STING is an essential mediator of the Ku70-mediated production of IFN-λ1 in response to exogenous DNA

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We previously identified Ku70, a subunit of a DNA repair protein complex, as a cytosolic DNA sensor that induces the production of interferon-λ1 (IFN-λ1) by human primary cells and cell lines. IFN-λ1 is a type III IFN and has similar antiviral activity to that of the type I IFNs (IFN-α and IFN-β). We observed that human embryonic kidney (HEK) 293T cells, which are deficient in the innate immune adaptor protein STING (stimulator of IFN genes), did not produce IFN-λ1 in response to DNA unless they were reconstituted with STING. Conversely, parental HEK 293 cells produced IFN-λ1 after they were exposed to exogenous DNA; however, when STING was knocked out in the HEK 293 cells through the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 genome editing system, they lost this response. Through confocal microscopy, we demonstrated that endogenous Ku70 was located in the nucleus and then translocated to the cytoplasm upon DNA exposure to form a complex with STING. Additionally, the DNA binding domain of Ku70 was essential for formation of the Ku70-STING complex. Knocking down STING in primary human macrophages inhibited their ability to produce IFN-λ1 in response to transfection with DNA or infection with the DNA virus HSV-2 (herpes simplex virus–2). Together, these data suggest that STING mediates the Ku70-mediated IFN-λ1 innate immune response to exogenous DNA or DNA virus infection.

INTRODUCTION

Immune recognition of pathogens is mediated by germ-line–encoded pattern recognition receptors (PRRs), which recognize conserved microbial structures termed pathogen-associated molecular patterns (PAMPs) (1, 2). PRR families include the Toll-like receptors (TLRs), the retinoic acid–inducible gene 1 (RIG-I)–like receptors, and a diverse family of cytosolic DNA sensors (3–7). Once engaged by PAMPs, PRRs stimulate intracellular signaling pathways and activate transcription factors, such as nuclear factor κB and interferon (IFN) regulatory factor 3 (IRF3), which lead to the increased production of proinflammatory cytokines, such as tumor necrosis factor–α and interleukin-1β (IL-1β), and of the antiviral type I IFNs, IFN-α or IFN-β (8, 9).

Considerable effort has been made to try to elucidate the initial type I IFN signaling events that enable cells to detect the presence of cytosolic DNA. This search has led to the identification of multiple DNA sensors. The IFN-inducible protein DAI (DNA-dependent activator of IRFs) is encoded by the DAI gene (10). DAI is a type III IFN and has antiviral activity against DNA viruses including HSV-1 and HSV-2 (11–15). Furthermore, DAI can sense cytosolic DNA leading to IFN production (16–18) and activates the IFN-γ–inducible gene 16 (19). DAI is a DNA sensor that triggers the production of type III IFNs (20–22). DAI activates type III IFN production through a signaling pathway involving TANK-binding kinase 1 (TANK) and IFN-λ1 (23). The lack of IFN-λ1 production leads to a loss of antiviral activity in the context of DNA infection (24). Therefore, DAI’s role in type III IFN production is critical for the antiviral response triggered by cytosolic DNA. This includes components of the DNA-PK (DNA-dependent protein kinase) complex, which is composed of Ku70, Ku80, and DNA-PKcs as well as MRE11 (meiotic recombination 11 homolog A) (19, 20).

Type III IFNs are less well-characterized IFNs with a similar antiviral activity to that of the type I IFNs, and they include IFN-λ1, IFN-λ2, and IFN-λ3 (also known as IL-29, IL-28A, and IL-28B, respectively) as well as IFN-λ4 (21–26). Expression of the genes that encode IFN-λ proteins is inducible by infection with many types of viruses (27–29). However, analysis of the murine genome showed that the gene encoding the mouse ortholog of human IFN-λ1 lacks exon 2 entirely and contains a stop codon within exon 1 even in wild mice; thus, the Ifnl1 gene does not encode a functional IFN-λ1 protein (30). Therefore, any investigation of the physiological relevance of IFN-λ1 in a mouse model is restricted. Compared to the signaling pathways associated with the production of type I IFNs, the signaling pathways underlying the production of type III IFNs are poorly understood. We previously identified the DNA-PK component Ku70 as a DNA sensor that stimulates type III IFN production by human primary macrophages and human cell lines. Ku70, initially characterized as a DNA repair protein, specifically binds to cytosolic DNA (such as after transfection) or viral DNA and then activates the IFN transcription factors IRF1 and IRF7, leading to robust production of IFN-λ1 (31). Our previous study indicated that the induction of type III IFN production is distinct from that of type I IFN production; however, the identity of the adaptor protein downstream of Ku70 was unknown. We also reported that SV40 T-antigen–transformed human embryonic kidney (HEK) 293 cells (herein referred to as 293T cells) do not produce IFN-λ1 in response to DNA stimulation. The mechanism underlying this lack of response is unknown (32).

The adaptor protein STING (stimulator of IFN genes) was reported to play a pivotal role in responding to DNA by mediating TBK1 (TANK binding kinase 1)–dependent activation of IRF3 in response to cytosolic, double-stranded DNA (33–35). A wealth of information on the role of STING in DNA sensing and on the mechanisms whereby it contributes to signaling in the induction of type I IFN production has been discovered. In the signaling pathway to produce type I IFNs, STING acts

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downstream of several cytosolic DNA sensors, including DAI, DDX41, cGAS (cyclic GMP (guanosine monophosphate)–AMP (adenosine monophosphate) synthase), and IFI16 (10, 15, 17, 36). Other mediators involved in orchestration of the innate immune response include β-catenin, which functions downstream of LRRFIP1 to stimulate IFN production, and the adaptor protein MyD88 (myeloid differentiation primary response 88), which is involved in responding to cytosolic DNA through DHX9- or DHX36-mediated innate immune responses (11, 16, 18). Here, we have looked for host factor(s) involved in the Ku70-mediated induction of type III IFN production in response to exogenous DNA. Our findings offer a new perspective on DNA sensing and have implications for host defense, vaccine development, and autoimmunity.

