, TANK-binding kinase 1; TF, transcription factor; WT, wild-type.

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Published January 21, 2015, doi:10.4049/jimmunol.1402066

The Journal of Immunology

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1402066
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-γ 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-γ production. Knockdown of the constitutively expressed DNA sensor DEAD (Asp-Glu- Ala-Asp) box polypeptide 41 (DDX41) attenuates both poly(dA:dT)- and poly(I:C)-triggered IFN-β production and cGAS induction. We further show that induction of cGAS by the first wave of IFN-γ plays a role in the subsequent positive feedback regulation of DNA-triggered IFN-γ production. Our study not only demonstrates that cGAS is positively regulated by IFN-γ, but it also indicates that the induction of cGAS plays a role in the IFN-γ positive feedback loop.

Materials and Methods
Mice and reagents
Wild-type C57BL/6 (6–8 wk of age) and age-matched Ifnar1−/−, Stat1−/−, Myd88−/−, Trif−/−, Cardif−/−, Sting−/−, and Ifi3−/− 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. cGAS, polyinosinocytidylic 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 Signet (Boston, MA). Anti-ID4 #1 (H00051428) Ab was from Novus Biologicals (Littleton, CO). Anti-GAPDH (GT239) was from GeneTex (Irvine, CA). Recombinant human and mouse Ifnar-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. 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 used for experiments as BMMs. For J2 virus–immortalized macrophages (J2-BMMs), a cell line (called GG2E2E) 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). Femur and tibia from Ifnγ−/− mice (8 wk old, male, C57BL/6 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)−triggered IFN-β production and cGAS induction. We further show that induction of cGAS by the first wave of IFN-γ plays a role in the subsequent positive feedback regulation of DNA-triggered IFN-γ production. Our study not only demonstrates that cGAS is positively regulated by IFN-γ, but it also indicates that the induction of cGAS plays a role in the IFN-γ positive feedback loop.

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 fluorescein 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 Core (Los Angeles, CA). 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 TruSeq 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 immunobots
IFN-α and IFN-β in culture supernatant were quantified with a VeriKine mouse Ifnar-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 bichoninic 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 cGAM 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 plG4.20 (luc2/Puro) vector (Promega, Madison, WI) to generate WT-luc and ΔΔ3-luc reporter constructs. The IRES2#2, IRES2#, and SfiI binding sites of ΔΔ3-luc reporter were mutated to generate Δ3#3–luc–mut#2-luc, Δ3#3–mut–mut#1–luc, and Δ3–luc–mut–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 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 manufacturer’s instructions (Dual-Luciferase reporter assay system from Promega). The transfection of cGAS reporters constructs in HEK293T cells was carried out by transfection with polyethylene. At 24 h after transfection, the cells were lysed and the relative luciferase activity was measured 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-γ for 6 h before crosslinking for chromatin isolation. ChIP reactions were performed with anti–STAT1α 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, 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 pCDFlCMV-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^{−/−} J2-BMMs were transduced 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).

**FIGURE 1.** cGAS and DDX41 expression in BMMs during response to IFN-α and lipid A. (A) BMMs were treated with 62.5 U/ml IFN-α for 2.5 h, RNA was extracted, and gene expression profile was detected by Affymetrix 430.2 chips. cGAS mRNA level is shown as probe intensity from microarray. (B) WT or Ifnar1^{−/−} BMMs were stimulated with 100 ng/ml lipid A for indicated time points. RNA was extracted, and gene expression profile was detected by RNA-Seq. cGAS mRNA level is shown as fragments per kilobase of transcript per million fragments mapped from the RNA-Seq data as described in (A). (C) Ddx41 mRNA level is shown as probe intensity from microarray. (D) WT and Ifnar1^{−/−} BMMs were stimulated with 100 ng/ml lipid A for indicated time points. RNA was extracted, and gene expression profile was detected by RNA-Seq. cGAS mRNA level is shown as Fragments per kilobase of transcript per million fragments mapped. (E) WT and Ifnar1^{−/−} BMMs were transected 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 Rps32. **p < 0.01 (Student t test). Data are from three independent experiments (mean ± SEM).
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 coverts 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, Mys88<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, Cardf<sup>−/−</sup>, and Sting-gt/gt 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 S' 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 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 S' 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 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

