Positive feedback regulation of type I interferon by the interferon-stimulated gene STING

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Abstract

Stimulator of interferon genes (STING) is an important regulator of the innate immune response to cytoplasmic DNA. However, regulation of STING itself is largely unknown. Here, we show that STING transcription is induced by innate immune activators, such as cyclic dinucleotides (CDNs), through an IFNAR1-dependent pathway. We also identify a STAT1 binding site in the STING promoter that contributes to the activation of STING transcription. Furthermore, we show that induction of STING mediates the positive feedback regulation of CDN-triggered IFN-I. Thus, our study demonstrates that STING is an interferon-stimulated gene (ISG) and its induction is crucial for the IFN-I positive feedback loop.

Keywords: CDN; cGAMP; IFN; ISG; STING

Introduction

Stimulator of interferon genes (STING, also named MITA, MYP5, or ERIS) plays a critical role in sensing pathogenic nucleic acid as an adapter protein or as a DNA sensor [1–7]. STING not only can directly complex with DNA such as cyclic dinucleotides (CDNs) and pathogen-related ssDNA and dsDNA [6–8], but also can interact with other DNA sensors such as gamma-interferon-inducible protein16 (IFI16) and DEAD (Asp-Glu-Ala-Asp) box polypeptide 41 (DDX41) [9,10]. Following activation by pathogenic DNAs or its upstream DNA sensors, STING translocates with TANK-binding kinase 1 (TBK1) to perinuclear endosomes, leading to the activation of interferon regulatory factors (IRFs) and NFκB, which triggers the expression of type I interferon (IFN-I) and other immune response genes [1,2,5]. STING has emerged as central for DNA-induced IFN-I activation through the STING–TBK1–IRF3 signaling axis [1–10]. Although STING-dependent IFN-I pathway has been extensively studied, it is largely unknown how STING is regulated, particularly at the transcriptional level.

Rapid and robust induction of IFN-I is a critical event during viral and bacterial infections [11,12]. The initial production of IFN-I is further regulated by a positive feedback loop that is based on the ability of IFNβ and IFNα to induce numerous IFN-stimulated genes (ISGs). For example, induction of cytosolic RNA and DNA sensors such as RIG-I, MDA5, and IFI16 enhances the induction of IFN-I by sensing more pathogen-derived nucleic acids [12–14]. In addition, induction of transcription factor (TF) IRF7 drives numerous of IFNα genes expression and initiates IFN-I positive feedback through the well-established IRF3–IFNβ–IRF7–IFNα/β signaling axis [15–17]. Both the first wave induction and the subsequent positive feedback regulation of IFN-I are important for host innate immune responses. Several CDNs that can trigger IFN-I have been identified in recent studies [6,7,18]. Cyclic guanosine monophosphate (c-di-GMP) and cyclic diadenosine monophosphate (c-di-AMP) are two key secondary messengers with essential roles in regulating bacterial metabolism, motility, and virulence [19,20]. Cyclic GMP–AMP (cGAMP) is a metazoan endogenous second messenger derived from cytoplasmic DNA via the synthase activity of cyclic GMP–AMP synthase (cGAS) [6,21]. All these CDNs produced by bacteria and cellular cGAS could trigger IFN-I production through the STING-dependent pathway [6,7,18]. However, it is unclear whether the positive feedback loop is required for CDNs-triggered IFN-I production.

Here, our data indicate that STING expression could be induced by IFN-I via a STAT1 binding site in its promoter region; positive feedback regulation loop is required for optimal production of CDNs-triggered IFN-I. Furthermore, we show that induction of STING by the first wave of IFN-I plays a role in the subsequent positive feedback regulation of CDNs-triggered IFN-I production, and IRF7-dependent IFNα production further amplifies STING induction at the late stage.