**RESULTS**

**As compared to HEK 293 cells, 293T cells do not produce IFN-λ1 in response to DNA**

We previously reported that Ku70 is a cytosolic DNA sensor that induces IFN-λ1 production by primary human macrophages and some nonimmune cells, such as HEK 293 cells and human rhabdomyosarcoma cells (31), but not by 293T cells (32). Here, we transfected 293T cells and HEK 293 cells with plasmid encoding green fluorescent protein (GFP). One day later, the extent of transfection was monitored by fluorescence microscopy analysis. Similar amounts of GFP were detected in both cell lines (Fig. 1A). To determine the extent of expression of the genes encoding IFNA, IFNB, IFNL1, and IFNL2/3 in response to the transfection, we extracted cellular RNA from the transfected cells and measured the abundances of the appropriate mRNAs with a real-time reverse transcription polymerase chain reaction (RT-PCR) assay. Consistent with our previous report (32), HEK 293 cells showed a more than 200-fold increase in the abundance of IFNL1 mRNA after transfection with DNA. However, the abundances of IFNA, IFNB, and IFNL2/3 mRNAs were only 1.3-, 3.6-, and 61.3-fold greater, respectively, compared to those in untreated cells (Fig. 1B). In contrast, none of these mRNAs were increased in abundance in 293T cells after transfection with DNA (Fig. 1B). To further confirm induction of the IFN response, we used enzyme-linked immunosorbent assays (ELISAs) to measure the amounts of IFN-λ1, IFN-α, IFN-β, and IFN-λ2/3 proteins produced by the cells. IFN-λ1 was the major IFN produced by the HEK 293 cells, which had concentrations of 1393 ± 43.1 pg/ml in the culture medium, whereas the concentrations of IFN-λ2/3, IFN-α, and IFN-β were reduced at 371.0 ± 0.9, 61.3 ± 0.6, and 157.3 ± 1.9 pg/ml, respectively (Fig. 1C). Consistent with the gene expression data, we failed to detect any IFN-α, IFN-β, IFN-λ1, or IFN-λ2/3 production by the transfected 293T cells (Fig. 1C). Additionally, a time course of the production of IFN-α, IFN-β, IFN-λ1, and IFN-λ2/3 was measured in the transfected HEK cells (Fig. S1). On the basis of these data, we hypothesized that one or more important signaling factors were missing in the 293T cells.

**293T cells lack endogenous STING**

STING is an important mediator in several DNA-sensing pathways that are associated with the induction of type I IFN responses (10, 15, 17, 36). To determine whether differences in the amounts of STING or Ku70 might be associated with the differential ability of 293T and HEK 293 cells to produce IFN-λ1, we compared the amounts of STING and Ku70 mRNA and protein between the two cell lines. The abundance of STING mRNA was less in 293T cells than in HEK 293 cells (Fig. 2A). Consistent with this result, the abundance of STING protein, as measured by Western blotting analysis, was also markedly reduced in 293T cells compared to that in HEK 293 cells. There was no substantial difference in the amount of Ku70 protein between 293T and HEK 293 cells (Fig. 2B). As additional controls, we also assessed the amounts of β-catenin and MyD88 proteins in 293T and HEK 293 cells; however, they were similar in both cases (Fig. S2).

**STING is an essential mediator in Ku70-mediated induction of IFN-λ1 production in response to DNA**

To test the hypothesis that the absence of STING in 293T cells was responsible for their inability to produce IFN-λ1 in response to DNA

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*Fig. 1. Transfection of 293T cells with GFP-encoding plasmid DNA does not induce the production of IFN-α, IFN-β, IFN-λ1, or IFN-λ2/3 mRNA and protein. (A to C) HEK 293 and 293T cells were transfected with plasmid pCMV-GFP. (A) Twenty-four hours later, the cells were observed under a fluorescence microscope. Green fluorescence was shown in the context of total cells. Images are representative of three independent experiments. (B) Total RNA was extracted and the relative amounts of IFNA, IFNB, IFNL1, and IFNL2/3 mRNAs were measured by real-time RT-PCR. The relative expression of the indicated genes was compared to that in untreated cells. Data are means ± SD of three independent experiments. (C) Forty-eight hours after transfection, the cell culture medium was collected, and the concentrations of the indicated proteins were measured by ELISA. Data are means ± SD of three independent experiments.*
exposure, we established an IFN-λ1 reporter assay system. In this system, 293T cells were cotransfected with pGL4–IFN-λ1, a reporter plasmid containing the IFNL1 promoter region, and different amounts of plasmid encoding Ku70 with or without 100 ng of plasmid encoding STING. To fully induce Ku70 and STING, the transfected cells were incubated for 48 hours and were then further stimulated by being transfected with noncoding plasmid DNA, and IFNL1 promoter activity was quantitated by relative luciferase activity (Fig. 3A). We found that Ku70 induced IFNL1 promoter activity only in the presence of STING, and it did so in a concentration-dependent manner. Note that Ku70 alone was not able to induce IFNL1 promoter activity. We detected substantial amounts of STING in those 293T cells that were transfected with the STING-encoding plasmid (Fig. 3B); however, STING was undetectable in 293T cells that were transfected with an empty vector. To further confirm the effect of exogenous STING in 293T cells on IFNL1 expression, we performed real-time RT-PCR assay using cellular RNA isolated from DNA-stimulated, STING-overexpressing 293T cells. The abundance of IFNL1 mRNA in the STING-expressing 293T cells was increased in response to DNA stimulation (Fig. 3C), whereas 293T cells failed to induce IFNL1 expression if they were transfected with an empty plasmid, which was similar to the IFNL1 response observed in untreated 293T cells (Fig. 3C). We next investigated whether the removal of STING from HEK 293 cells would modify the DNA-stimulated induction of IFNL1 expression. For these experiments, STING knockout (STING KO) HEK 293 cells were generated through the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 genome editing system. STING was undetectable in the STING KO cells; however, it was detectable after the cells were transfected with a STING expression plasmid (Fig. 3D). The abundance of Ku70 was similar in all of the cell lines examined, suggesting that the loss of STING had no effect on Ku70 abundance. STING KO cells did not exhibit IFNL1 expression after transfection with DNA. However, the DNA-stimulated induction of IFNL1 expression was restored in cells transfected with the STING-encoding plasmid (Fig. 3E). Together, the results suggest that STING is an essential mediator of the DNA-stimulated induction of IFNL1.