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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 ISREs could be induced by both IFN-I and IFN-II (42–44). To test whether IRF7 is also required for viral DNA–triggered IFN-I production in macrophages, we compared the IFN-I induction of cGAS in Δ#3–mut#1 and Δ#3–mut#2 reporters in HEK293 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 dinucleotide, 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/TBK1/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 IFR3/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, Irf3−/−, and Irf7−/− J2-BMMs. Significantly attenuated induction of IFN-β mRNA was observed in Irf3−/− but not in Irf7−/− J2-BMMs activated by transfection of poly(dA:dT) for 4 h, whereas IFN-α mRNA induction was impaired in both Irf3−/− and Irf7−/− J2-BMMs at this time point (Fig. 4C, 4D). Significant less supernatant IFN-β and IFN-α were detected in Irf3−/− and Irf7−/− 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 Irf7−/− J2-BMMs (Fig. 4E, 4F). Consistent with the IFN-I production results, induction of cGAS was impaired in Irf3−/− J2-BMMs activated by transfection of

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**FIGURE 4.** Optimal production of viral DNA–triggered IFN-I requires IFNAR signaling. (A and B) WT, Ifnar1−/−, or Stat1−/− J2-BMMs were transfected with 1 μg/ml poly(dA:dT) for 12 h (A) or infected with HSV-1 (multiplicity of infection of 1) for 24 h (B). Supernatant IFN-β from these cells was measured by ELISA. (C and D) WT, Irf3−/−, or Irf7−/− J2-BMMs were transfected with 1 μg/ml poly(dA:dT) for 4 h, and Ifnb (C) and Ifna4 (D) mRNA level in these cells was detected by qPCR and normalized to Rpl32. (E and F) WT, Irf3−/−, or Irf7−/− J2-BMMs were transfected with 1 μg/ml poly(dA:dT) for 12 h, and supernatant IFN-β (E) and IFN-α (F) from these cells was measured by ELISA. (G) WT, Irf3−/−, or Irf7−/− J2-BMMs were transfected with 1 μg/ml poly(dA:dT) for indicated time points, and cGas mRNA level in these cells was detected by qPCR and normalized to Rpl32. Data are from three independent experiments (mean ± SEM). *p < 0.05, **p < 0.01 (Student t test).
Knockdown of DDX41 attenuated poly(dA:dT)-triggered IFN-I production and subsequent cGAS induction

Given that DDX41 is constitutively expressed and cGAS is inducible by IFN-I in BMMs, we hypothesized that the first wave of IFN-I production was triggered by DDX41-dependent signaling and that induction of cGAS by IFN-I mediated the subsequent robust IFN-I production in viral DNA–activated BMMs. Consistent with our hypothesis, we found that knockdown of DDX41 significantly reduced induction of IFN-I 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-I production (7). Knockdown of p204 significantly reduced induction of IFN-I 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–C). Together, these data suggest that DDX41 not only regulates the production of IFN-I by DNA, but it also affects the induction of the IFN-inducible sensor cGAS. Furthermore, we found that the induction of IFN-I 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-I production before induction of cGAS expression (Fig. 5G).

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

To determine whether the induction of cGAS by the first wave production of IFN-I plays a role in the positive feedback loop of DNA-triggered IFN-I 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-
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.
nificantly 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 cytoplasmic 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 Ifnar−/− BMMs, which indicates that DDX41 contributes to the subsequent positive feedback loop of IFN-I.

Additionally, more evidence is required to support the model in which DNA is sensed more DNA and producing more cGAMP. Furthermore, cGAS is also an ISG. DDX41 is also constitutively expressed in BMMs. Knockdown of 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 Ifnar−/− BMMs, which indicates that DDX41 contributes to the subsequent positive feedback loop of IFN-I.


disclosures

The authors have no financial conflicts of interest.

Acknowledgments

We thank Kislav Parvatiyar and Amir Ali Ghaffari for helpful discussions, and we thank Dr. Tadatsugu Taniguchi (University of Tokyo) and Dr. Michael S. Diamond (Washington University) for sharing Ifi35 and Ifit7−/− mice. We appreciate Neda Arora for excellent technical support and Jing Zhu for editing the manuscript.

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