Results and Discussion

STING is an ISG

By analyzing the gene expression profile of the IFN-I- and IFN-II-stimulated bone marrow-derived macrophages (BMMs) [14], we
found that STING mRNA expression was significantly upregulated in IFNα-treated BMMs, as the well-known IFN-inducible TF IRF7 (Fig 1A). However, other genes in the downstream of STING signaling pathway, such as Tbk1, Irf3, Iftai, and Iftar2, could not be induced by IFNα (Fig 1A and Supplementary Fig S1A). Consistent with the STING mRNA expression data, STING protein level was significantly elevated in IFNα-treated BMMs (Fig 1B). STING also could be induced by IFNα in human macrophages and dendritic cells (DCs) (Fig 1C and D). To further determine whether STING is an ISG, we transfected polyIC and polydA:dT, the mimics of pathogen-derived nucleic acid which trigger large amount of IFN-I production in immune cells, into different BMMs and compared the STING expression. Higher STING mRNA was detected in WT BMMs than Ifnar1−/− BMMs activated by either polyIC or polydA:dT (Fig 1E). In addition, polyIC triggered more STING transcripts in WT BMMs than Trif−/− BMMs, while polydA:dT triggered similar amount of STING mRNA in WT and Trif−/− BMMs (Fig 1F). Given that polyIC-triggered IFN-I is mainly TRIF dependent while polydA:dT activates IFN-I in a TRIF-independent manner [22], our data here suggest that induction of STING by polyIC or polydA:dT requires IFN-I production and its downstream signaling. Furthermore, we found that cGAMP induced STING expression in a dose-dependent manner in WT BMMs but not in Ifnar1−/− cells (Fig 1G). Consistently, STING protein was induced by cGAMP in WT BMMs but not in Ifnar1−/− BMMs (Fig 1H). Interestingly, there is no upregulation of STING mRNA in LPS-stimulated WT BMMs, though LPS also could trigger IFN-I production via TRIF-dependent pathway (Fig 1I). LPS induced STING mRNA in Myd88−/− BMMs in a time-dependent manner (Fig 1J), which implied that LPS could suppress STING expression through the Myd88-dependent pathway and induce STING expression through the TRIF-dependent pathway. Furthermore, by analyzing the STING mRNA level in LPS-activated WT and Trif−/− BMMs, we found that STING expression was suppressed by LPS after 4 h stimulation and recovered after 12 h stimulation in WT BMMs, while STING mRNA was suppressed at both time points examined in Trif−/− BMMs (Fig 1J and Supplementary Fig S1B). The TLR2 ligand, Pam3CSK4, activates Myd88 pathway but does not trigger IFN-I production [23]. STING expression was suppressed in Pam3CSK4-activated WT BMMs but not in Myd88−/− BMMs (Supplementary Fig S1C), which further suggested that STING could be suppressed by Myd88-dependent signaling. Taken together, we have demonstrated that STING transcripts could be induced by IFN-I and most of the ligands which could trigger IFN-I in BMMs, therefore demonstrating that STING is an ISG. LPS plays dual roles in controlling STING expression. In LPS-activated BMMs, TRIF-dependent signaling induces STING expression by triggering IFN-I production, while Myd88-dependent pathway suppresses STING expression via an unknown mechanism.

Several studies have described the posttranslational modifications of STING. Phosphorylation of STING on Ser358 by Tbk1 is required for STING-mediated activation of IRF3 [5]. However, phosphorylation of STING at Ser36 by UNC-51-like kinase (ULK1) suppressed IRF3 activation [24]. K63-linked ubiquitination of STING by TRIM56 and TRIM32 induced STING dimerization, which is a prerequisite for recruitment of TBK1 and subsequent induction of IFN-I [25,26]. However, K48-linked ubiquitination of STING by RNF5 mediates degradation of STING [27]. Besides these posttranslational regulations of STING, our results here have added a new layer of regulation of STING at transcription level.