To exclude the possibility that the role of STING in facilitating IFN-λ1 production in response to DNA was cell type–dependent, we performed similar experiments with the THP-1 cell line. THP-1 cells are human leukemia monocytes, which have been extensively used to study monocyte and macrophage functions, mechanisms, and signaling pathways as well as nutrient and drug transport (37). Western blotting analysis confirmed that THP-1 cells have endogenous Ku70 and STING. Transfection of the cells with small interfering RNAs (siRNAs) specific for Ku70 and STING reduced the abundances of the target proteins by 51 and 66%, respectively, compared to those in cells transfected with control siRNA (Fig. 3F). Using the cells in which Ku70 or STING were knocked down, we further performed transfections with plasmid DNA and assessed the expression of IFN-λ1. Similar to HEK 293 cells, THP-1 cells exhibited a substantial (12,131-fold) increase in IFNL1 mRNA in response to transfection with DNA; however, this induction was inhibited in cells in which the abundances of either Ku70 or STING were knocked down by 59 and 78%, respectively (Fig. 3G). In cells transfected with both siRNA-Ku70 and siRNA-STING, the induction of IFNL1 expression was inhibited by 88% compared with that in the cells that were not exposed to siRNA (Fig. 3G). Consistent with these data, the abundance of IFN-λ1 protein in the THP-1 cells in which the abundance of Ku70 and STING was reduced by 96% compared to that in the cells that were not exposed to siRNA (Fig. 3H), suggesting that the Ku70-STING pathway plays a role in the DNA-stimulated induction of IFNL1 production in THP-1 cells (Fig. 3, G and H). The knockdown of Ku70 and STING in the THP-1 cells also suppressed the production of IFN-α, IFN-β, and IFN-λ2/3 (fig. S3).

We previously reported that cross-talk between signaling by the DNA sensor IFI16 and the RNA sensor RIG-I mediates the type III IFN response. Here, we investigated whether IFI16 was also involved in the Ku70-mediated production of IFN-λ1 in response to exogenous DNA. We knocked down IFI16 by transfecting the cells with IFI16-specific siRNA and confirmed the efficiency of knockdown by Western blotting analysis (fig. S4A). However, the extent of IFNL1 induction was not changed in the IFI16-knockdown cells compared with that in the cells that were not treated with siRNA (fig. S4B).

Ku70 translocates from the nucleus to the cytoplasm and forms a complex with STING in response to DNA

Our findings led us to investigate the physical relationship between STING and Ku70. The locations of endogenous Ku70 and STING were analyzed by immunofluorescence staining and confocal microscopy analysis of unstimulated and DNA-stimulated cells (Fig. 4A). The cellular localization was verified by counterstaining nuclei with 4′,6-diamidino-2-phenylindole (DAPI). In unstimulated cells, Ku70 was detected in the nucleus, whereas STING was found in the cytoplasm (Fig. 4A, top). After the cells were stimulated with DNA, we found that Ku70 translocated from the nucleus to the cytoplasm, whereas STING remained in the cytoplasm (Fig. 4A, bottom). This translocation of Ku70 with STING was confirmed by Western blot analysis (fig. S4C). The relative abundance of Ku70 and STING was reduced by 96% compared to that in the unstimulated control cells (Fig. 4B). Together, these results showed that Ku70 and STING form a complex in response to DNA stimulation.
Ku70 from the nucleus to the cytoplasm provided an opportunity for STING and Ku70 to interact with each other.

We further designed a coimmunoprecipitation assay to confirm whether STING interacted with Ku70 in the context of stimulation with DNA. The results demonstrated that in the absence of exogenous DNA, FLAG-tagged Ku70 did not interact with exogenous STING. However, Flag-tagged Ku70 coimmunoprecipitated with STING when the cells were treated with DNA (Fig. 4B). We also performed the coimmunoprecipitation assay with an anti-Myc antibody to pull down STING (Fig. 4C). The results of these experiments consistently supported the hypothesis that STING and Ku70 are found in the same complex only after the cells are transfected with DNA (Fig. 4, B and C).

The DNA binding domain of Ku70 is required for Ku70 to form a complex containing STING

Ku70 is composed of an α/β domain (I), a β-barrel DNA binding domain (II), a linker (III), and a C-terminal domain (IV) (Fig. 5A). To analyze which domain is involved in the binding of Ku70 to STING, we constructed several truncated, Flag-tagged Ku70 mutants, containing the I, II, and III domains (I.I.I.III), the I and II domains (I.II), or the I domain alone (I). We then performed coimmunoprecipitation assays with 293T cells transfected with plasmids encoding the Flag-tagged Ku70 mutants and detected which of these bound to STING in response to DNA stimulation (Fig. 5B). The DNA binding domain of Ku70 is required for Ku70 to form a complex containing STING. In independent experiments, Ku70 mutants with I.II.III, I.II, or II did not coimmunoprecipitate with STING (Fig. 5B) confirming the importance of this region of Ku70 in binding to STING.

A heterodimer of Ku proteins (Ku70 or Ku80) and the catalytic subunit DNA-PKcs are the components of the DNA-PK complex. We additionally confirmed whether Ku80 and DNA-PKcs were present in the

**Fig. 3. STING is critical for the DNA-stimulated, Ku70-mediated induction of IFN-λ1 production.** (A) 293T cells were cotransfected with an IFNL1 reporter plasmid (pGL4-IFN-λ1), a Renilla luciferase plasmid (pRL-TK), and different amounts of the Ku70 expression plasmid with or without transfection with 100 ng of the STING expression plasmid, as indicated. Forty-eight hours after transfection, the cells were then stimulated with 1 μg of noncoding plasmid DNA. Twenty-four hours after stimulation, the cells were collected for luciferase assays. Data are means ± SD of three independent experiments. (B) Western blotting analysis was performed to confirm the presence of STING in the transfected 293T cells. Untreated 293T cells and 293T cells transfected with empty plasmid were included as controls. Whole-cell lysates were analyzed with anti-STING and anti-Ku70 antibodies. β-Actin was detected as a loading control. Western blots are representative of three independent experiments. (C) 293T cells were left untreated or were transfected with either an empty plasmid or a STING expression plasmid and then were stimulated with 1 μg of plasmid DNA. One day later, IFNL1 mRNA abundances were measured by real-time RT-PCR. Relative values were determined by comparison with untreated 293T cells. Data are means ± SD of three independent experiments. **P < 0.001. (D) Western blotting analysis was performed to confirm the knockout of STING in the STING KO cells. Whole-cell lysates were analyzed with anti-STING and anti-Ku70 antibodies. β-Actin was used as a loading control. Western blots are representative of three independent experiments. (E) STING KO cells were transfected with an empty vector or a STING expression vector, and the cells were then stimulated by transfection with 1 μg of noncoding plasmid DNA. Twenty-four hours after stimulation, total cellular RNA was extracted and the relative abundance of IFNL1 mRNA was measured. The values were compared to the amount of IFNL1 mRNA in HEK cells that were transfected with empty vector without additional DNA stimulation. Data are means ± SD of three independent experiments. **P < 0.001. (F) Western blotting analysis was performed to confirm the knockout of Ku70 or STING in THP-1 cells. THP-1 cells were transfected with or without the indicated siRNAs and then were transfected with DNA 1 day later. Whole-cell lysates were collected and incubated with anti-STING and anti-Ku70 antibodies. β-Actin was used as a loading control. Western blots are representative of three independent experiments. (G) Twenty-four hours after the indicated THP-1 cells were stimulated by transfection with DNA, the abundance of IFNL1 mRNA was measured by real-time RT-PCR. Relative values were determined by comparison with untreated THP-1 cells. Data are means ± SD of three independent experiments. (H) Forty-eight hours after the indicated THP-1 cells were stimulated by transfection with DNA, the cell culture medium was collected and the amounts of IFN-λ1 were determined by ELISA. Data are means ± SD of three independent experiments.
Ku70-STING complex. The proteins that coimmunoprecipitated with the DNA binding domain of Ku70 were analyzed by Western blotting with anti-DNA-PKcs, anti-STING, and anti-Ku80 antibodies, and the result showed that Ku80 was present in the complex but that DNA-PKcs was not necessarily required for the interaction of the DNA binding domain of Ku70 with STING (fig. S5A). An IFNL1 promoter reporter assay further suggested that the DNA binding domain alone, like full-length Ku70, dose-dependently activated IFNL1 promoter activity (fig. S5B). Ku80, even if present in the Ku70-STING complex, was not functional in the DNA-mediated induction of IFN-α1 production (fig. S5B).

The activation of the transcription factors IRF3, IRF1, and IRF7 is required for the Ku70- and STING-mediated production of IFN-α1 in response to exogenous DNA

Having identified STING as a critical protein in the Ku70-mediated production of IFN-α1, we then determined which transcription factor(s) was involved. First, we tested the effect of knocking down IRF1, IRF3, or IRF7 on the induction of IFN-encoding genes. We transfected HEK 293 cells with siRNAs targeting IRF1, IRF3, or IRF7. On the following day, the cells were then transfected with DNA. Whole-cell lysates were then collected and analyzed by Western blotting to determine IRF1, IRF3, and IRF7 abundance, and total RNA was extracted to measure the relative amounts of IFN mRNAs by real-time RT-PCR analysis. In the absence of exogenous DNA, the abundance of IRF3 in HEK 293 cells was greater than that of either IRF1 or IRF7 (Fig. 6A). In response to transfection with DNA, the amounts of IRF1 and IRF7, but not that of IRF3, were increased (Fig. 6A). Transfection of the cells with IRF1-specific siRNA not only efficiently reduced the abundance of IRF1 but also decreased that of IRF7 (Fig. 6A). In addition, the abundances of both IRF7 and IRF1 were reduced in cells transfected with IRF7-specific siRNA. Furthermore, transfection of the cells with IRF3-specific siRNA not only reduced the abundance of IRF3 but also reduced the amounts of IRF1 and IRF7 (Fig. 6A). Our real-time RT-PCR analysis showed that the induction of IFNL1 expression and IFN-α1 protein production were inhibited in cells in which IRF1, IRF3, or IRF7 was knocked down (Fig. 6, B and C). Similar results were shown for IFN-β, and IFN-α2/3 (fig. S6, A and B).

Given that IRF3 and IRF7 reside in the cytosol and undergo serine phosphorylation of their C-terminal regions in response to viral infection, which enables them to dimerize and translocate to the nucleus (38), we next evaluated the nuclear accumulation of IRF1, IRF3, and IRF7 in HEK 293 cells and STING KO cells in response to stimulation DNA. We transfected the cells with DNA and then prepared nuclear fractions at various times (Fig. 6D). Western blotting analysis showed that the abundances of IRF1 and IRF7, but not that of IRF3, were increased in the nucleus of HEK 293 cells 48 hours after transfection. In contrast, no increase in the nuclear accumulation of these transcription factors was observed in the STING KO cells (Fig. 6D). The results demonstrated that the DNA-dependent activation of IRF1 and IRF7 occurred only in the presence of STING.