A STAT1 binding site is critical for induction of STING by IFN-I

To determine how STING is induced by IFN-I, we analyzed the potential TF binding sites in STING 5′-UTR region. Mouse STing locates in chromosome 18 and is encoded by the negative strand of DNA (Fig 2A). Among all the predicted TF binding sites around the STing transcription start site (TSS), there are two potential STAT1 binding sites accounting for induction of STING by IFN-I. Both sites got high matrix score in the TRANSFAC software [28,29], and the sequences of the two STAT1 binding sites are conserved in mouse, rat, and human STING (Fig 2B). To verify Stat1 binding at the STing promoter, we downloaded and processed Stat1 ChIP-seq (chromatin immunoprecipitation sequencing) data in BMMs [30,31]. Interestingly, we found a significant Stat1 binding peak around the Stat1#1 region of the STing promoter in the BMMs treated with IFNα and IFNγ (Fig 2C). In addition, we observed the dynamic change of the Stat1 binding in this region in BMMs treated with IFNα for different time points (Supplementary Fig S2A). We further verified the ChIP-qSeq data by ChIP-qPCR assay and detected significantly higher Stat1 binding in IFNα- or IFNγ-treated BMMs comparing with the untreated BMMs (Fig 2D). Three reporter constructs were made to verify the potential function of the two STAT1 binding sites (Fig 2E). IFNα activated both WT and Δα2 luciferase reporters, but not the Δα2-mut#1 reporter in RAW264.7 cells (Fig 2F), which suggested that Stat1#1 binding site was a major site of STING regulated by IFN-I. IPS1, TBK1, and IRF1 trigger IFN-I production in HEK293T cells [32,33]. Consistent with the results from RAW264.7 cells, all of the IPS1, TBK1, and IRF1 could activate WT and Δα2 luciferase reporters, but not the Δα2-mut#1 reporter in HEK293T cells (Fig 2G). Furthermore, IFNα treatment or STAT1 transfection plus IFNα treatment could significantly activates WT and Δα2 luciferase reporters, but not the Δα2-mut#1 reporter in HEK293T cells (Fig 2H). Considering that STAT1 is a common TF activated by both IFN-I and IFN-II, we checked the STING mRNA in IFNγ-stimulated BMMs. IFNγ stimulation also significantly induced STING mRNA in BMMs (Supplementary Fig S2B) and THP1 cells (Supplementary Fig S2C), which further suggested that activation of STAT1 was required for induction of STING. Our data therefore suggest that both IFN-I and IFN-II can induce STING expression at transcription level through a STAT1 binding site in the STING promoter.

Activation of IFNAR by IFN-I leads to the formation of STAT1–STAT2–IRF9 (known as ISGF3, IFN-stimulated gene factor 3) complexes and STAT1–STAT1 homodimers, which bind to IFN-stimulated response elements (ISREs) and IFNγ-activated site (GAS) elements, respectively. Activation of IFNγ receptor (IFNGR) by IFNγ only triggers dimerization of STAT1 and induces the genes with GAS elements in their promoters [34]. “TTC/ANNCG/TAA” is the typical GAS motif [34]. The Stat1#1 site in the STing promoter harbors the core sequence “TTCGGGAAA”, a GAS motif. This motif gives the reason for why STING could be induced by both IFN-I and IFN-II. Considering that IFNγ treatment only leads to dimerization
STING is an interferon-stimulated gene (ISG).

A BMMs were treated with 62.5 U/ml IFNα for 2.5 h. RNA was extracted and gene expression profile was detected by Affymetrix 430.2 Chip. Sting, If3, and If7 mRNA levels are shown as probe intensity from microarray.

B BMMs were treated with 100 U/ml IFNα for the indicated time, and the protein level of Sting in these cells was detected by WB. α-tubulin are shown as a loading control.

C, D Human macrophage (C) and dendritic cells (D) were treated with 100 U/ml recombinant human IFNα for the indicated time, and STING mRNA level in these cells was detected by qPCR and normalized to Rpl32.

E, F WT and Ifnar1−/− (E) or Trif−/− (F) BMMs were transfected with 1 μg/ml polyI:C or polydA:dT for 4 h, and Sting mRNA level in these cells was detected by qPCR and normalized to Rpl32.

G WT and Ifnar1−/− BMMs were transfected with the indicated amount of cGAMP for 4 h, and Sting mRNA from these cells was detected by qPCR and normalized to Rpl32.

H WT and Ifnar1−/− BMMs were transfected with 3 μg/ml cGAMP for the indicated times, Sting protein level was detected by WB, and α-tubulin are shown as a loading control.

I, J WT and Myd88−/− BMMs were stimulated with 100 ng/ml LPS for 4 h or 12 h, and supernatant IFNβ was measured by ELIAS (I). Sting mRNA from these cells was detected by qPCR and normalized to Rpl32 (J).

Data information: Data of (A, C–G, I, J) are from three independent experiments (mean ± s.e.m.), *P < 0.05, **P < 0.01 (Student’s t-test). Data of (B) and (H) are from one representative experiment. Similar results were obtained in three independent experiments.
of STAT1, while IFNα or IFNβ treatment results in two STAT1-containing complexes, it is reasonable that IFNγ induces slightly more STING transcripts and triggers more STAT1 binding in the STING promoter region than IFN-I, as the results shown in Fig 2D and Supplementary Fig S2C.