To further elucidate the role of STING in the activation of IRF1 and IRF7 after transfection of cells with DNA, we used confocal microscopy to monitor the nuclear translocation of endogenous IRF1 and IRF7 in HEK 293 and STING KO cells (Fig. 6, E to H). Forty-eight hours after transfection of the cells with DNA, we observed substantial accumulation of both IRF1 and IRF7 in the nuclei of HEK 293 cells (Fig. 6, E and G). Similar changes were not observed in the STING KO cells (Fig. 6, F and H). These results are consistent with STING playing a key role in activating these transcription factors upstream of IFN gene induction. In summary, the absence of STING dampened the activation of IRF1 and IRF7 and consequently resulted in reduced IFN-α1 production.
Loss of STING inhibits IFN-\(\lambda\) production by human primary macrophages in response to transfection with DNA or infection with herpes simplex virus–2

To further demonstrate the physiologic relevance of STING in the Ku70-mediated production of IFN-\(\lambda\) in response to DNA or DNA virus, we transduced human monocyte-derived macrophages (MDMs) with lentiviruses encoding short hairpin RNA (shRNA) targeting the STING expression cassette. The transduced cells were then transfected with DNA plasmids or infected with herpes simplex virus–2 (HSV-2). Twenty-four or 48 hours later, the cells were collected to analyze gene expression, and the culture medium was harvested to measure the abundances of IFN proteins. We first showed that transduction with the lentivirus led to a substantial reduction in the abundance of STING protein; however, no changes in STING abundance were observed in untransduced cells or in cells transduced with lentivirus encoding control shRNA (Fig. 7A). We further found that the DNA-increased expression of IFNL1 was inhibited by up to 80% when STING was knocked down by shRNA (Fig. 7B). Analysis of IFN-\(\lambda\) protein abundance in the cell culture medium gave consistent results (Fig. 7C).

In addition, we also evaluated the effect of knocking down STING on IFN-\(\lambda\) production in cells infected with HSV-2. The results of this experiment showed that knocking down STING resulted in a reduction in the amounts of IFNL1 mRNA and protein (Fig. 7, D and E). Furthermore, knocking down STING also suppressed the induction of IFNA, IFNB, and IFNL2/3 (fig. S7). Together, these data suggest that STING mediates the Ku70-dependent production of IFN in response to exogenous DNA or infection with a DNA virus.

DISCUSSION

The innate immune system is the first line of defense against invading pathogens. It is well known that microbial nucleic acids stimulate the production of type I IFNs, such as IFN-\(\alpha\) and IFN-\(\beta\), as a key host defense strategy to limit the replication of invading microorganisms (39). Many of these pathways and their capacity to induce type I IFN production have been extensively studied (40). Accumulated evidence suggests that non-self nucleic acids can also stimulate type II and III IFN responses (21, 23, 31, 32); however, the molecular mechanisms involved are poorly understood.

The DNA repair protein Ku70 also acts as a DNA sensor to induce the production of IFN-\(\lambda\), as opposed to IFN-\(\alpha\) or IFN-\(\beta\), in primary cells or cell lines, such as HEK 293 cells, but not 293T cells (31, 32). However, the downstream mediator of Ku70 was unclear. Here, by comparing the properties of HEK 293 and 293T cells, we identified STING, located downstream of Ku70, as an essential mediator of the production of IFN-\(\lambda\) in response to exogenous DNA. The inability of 293T cells to produce IFN-\(\lambda\) after transfection with DNA was...
Fig. 6. The activation of IRF1, IRF3, and IRF7 involves the Ku70-mediated induction of IFN-λ1 production in response to exogenous DNA. (A) HEK 293 cells were left untreated or were transfected with the indicated siRNAs. The cells were then transfected with DNA 1 day later. Twenty-four hours after transfection with DNA, whole-cell lysates were analyzed by Western blotting with antibodies against the indicated proteins. Untreated cells were included as a control, and β-actin was used as a loading control for each condition. Western blots are representative of three independent experiments. (B) Total RNA was extracted 24 hours after DNA transfection, and the relative abundance of IFNL1 mRNA was measured by real-time RT-PCR. The relative values were determined by comparison with untreated cells. Data are means ± SD of three independent experiments. (C) Forty-eight hours after DNA transfection, the cellular culture medium was collected and the amounts of IFN-λ1 protein were detected by ELISA. Data are means ± SD of three independent experiments. (D) HEK 293 and STING KO cells were stimulated by transfection with 1 μg of plasmid DNA. Nuclear fractions of the cells were isolated at the indicated times after transfection and were analyzed by Western blotting with antibodies against the indicated proteins. NUP98 was measured as a loading control. Western blots are representative of three independent experiments. (E to H) HEK 293 and STING KO cells were grown on glass-bottom, 35-mm culture dishes. The cells were transfected with plasmid DNA. Forty-eight hours later, the cells were fixed and stained with antibodies against IRF1 (green) (E and F) or IRF7 (red) (G and H) and visualized by confocal microscopy. Nuclei were visualized with DAPI (blue). Scale bars, 5 μm. Original magnification was ×60. Images are representative of three independent experiments.
Confocal microscopy analysis showed that Ku70 was found in the nuclei of unstimulated cells. We found that Ku70 underwent a translocation from the nucleus to the cytoplasm when the cells were transfected with a DNA plasmid. In contrast, STING was located in the cytoplasm in both unstimulated and stimulated cells. These results suggested that the translocation of Ku70 prompted an interaction between Ku70 and STING. Further work is necessary to uncover the mechanisms about the translocation of Ku70. In addition, coimmunoprecipitation experiments demonstrated that Ku70 formed a complex with STING in response to transfection with DNA. Ku70 is a component of the heterotrimeric protein complex DNA-PK, which also contains Ku80 and the catalytic subunit DNA-PKcs. DNA-PK acts as a PRR, binding to cytoplasmic DNA and stimulating the expression of genes encoding type I IFNs (19). This study also showed that Ku70 and STING form a complex from which Ku70 dissociates 3 hours after stimulation with DNA. Ferguson et al. (19) stated that the dissociation of Ku70 and STING activated downstream signaling and induction of the expression of genes encoding type I IFNs. Our data are complementary to these findings in demonstrating that prolonged association of STING and Ku70 may lead to the production of type III IFN as a late event after exposure to DNA. Here, we focused on the late stage of the IFN immune response. This time frame is consistent with the time course of IFN-λ1 production in response to stimulation with DNA (fig. S1), which is also consistent with our previous study (31). The result from ELISA demonstrated that there was a marked increase in the amount of IFN-λ1 protein secreted into the cell culture medium 24 to 48 hours after transfection. Furthermore, these experiments showed that detectable amounts of IFN-α, IFN-β, and IFN-λ-2/3 were found in the cell culture medium but at a much lower abundance than that of IFN-λ1 (fig. S1). Thus, the Ku70-mediated production of IFN-λ1 in response to exposure to DNA or infection with a DNA virus is a dominant response in HEK 293 cells, THP-1 cells, and primary human macrophages.

Ku70 is composed of four major domains: the α/β domain, the DNA binding domain (β-barrel), the linker domain, and the C-terminal domain (41, 42). Here, we identified that the DNA binding domain was essential for the binding of Ku70 to STING. Ku70 and Ku80 form heterodimers, and the absence of one subunit destabilizes the other (43, 44). Both the Ku heterodimer (42) and DNA-PKcs (45) can bind directly to DNA; however, in the absence of either Ku70 or Ku80, the affinity of DNA-PKcs for DNA is substantially reduced (46). These findings suggest that each component in the DNA-PK complex has an important role to play. However, we further found that Ku80, even when present in the Ku70-STING complex, was not functional in the DNA-mediated induction of IFN-λ1 production (fig. S5). DNA-PKcs was not necessarily required for the interaction of the DNA binding domain of Ku70 with STING (fig. S5A), whereas this DNA binding domain itself was functional in the DNA-mediated induction of IFN-λ1 production (fig. S5B). In summary, although Ku80 or DNA-PKcs was present in the complex containing Ku70 and STING, they were not functional in the induction of IFN-λ1 production in response to DNA.

Previous studies identified the STING-TBK1-IRF3 pathway as playing an important role in the induction of type I IFN production in response to different forms of nucleic acids (47, 48). Here, we provided further evidence to show that the transcription factors IRF1, IRF7, and IRF3 were all involved in the DNA-dependent induction of IFN-λ1 production. A major distinction between IRF3 on the one hand and IRF1 and IRF7 on the other is that IRF3 is constitutively found in most cell types, whereas IRF1 and IRF7 are found in cells only after the exposure of cells to type I IFNs (49). Our data confirmed that production...
of the IRF1 and IRF7 proteins was induced in DNA-transfected cells and was inhibited by knockdown of IRF3, indicating that IRF1 and IRF7 production required IRF3. Our data further showed that there was a certain amount of IRF3 that accumulated in the nucleus, but the intensity of the signal did not show differences between DNA-stimulated and unstimulated HEK 293 cells or STING KO cells. This finding suggests that IRF3 might be activated at an early stage but that IRF3 is not responsible for the late-stage induction of IFN-λ1 production. The genes encoding IRF1 and IRF7 were induced through the activation of IRF3 at an early stage and then contributed to the abundant induction of IFN-λ1 production at a later stage. This observation led us to divide the model of Ku70-mediated IFN-λ1 production into two distinct steps. First, the immediate or early response involved a very low (or undetectable) amount of IFN produced through the activation of IRF3. Second, a low level of synthesis of IFNs from the first step results in positive autocrine feedback by the production of IRF1 and IRF7, which provides efficient amplification of IFN-λ1 production at a later stage (49). This two-stage model is consistent with the time course of DNA-stimulated, Ku70-mediated IFN production (fig. S1). In cells transfected with DNA, we were unable to detect the expression of IFNA, IFNB, or IFNL2/3 at earlier times (3 and 6 hours), and we detected IFN-λ1 at low abundance. Robust amounts of IFN-λ1 were produced at a later stage, that is, at 24 to 48 hours after the cells were stimulated with DNA. This result further suggested that the production of IFN-α, IFN-β, and IFN-λ2/3 was simultaneously induced together with that of IFN-λ1. The same induction kinetics implied that the induction of production of these four IFNs shared a similar signaling transduction pathway in response to exogenous DNA, in which Ku70 and STING played an important role in the dominant induction of IFN-λ1 production with the simultaneous induction of IFN-α, IFN-β, and IFN-λ2/3 production to a much lesser extent. In addition, this Ku70-STING-IRF3-, IRF1-, and IRF7-mediated pathway to induce IFN-λ1 production differs from the IFI16-STING-IRF3-mediated IFN-λ1 pathway, which we have previously reported (32), because IFI16-induced IFNL1 signaling peaked at 6 hours after stimulation with DNA stimulation, and IRF1 and IRF7 are not involved (32). Note that the exogenous DNA that we used for the induction of IFN-λ1 in the Ku70 pathway is distinct from the DNA that was used for the stimulation of the IFI16 pathway. In our current study of the Ku70 pathway in the induction of IFN-λ1 production, linearized DNA and HSV-2 were used, whereas in the study of the IFI16-mediated pathway for the induction of IFN-λ1 production, circular DNA and HSV-1 virus were used (15, 32). The knockdown of IFI16 has no effect on the DNA-induced induction of IFN-λ1, indicating that IFI16 is not involved in the Ku70-mediated IFN-λ1 induction pathway (fig. S4). Therefore, it is still an interesting research area to discover the mechanism of distinct pathogen recognition patterns in host cells in response to very similar foreign invasion. Together, our results suggest that STING is an essential mediator downstream of Ku70 for the DNA-induced production of IFN-λ1. This finding advances our understanding of the regulation of the innate immune response and expands the current repertoire of DNA-sensing mechanisms. These findings may be of value in furthering our understanding of the function of the immune system in health and disease.