Optimal production of CDNs-triggered IFN-I requires IFNAR signaling

In response to cytosolic DNA, host cells produce cGAMP to trigger IFN-I production utilizing the STING–TBK1–IRF3 signaling axis [6].
Figure 2. A STAT1 binding site is critical for the induction of STING by IFN-I.

A The chromosome location of Sting and its nearby genes. The diagram was modified from NCBI gene (Gene ID: 72512).

B The potential Stat1 binding sites in the promoter of Sting. Transcription factor binding site prediction was performed by TRANSFAC. The location of the Stat1 binding sites and the matrix score are shown. The conservation comparison of the predicted Stat1 binding sites between human, rat, and mouse was according to the sequence from Ensembl Genome Browser. TSS, transcription start site; CDS, coding DNA sequence.

C BMFs were treated with 100 U/ml IFNβ and IFNα for 6 h, Stat1 CHIP-Seq data were analyzed, and the Stat1 binding region in Sting promoter is shown. The Stat1 CHIP-Seq raw data were downloaded from GEO (accession no. GSE39313).

D BMFs were treated with 100 U/ml IFNα or IFNβ for 2 h, and the binding of Sting promoter region with Stat1 (or IgG) was detected by ChIP-qPCR. The data are shown as fold change of Stat1/IgG.

E The sequence of the mutated Stat1#1 binding site and the schematic diagram of the Sting promoter reporter plasmids.

F Control vector (pGL3.2) and indicated Sting promoter reporter constructs expressing firefly luciferase were transfected into RAW264.7 cells by nucleofection system. pRL-TK-luc vector expressing renilla luciferase was co-transfected as a control for transfection efficiency. Data are shown as the relative luciferase activity.

G Flag, IPS1, TBK1, or IRF1 was co-transfected with the indicated Sting promoter reporter constructs and pRL-TK-luc vector into HEK293T cells. Data are shown as the relative luciferase activity.

H Flag or Stat1 was co-transfected with the indicated Sting promoter reporter constructs and pRL-TK-luc vector into HEK293T cells for 12 h, and these cells were treated with control or 500 U/ml IFNα for another 12 h. Firefly and Renilla luciferase activities were measured and relative luciferase activity are shown.

Data information: Data of (D) are from three independent experiments (mean ± s.e.m.), **P < 0.01 (Student’s t-test). Data of (F–H) are from one representative experiment (mean ± s.d., n = 6), *P < 0.05, **P < 0.01 (Student’s t-test). Similar results were obtained in three independent experiments.

Interestingly, more induction of IFNβ and IFNα4 transcripts was detected in cGAMP-activated WT BMFs than Irfna1+/− BMFs (Fig 3A and B). More, higher supertransient IFNβ and IFNα were detected in cGAMP-activated WT BMFs than Irfna1+/− BMFs (Fig 3C and D). Similar studies in J2 virus-immortalized macrophage cell line (J2-BMMs) supported that cGAMP-induced IFN-I transcripts and protein production were positively regulated by Irfna1 and STAT1 (Fig 3E–H), which suggested that optimal production of cGAMP-triggered IFN-I requires IFNAR signaling.

To further determine the requirement of IFNAR signaling in cGAMP-triggered IFN-I induction, BMFs were pretreated with IFNα and then activated by cGAMP. Priming of BMFs with IFNα significantly enhanced cGAMP-mediated induction of IFNβ and IFNα4 transcripts in a dose-dependent manner (Fig 3I and J). Similarly, significantly higher IFNβ protein was produced after IFNα pretreatment, both by low-dose and high-dose cGAMP-activated BMFs (Fig 3K).

Bacterial CDNs such as c-di-AMP and c-di-GMP are also able to elicit a host IFN-I innate immune response [7,18]. Transfection with c-di-AMP and c-di-GMP triggers IFNβ mRNA expression in a dose-dependent manner (Supplementary Fig S3A and B). Induction of IFNβ transcript and protein by either c-di-AMP or c-di-GMP was impaired in Irfna1−/− BMFs and J2-BMMs (Supplementary Fig S3). Taken together, these data indicated that optimal production of both endogenous and bacterial CDNs-triggered IFN-I requires IFNAR signaling, which suggested that the positive feedback loop played a role in the CDNs-triggered IFN-I. Given that the induction of cytosolic RNA and DNA sensors such as RIG-I, MDA5, and IFI16 by IFN-I positively regulates IFN-I production by sensing more pathogen nucleic acids [12–14], we hypothesized that the induction of STING could increase the host’s ability of sensing CDNs and positively regulate CDNs-triggered IFN-I production.

IRF7-dependent IFNα mediates the late stage of STING induction in cGAMP-activated BMFs

IRF7 is a well-known ISG that mediates IFN-I positive feedback loop mainly by inducing IFNα during viral infection [15–17]. Therefore, we tested whether induction of STING by cGAMP requires IRF7 and whether the classical IRF3–Irfna1/Irf7–IFNα/β signaling axis plays a role in the positive feedback loop of cGAMP-triggered IFN-I production. Firstly, we found that IRF3 expression determined basal and inducible level of IRF7 in untreated and cGAMP-activated J2-BMMs (Fig 4A), which supported that IRF7 was an ISG and induction of IRF7 was IRF3 dependent in cGAMP-activated macrophages. To test whether IRF7 is required for cGAMP-triggered IFN-I induction, we compared the induction of IFNβ and IFNα4 in WT, Irf3−/−, and Irf7−/− J2-BMMs triggered by cGAMP. Significantly attenuated induction of IFNβ mRNA was observed in Irf3−/− but not in Irf7−/− J2-BMMs activated by transfection of cGAMP for 4 h, while IFNα4 mRNA induction was impaired in both Irf3−/− and Irf7−/− J2-BMMs (Fig 4B and C). Significantly less supertransient IFNβ and IFNα were detected in Irf3−/− and Irf7−/− J2-BMMs activated by transfection of cGAMP for 16 h, although the downregulation of IFNβ was not as drastic as IFNα in Irf7−/− J2-BMMs (Fig 4D and E). Consistent with the Irf3-I production results, induction of STING was impaired in Irf3−/− J2-BMMs activated by transfection of cGAMP for 4 h and 16 h, while modest but significant downregulation of STING induction was observed in Irf7−/− J2-BMMs activated by transfection of cGAMP for 16 h (Fig 4F). These data indicate that IRF7 is critical for cGAMP-triggered IFNα production and modestly regulates IFNβ production at the late stage. However, IRF7 is dispensable for early stage of IFNβ induction in cGAMP-activated macrophages. Classic IFN-I positive feedback loop via IRF3–Irfna1–IRF7–IFNα/β signaling axis plays a role in cGAMP-triggered total IFN-I production. However, considering that induction of STING is IFNAR dependent but IRF7 independent at the early stage of cGAMP transfection, induction of DNA sensors such as STING by the first wave production of IFN-I is an alternative mechanism for IFN-I positive feedback, particularly at the early stage.

Overexpression of STING abolishes the difference of CDNs-triggered IFN-I production between WT and Irfna1−/− BMFs

To determine whether the induction of STING by IFN-I plays a role in the positive feedback loop of CDNs-triggered IFN-I production, we overexpressed mouse Sting in both WT and Irfna1−/− J2-BMMs by lentiviral gene transduction to get a similar level of Sting expression in these cells. Sting mRNA was elevated dramatically after transducing with Sting-overexpressing lentiviruses, and the expression levels of Sting were similar between Sting-overexpressing WT and Irfna1−/− J2-BMMs (Fig 4A). In addition, the Sting protein level is
comparable between J2-BMMs transduced with LV-Sting and BMMs triggered by cGAMP (Supplementary Fig S4A). Considering the almost-saturated Sting expression in these Sting-overexpressing cells, CDNs-triggered IFN-I may not affect the STING expression in these cells. We found less difference of cGAMP-triggered IFNβ production between Sting-overexpressing WT and Ifnar1−/−
J2-BMMs, comparing to LV-Empty transduced WT and Ifnar1−/− J2-BMMs (Fig 4B and C). Overexpression of Sting rescues the impaired cGAMP-triggered IFNβ production but not the defect of cGAMP-triggered IFNα production in Irf7−/− macrophages (Supplementary Fig S4B–D). These results indicated that overexpression of Sting rescued the defect of IFNβ production in Ifnar1−/− macrophages and reduced the difference of CDNs-triggered IFNβ production between WT and Ifnar1−/− J2-BMMs, which suggested that induction of Sting by IFN-I contributed to the positive feedback loop of IFN-I production. Classical IRF3–IFNβ–IRF7–IFNα/β signaling pathway plays a role at the late stage of STING induction by triggering IFNα production, which accounts for how IRF7 and STING work together to play roles in type I IFN positive feedback loop (Fig 5D).