**MATERIALS AND METHODS**

**Cells, antibodies, and viruses**

HEK 293 cells, 293T cells, and THP-1 cells were obtained from the American Type Culture Collection (ATCC) and maintained according to the manufacturer’s instructions. CD14+ monocytes were purified from the peripheral blood mononuclear cells of healthy donors using CD14 MicroBeads (Miltenyi Biotec) according to the manufacturer’s instructions, as previously described (50). To generate MDMs, isolated CD14+ monocytes were plated on a 10-cm petri dish at 10 × 10⁶ cells per dish. Monocytes were stimulated with macrophage colony-stimulating factor (25 ng/ml) (R&D Systems) in macrophage serum-free medium (Thermo Fisher Scientific) for 7 days. MDMs were then maintained in Dulbecco’s modified Eagle’s medium (Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS) (HyClone Laboratories), 25 mM Hepes (Quality Biology), and gentamicin (5 μg/ml) (Thermo Fisher Scientific) before they were used in experiments. HSV-2 was obtained from Advanced Biotechnologies Inc., and viral titers were determined by plaque-forming assays with Vero cells (ATCC) (51). Antibodies used in this study were as follows: anti-STING, anti-MycD88, anti-β-catenin, anti-Ku80, anti-IRF1, anti-IRF7, and anti-Flag antibodies were from Cell Signaling Technology; anti-Ku70 and anti–DNA-PKcs antibodies were from Abcam; and anti-IRF3 antibody was from OriGene. Alexa Fluor 488– and Alexa Fluor 555–labeled secondary antibodies were purchased from Cell Signaling Technology.

**Gel electrophoresis and Western blotting analysis**

Cell lysates were prepared with radioimmunoprecipitation assay buffer (Boston BioProducts) in the presence of protease inhibitor cocktail (Sigma-Aldrich) and Halt phosphatase inhibitor cocktail (Thermo Fisher Scientific). Nuclear protein was extracted with the Nuclear Extract Kit (Active Motif) according to the manufacturer’s instructions. The protein concentrations of the cell lysates were quantified with the bicinchoninic acid protein assay (Thermo Fisher Scientific) to ensure that equal amounts of total protein were loaded in each well of NuPAGE 4 to 12% bis-tris gels (Thermo Fisher Scientific). Proteins were transferred onto a nitrocellulose membrane and analyzed by Western blotting with the appropriate antibodies, which was followed by incubation with horseradish peroxidase–conjugated secondary antibodies and detection of bands with ECL plus Western blotting detection reagents (GE Healthcare). Band intensities were analyzed with National Institutes of Health Image J software (http://rsbweb.nih.gov/ij/).

**Plasmids**

The PCR2.1 plasmid (Thermo Fisher Scientific) was digested with Eco RI, and this digested plasmid was used as a noncoding DNA stimulant. The Myc-tagged STING expression vector was constructed as follows. STING-encoding complementary DNA (cDNA) was synthesized from the total cellular RNA of HEK 293 cells using the Superscript First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific). The reverse-transcribed cDNA encoding STING was subcloned into pEF6/Myc-His (Thermo Fisher Scientific). The insertion was confirmed by DNA sequencing with BigDye version 3.0 (Applied Biosystems). Other expression vectors used in this study were constructed with a similar method. The cDNA encoding Ku70 was cloned into pEF1-Flag (Thermo Fisher Scientific). The DNA encoding the full-length IFNL1 promoter region [993 base pairs (bp)] was amplified from HEK 293 germline DNA with the Expand high-fidelity PCR system (Roche Molecular Biochemical) with a primer pair (5′-GAGCTCAAAAC-CAATGGCGAAGCTCC-3′ and 5′-AGATTCTTGCTAAATCG-CAACCTGCTTCC-3′). The 993-bp fragment was subcloned and inserted into the vector pGL4.10 (Promega). The presence of the intended fragment without any unexpected mutations was confirmed by DNA sequencing, and this vector was named pGL4–IFN-λ1.
plasmid pTK-RL, which expresses Renilla luciferase under the control of HSV thymidine kinase promoter, was obtained from Promega and used to normalize experimental variations.

**IFN-α1 reporter assay**

293T cells (30 × 10^3 cells per well) were seeded in six-well plates and cotransfected with 100 ng of pGL4–IFN-α1, 10 ng of pTK-RL, and the appropriate amounts of STING expression vector, wild-type Ku70 expression vector, or mutant Ku70 expression vector. The cells were then transfected on day 2 with 1 μg of linearized DNA (as a stimulant), and the cells were then collected for the luciferase assay. The luciferase assay was performed with Dual-Glo luciferase assay system reagents (Promega). Relative luciferase activity was calculated on the basis of the ratio of the luminescence of firefly luciferase to that of Renilla luciferase.

**RNA extraction and real-time RT-PCR**

Total cellular RNA was isolated from cells with the RNeasy isolation kit (Qiagen). The cDNA was synthesized from total RNA with TaqMan reverse transcription reagents (Thermo Fisher Scientific) with random hexamers as primers, according to the manufacturer’s instructions. The relative abundance of IFNLI mRNA was measured by quantitative RT-PCR on a CFX96 real-time system (Bio-Rad); a two-temperature cycle of 95°C for 15 s and 60°C for 1 min (repeated for 40 cycles) was used. Relative quantities of IFNLI transcripts were calculated with the ΔΔCt method with GAPDH mRNA as a reference. Normalized samples were expressed relative to the average ΔCt value for controls to obtain relative fold changes in mRNA abundance.