In summary, our study not only has demonstrated a novel mechanism by which STING is induced by IFN-I via a STAT1 binding site in the promoter region of STING, but also suggested a role of the induction of STING in IFN-I positive feedback regulation loop. Although we have described a new layer regulation of STING at transcription level other than the previous reported regulation at posttranslational level [5,24–27], it seems that the regulation of STING during innate immune responses is much more complicated than we think. LPS positively regulates STING expression via TRIF-dependent signaling while negatively suppresses STING expression through MyD88-dependent pathway. Our results indicate that cGAMP induces STING mRNA and protein in macrophage by triggering IFN-I production, while cGAMP destabilizes STING protein by triggering ULK1 phosphorylation in primary MEF and hTERT-BJ1 cells [24]. These data suggested that STING is fine-regulated during host innate immune responses. Activation of STING by CDNs results in the induction of numerous genes that suppress pathogen replication and facilitate adaptive immunity [1–5]. However, persistent transcription of innate immune genes causes inflammatory disorders and autoimmune diseases [35–37]. Thus, STING expression should be tightly regulated at multiple levels to maintain functional host innate immune responses and homeostasis.

IRF3–IFNβ–IRF7–IFNα/β signaling axis is a well-established loop for IFN-I positive feedback during viral infections. In our study, we
Figure 5. Overexpression of STING abolishes the difference of cGAMP-triggered IFNβ production between WT and Ifnar1−/− macrophages.

A WT and Ifnar1−/− J2-BMMs were transduced with empty or Sting-expressing lentivirus (LV-Empty or LV-Sting) for 3 days. Sting mRNA was detected by qPCR.

B WT and Ifnar1−/− J2-BMMs were transduced with LV-Empty or LV-Sting for 3 days; then, the medium was replaced and the cells were left untreated or transfected with 3 μg/ml cGAMP for another 16 h. IFNβ in the supernatant of these cells was measured by ELISA.

C J2-BMMs were treated and induction of IFNβ was measured as described in (B). IFN-β/(IFN-β)WT in percent was calculated and compared for LV-Empty- and LV-STING-transduced cGAMP-activated cells.

D Induction of STING plays a role in the positive feedback loop of CDNs-triggered IFN-I production. Viral DNA such as HSV and HIV DNA is recognized and converted into cGAMP by cGAS. Bacteria such as Listeria monocytogenes and Mycobacterium tuberculosis can produce the other CDNs, for example, c-di-AMP and c-di-GMP. These CDNs are recognized by STING and trigger the STING–TBK1–IRF3 signaling axis to produce the first wave of IFN-I. Initial production of IFN-I induces the IFN-AR signaling, which further amplifies the CDNs recognition and the IFN-I production, as shown in step (1). In addition, induction of transcription factor IRF7 by first wave IFN-I drives the production of multiple IFNs, which mediates the further induction of STING and IFNα at the later stage, as shown in step (2).

Data information: Data of (A–C) are from three independent experiments (mean ± s.e.m.), **P < 0.01 (Student’s t-test).
show that induction of STING is IRF3 dependent but IRF7 independent in cGAMP-activated BMMs at the early stage, which suggest that STING-dependent IFN-I positive feedback loop is independent of the classical IRF7-dependent IFN-I positive feedback pathway, particularly at early stage. It is interesting to investigate whether other IFN-inducible DNA sensors and RNA sensors such as cGAS and RIG-I mediate IFN-I positive feedback independent of IRF3–IFNβ–IRF7–IFNα/β axis or not. Our study here focuses on the positive regulation of STING expression, and we suggest that this positive regulation leads to more induction of CDNs-triggered IFN-I. It is interesting that STING-dependent IFN-I production facilitates host clearance of VSV and HSV-1 infections while inhibits cell-mediated immune responses against certain pathogens, and also the roles of this loop in inflammatory disorders and autoimmune diseases. Further experiments are required for fully understanding the physiological or pathophysiological significance of the mechanism proposed by this study.

Materials and Methods

Mice and reagents

Wild-type C57BL/6 (6–8 weeks of age) and age-matched Ifnar1−/−, Stat1−/−, MyD88−/−, and Trif−/− male mice were purchased from Jackson Laboratory. Ifnγ−/− mice were from Dr. Tadatsugu Taniguchi’s laboratory (University of Tokyo). All mice experiments were performed in accordance with guidelines from the University of California, Los Angeles, Institutional Animal Care and Use Committee. TLR2 ligand Pam3CSK4, cGAMP, c-di-AMP, c-di-GMP, polyI:C, and polydA:dT were purchased from InvivoGen (San Diego, CA). TLR4 ligand LPS (Escherichia coli 0111:B4) and anti-β-tubulin antibody were from Sigma-Aldrich (St. Louis, MO). Anti-STING antibody (#3337) was from Cell Signaling Technology (Danvers, MA). Recombinant human and mouse IFNα was from PBL interferon source (Piscataway, NJ), and recombinant mouse IFNγ 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 RPMI1640 supplemented with 10% FBS and 1% penicillin/streptomycin. For human macrophage and DC differentiation, CD14+ human PBMCs purified from whole blood (obtained from heath donors, with informed consent, UCLA I.R.B. #92-10-591-31) were cultured for 7 days with 800 U/ml GM-CSF (Immunex) and 500 U/ml IL-4 (Peprotech) for DC differentiation, or with 50 ng/ml M-CSF (R&D Systems) for macrophage differentiation. Cytokines were replenished at day 3, and the media was replaced on day 7 prior to activation of the cells. For mice bone marrow-derived macrophage (BMM) differentiation, bone marrow was harvested from wild-type or indicated knockout mice and differentiated in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 10 ng/ml of M-CSF for 7 days. The media was replaced on day 3 and day 6, and the cells were used for experiments as BMMs on day 7. For J2 virus-immortalized macrophages (J2-BMMs), a cell line transformed by retrovirus expressing v-raf and c-myc was established (called GG2EE) and grown in RPMI1640 (10 mM HEPES pH 7.8, 10% FBS, 1% penicillin/streptomycin). The supernatant containing J2 viruses was harvested and filtered through 0.22-μm filter. Bone marrow cells were infected with the J2 virus and immortalized as described previously [39,40]. Femur and tibia from Ifnγ−/− mice (8 weeks, male, C57BL/6 background) were overnight-shipped from Michael S. Diamond’s laboratory (Washington University). Ifnγ−/− bone marrow cells were differentiated into BMMs and immortalized to Ifnγ−/− J2-BMMs. To activate BMMs or J2-BMMs, 100 ng/ml LPS was added into culture medium, or an indicated amount of cGAMP, c-di-AMP, c-di-GMP, polyI:C, or polydA:dT was transfected into cells by Lipofectamine 2000 (Life Technologies). The ratio of transfection reagent to ligands was 2.5 (μl/μg). Detailed Lipofectamine 2000 transfection protocol was followed as described in the manufacturer’s instructions.

RNA isolation and quantitative PCR (qPCR)

Total RNA was extracted with TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. 1 μg RNA from each sample was reverse-transcribed by using iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad). Real-time quantitative RT–PCR analysis was performed by using SensiFAST SYBR & Fluorescein Kit (Bioline) and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The relative mRNA expression level of genes was normalized to the internal control ribosomal protein gene Rp32 by using 2−ΔΔCt cycle threshold method [41]. The primer sequences for qPCR were from primer bank [42], and they are available upon request.

Microarray

On day 7, the wild-type BMMs were stimulated with 62.5 U/ml IFNα or 1 U/ml IFNγ for 2.5 h, and the total RNA was extracted for microarray analyses. Microarrays were done on the Affymetrix 430.2 Chip (University of California, Los Angeles Genotyping and Sequencing Core). The microarray data analysis was performed using GeneSpring software (Agilent Technologies) [14]. The micro-array data have been deposited in the Gene Expression Omnibus (GEO) database (accession no. GSE35825).