**Generation of STING KO cells**

STING KO cells were generated from HEK 293 cells with the CRISPR/Cas9 genomic editing kit (OriGene) according to the manufacturer’s instructions. HEK 293 cells (5 × 10^5) were transfected with 600 ng of hCas9 target guide RNA and 600 ng of donor vector in a six-well tissue culture plate. The cells were split at a 1:1 dilution 2 days after transfection. After an additional 3 days in culture, the cells were split again at a 1:10 dilution. This procedure was repeated seven times. The cells were then transfected to 10-cm dishes and cultured with medium containing puromycin (2 μg/mL) (Thermo Fisher Scientific). The culture medium was changed every 2 to 3 days. Individual cell colonies were isolated by limiting dilution. After 1 to 2 weeks, the cells were observed under the microscope, and cells from those wells containing only one cell colony were selected and allowed to expand from a 96-well to a 6-well plate.

**Coimmunoprecipitation assays**

HEK 293T cells (1 × 10^5) were seeded onto a 100-mm dish and transfected with a Flag-tagged Ku70/Ku70 mutant expression vector and a Myc-tagged STING expression vector using a TransIT-293 Transfection Kit (Mirus Bio LLC). The cells were further transfected on day 2 with 10 μg of linearized DNA (as a stimulant). The cells were lysed in Pierce IP lysis buffer (Thermo Fisher Scientific) with 10% glycerol and 10 mM PMSF at 4°C to remove cell debris. The supernatants were immunoprecipitated with an anti-Flag agarose (Sigma-Aldrich) or anti–c-Myc agarose (Thermo Fisher Scientific) and then analyzed by Western blotting with anti-FLAG or anti-STING antibodies.

**Confocal microscopy**

Cells grown on 35-mm glass bottom dishes were fixed in 4% paraformaldehyde, blocked in blocking buffer [1× phosphate-buffered saline (PBS), 5% normal goat serum (Cell Signaling), and 0.1% saponin (Alfa Aesar)] for 1 hour, stained overnight with primary antibodies (diluted at 1:200) at 4°C, and then incubated with Alexa Fluor 488- or Alexa Fluor 555–labeled secondary antibodies (1:1000) for 1 hour. Bottom-coated coverslips were mounted with ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific). Images were captured on an LSM 710 scanning confocal microscope.

**Lentivirus packaging and transduction**

A lentiviral plasmid containing an expression cassette encoding STING-specific shRNA was purchased from OriGene. Lentiviral particles were packaged in 293T cells according to the manufacturer’s instructions. Harvested lentiviruses-containing cell culture medium was concentrated with a lenti concentrator (OriGene). The lentiviral particles were titrated on the basis of their abundance of the HIV p24 antigen measured with an HIV-1 p24 ELISA kit (PerkinElmer). For lentiviral transductions, human macrophages were seeded on six-well plates to 70% confluence and inoculated with lentiviruses at an MOI of 50 in culture medium containing 2% FBS in the presence of polybrene (8 μg/ml). Twenty-four hours after the cells were transduced, the medium was replaced by fresh complete culture medium, and the cells were then incubated for a further 72 hours to enable protein knockdown to occur.

**Enzyme-linked immunosorbent assay**

The amounts of IFN-α, IFN-β, IFN-λ1, and IFN-λ2/3 proteins in cell culture medium were measured with the VeriKine-HS Human IFN-α All Subtype ELISA Kit (PBL Assay Science), the VeriKine-HS Human IFN-β Serum ELISA Kit (PBL Assay Science), the human IL-29/IFN-λ1 DuoSet ELISA (R&D Systems), and the DIY Human IFN-λ2/3 (IL-28A/B) ELISA (PBL Assay Science) kits, respectively, according to the manufacturers’ protocols. The minimum detectable concentrations of IFN-α, IFN-β, IFN-λ1, and IFN-λ2/3 were 1.95, 1.2, 62.5, and 125 pg/ml, respectively.

**Statistical analysis**

Results were representative of at least three independent experiments. The values are expressed as means ± SD of individual samples. Statistical significance was determined by Student’s t test. P < 0.05 was considered to indicate a statistically significant difference between the experimental groups.

**SUPPLEMENTARY MATERIALS**

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Fig. S1. Time course of the DNA-stimulated induction of IFN-1 production.

Fig. S2. Comparison of the endogenous amounts of IFN-α, IFN-β, IFN-λ1, and IFN-λ2/3 production.

Fig. S3. Analysis of the production of IFN-1, IFN-2, and IFN-3 production in response to exogenous DNA.

Fig. S4. The activation of IRF1, IRF3, and IRF7 involves the Ku70-mediated induction of IFN-1 production in response to exogenous DNA.

Fig. S5. Ku80 and DNA-PKcs are not involved in the Ku70-mediated induction of IFN-1 production in response to exogenous DNA.

Fig. S6. The activation of IRF1, IRF3, and IRF7 involves the Ku70-mediated induction of IFN-1 production in response to exogenous DNA.

Fig. S7. Knockdown of STING inhibits the production of IFN-1, IFN-2, and IFN-3 production by human MDMs transfected with DNA or infected with HSV-2.

**REFERENCES AND NOTES**


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STING is an essential mediator of the Ku70-mediated production of IFN-λ1 in response to exogenous DNA

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STINGing viruses with IFN-λ1

When cells sense cytosolic DNA, such as occurs during viral infection, they produce type I interferons (IFNs) as part of the antiviral response. Various cytosolic DNA sensors and their downstream effectors stimulate activation of the transcription factor IFN regulatory factor 3 (IRF3) to induce expression of genes encoding type I IFNs. Sui et al. investigated the mechanism by which the DNA protein kinase (DNA-PK) component Ku70, a DNA repair protein, induced production of the type III IFN IFN-λ1 in human cells exposed to cytosolic DNA or infected with the DNA virus HSV-2. Ku70 translocated from the nucleus to the cytosol to interact with the adaptor protein STING, a well-characterized mediator of type I IFN production. However, in the Ku70 pathway, IRF1 and IRF7 were required in addition to IRF3, and type III IFN production was slower than that of type I IFN. Together, these data suggest a role for STING in the late phase of the antiviral IFN response to DNA viruses.