ChIP-Seq data analysis and Stat1 ChIP

Stat1 ChIP-Seq raw data for BMMs were downloaded from the GEO (accession no. GSE33913). BMMs cells differentiation and activation were described previously [30]. Sequenced reads were aligned to mouse genome (mm9) allowing up to two mismatches using Bowtie [43]. The data were processed as previously described [44]. For peak calling, mouse genome was divided into 100-bp windows. A P-value for Poisson distribution of enriched ChIPed DNA over input DNA for each window was calculated. Significant peaks were defined as the windows with significant P-value less than 10−3 and with two neighboring windows at the same significance. Stat1 ChIP
assays were performed according to the protocol of the ChiP assay kit (Magna ChiP™ HiSens kit, catalog No. 17-10460, Millipore). Anti-STAT1 antibody (sc-592x) from Santa Cruz Biotechnology was used for ChiP assay. Primer pair specific for the STING promoter Stat1#1 region was 5’-TGAGCTATCGAGCCCTGAC-3’ (forward) and 5’-ACACCTTTTTCGGGAAAT-3’ (reverse). STING promoter region in both input and immunoprecipitated genomic DNA was detected by qPCR. The percentage of Sting promoter region immunoprecipitated by anti-STAT1 or its isotype IgG relative to input DNA was calculated, and the data are shown as the enrichment of the Sting promoter region by STAT1.

ELISA and immunoblot

IFNα and IFNβ in cell culture supernatant were quantified with VeriKine Mouse Interferon Alpha and Beta ELISA Kit (PBL interferon source), respectively. The ELISAs were performed 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 BCA assay (Thermo Scientific) and equalized with the lysis buffer. Equal amounts of the extracts were loaded and subjected to SDS-PAGE, transferred onto PVDF membranes (Millipore), and then blotted with enhanced chemiluminescence (Pierce) or Odyssey Imaging Systems (LI-COR Biosciences).

STING promoter reporter and dual-luciferase reporter assay

The potential transcription factor (TF) binding sites in the mouse Sting promoter region were predicted by TRANFAC [28,29]. The conservation of the TF binding sites among the mammalian species was analyzed by UCSC genome browser (http://genome.ucsc.edu/). Different length of Sting promoters were amplified from C57BL/6 genome DNA and subcloned into the pGL4.20 [luc2/Puro] vector (Promega, Madison, WI) to generate wild-type Sting promoter reporter (WT-luc) and Stat1#2-deficient Sting promoter reporter (ΔStat1-luc). The Stat1#1 region of the Δ2-luc reporter was mutated to generate Δ2-luc-mut#1 reporter construct via QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). The indicated STING promoter reporter construct was co-transfected with Renilla luciferase reporter (pRL-TK-luc) into RAW264.7 cells by Amaxa Cell Line Nucleofector Kit V (Lonza). 12 h posttransfection, the medium was replaced and the cells were stimulated with 500 U/ml IFNα for another 12 h; then, the cells were lysed by passive lysis buffer, and the firefly luciferase activity of the STING promoter reporters was measured and normalized by Renilla luciferase activity according to the manufacturer’s instructions of the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). HEK293ST cells were co-transfected with indicated vectors (Flag, TBK1, IPS1, IRF1, or Stat1), STING promoter reporter constructs, and pRL-TK-luc according the manufacturer’s instructions of Jet-PEI (Polyplus-transfection). 24 h posttransfection (in some experiments, cells were transfected for 12 h and treated with 500 U/ml IFNα for another 12 h), the cells were lysed and the relative luciferase activity was measured as in RAW264.7 cells.

Lentivirus packaging and lentiviral transduction

Full-length mouse Sting gene was cloned into the lentiviral vector pCDEF1-CMV-MCS2-EF1-copGFP (CD11B-1; System Bioscience) to make the expression constructs LV-Sting. LV-Empty or LV-Sting vector was co-transfected into HEK293T cells with the pPACKF1 Packaging plasmids mix (LV100A-1, System Bioscience). Control or Sting-overexpressing lentiviruses were produced, and the WT, Ifnar1−/−, or Irf7−/− J2-BMM cells were transduced by these lentiviruses according to the user’s manual (System Bioscience) and the previous study [45].

Supplementary information for this article is available online: http://embor.embopress.org

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Author contributions

FM and GC designed the study. FM, BL, YY, SSI, and MS performed the experiments and analyzed the data. FM wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

STING is an interferon-stimulated gene

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