Cutting Edge: Ku70 Is a Novel Cytosolic DNA Sensor That Induces Type III Rather Than Type I IFN

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Cytosolic foreign DNA is detected by pattern recognition receptors and mainly induces type I IFN production. We found that transfection of different types of DNA into various untreated cells induces type III IFN (IFN-λ1) rather than type I IFN, indicating the presence of uncharacterized DNA sensor(s). A pull-down assay using cytosolic proteins identified that Ku70 and Ku80 are the DNA-binding proteins. The knockdown studies and the reporter assay revealed that Ku70 is a novel DNA sensor inducing the IFN-lambda1 activation. The functional analysis of IFNL1 promoter revealed that positive-regulatory domain I and IFN-stimulated response element sites are predominantly involved in the DNA-mediated IFNL1 activation. A pull-down assay using nuclear proteins demonstrated that the IFN-λ1 induction is associated with the activation of IFN regulatory factor-1 and -7. Thus, to our knowledge, we show for the first time that Ku70 mediates type III IFN induction by DNA. The Journal of Immunology, 2011, 186: 000–000.

In the activation of innate immune responses triggered by infection with bacterial or viral pathogens, microbe-specific molecular patterns in the pathogens are detected by pattern-recognition receptors (PRR). This ligand–receptor interaction triggers the activation of the innate immune system (1–3). Foreign DNA is recognized by membrane-bound PRR and cytoplasmic PRR. Recent studies demonstrate that DNA-dependent activator of IFN-regulatory factor (DAI) (4), absence in melanoma 2 (AIM-2) (5), leucine-rich repeat flightless-interacting protein 1 (LRRFIP1) (6), RNA polymerase III (7), and IFN-γ–inducible protein 16 (IFI16) (8) are cytoplasmic PRRs and induce production of type I IFN or II-1β. In this study, we report that Ku70, a component of a heterodimeric Ku protein, which is required for a variety of nuclear processes, including nonhomologous end-joining DNA repair, V(D)J recombination, and telomerase maintenance (9, 10), also functions as a cytosolic PRR recognizing DNA and induces the production of IFN-λ1 (a member of type III IFN) (11, 12) rather than type I IFN. The induction is mediated via the activation of IFN regulatory factor (IRF)-1 and IRF-7.

Materials and Methods

Cell culture, mice, and HIV replication assay

Human embryonic kidney cell line 293 (HEK293), HEK293T, human rhabdomyosarcoma cell line (RD), and HeLa cells were obtained from American Type Culture Collection. Monocyte-derived macrophages (MDMs) and dendritic cells (DCs) were prepared as previously described (13, 14). HIV replication assay was performed as previously described (13). Female wild-type (WT) C57/B6.129 mice were provided by the National Cancer Institute (NCI)-Frederick. Ku70-deficient mice on a C57/B6.129 background (15) were provided by Dr. Andre Nussenzweig (NCI, Bethesda, MD). All experiments with mice were performed in compliance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals and were approved by NCI-Frederick Animal Care and Use Committee.

Preparation of plasmid DNA and genomic DNA

All plasmids were purified using the Endofree Plasmid Maxi kit (Qiagen). Genomic DNA was extracted from HEK293 cells using QIAamp DNA mini kit (Qiagen).

Transfection

HEK293 cells (100 × 10^3 cells in 3 ml/well of 6-well plates) were transfected with 1 μg DNA or 5 nM siRNA using TransIT-293 (Mirus Bio) or RNAiMAX (Invitrogen), according to the manufacturer’s instructions. The online version of this article contains supplemental material.

Abbreviations used in this article: AIM-2, absence in melanoma 2; DAI, DNA-dependent activator of IFN-regulatory factor; DC, dendritic cell; HA, hemagglutinin; HEK293, human embryonic kidney cell line 293; IFI16, IFN-γ–inducible protein 16; IRF, IFN regulatory factor; ISRE, IFN-stimulated response element; LRRFIP1, leucine-rich repeat flightless-interacting protein 1; MDM, monocyte-derived macrophage; NCI, National Cancer Institute; PRDI, positive-regulatory domain I; PRR, pattern-recognition receptor; qRT-PCR, quantitative real-time RT-PCR; RD, human rhabdomyosarcoma cell line; si-Ctrl, siRNA-control; si-Ku70, siRNA-Ku70; si-Ku80, siRNA-Ku80; siRNA, small interfering RNA; WT, wild-type.
small interfering RNA (siRNA)-transfected HEK293 cells were cultured for 48 h followed by DNA transfection for 24 h. Primary monocytes were transfected with siRNA using a Nucleofector Transfection kit (Lonza) and then differentiated into MDM as described earlier. DNA transfection into MDM and mouse spleen cells was performed using the Nucleofector Transfection kit. All siRNAs were obtained from Ambion (Supplemental Table I).

Quantitative real-time RT-PCR
The quantitative real-time RT-PCR (qRT-PCR) was performed as previously described (13). All probes were obtained from Applied Biosystems (Supplemental Table II).

Microarray analysis
Gene expression profiles of DNA-transfected cells were analyzed using the Affymetrix Chip, as previously described (13).

Preparation of cytosolic fraction and nuclear extract
The cytosolic and nuclear proteins were extracted from HEK293 cells using a Nuclear Extraction kit (Active Motif).

Pull-down assay
A pull-down assay was performed using DNA or oligonucleotide-conjugated agarose beads as previously described (16).

Mass spectrometry analysis
Cytosolic proteins bound to beads were analyzed by mass spectrometer (LTQ XP; Thermo Finnigan) as previously described (17).

Western blot
Western blot analysis was performed as previously described (13), using anti-Ku70, anti-DAL, and anti–AIM-2 Abs (Abcam); anti-Ku80, anti–IRF-1, anti–NF-kB p65, and anti–NF-kB p50 Abs (Cell Signaling Technology); or anti–β-actin, anti–IRF-3, and anti–IRF-7 Abs (Santa Cruz Biotechnology).

Reporter assay
Luciferase activity was measured using the Dual-Glo luciferase reporter assay system (Promega) and normalized against Renilla luciferase activity following the manufacturer’s protocol.

Statistics
All results are representative of at least three independent experiments. All values are expressed as the mean and SD of individual samples. Samples were analyzed using the Student t test.

Results and Discussion
Transfection of various types of DNA induces activation of IFNL1 in different human cells
We have previously reported that IL-27 inhibits replication of HIV-1 and hepatitis C virus (13, 18). In studies designed to better understand the role of IL-27 in host defense, we constructed an expression vector encoding the human IL-27 gene (pCMV9.IL27) and transiently transfected it into HEK293 cells. As controls, mock and a noncoding empty plasmid (pCMV9) were used. On 3 d after transfection, the culture supernatants were collected and then studied to determine their ability to inhibit HIV-1 replication in MDM. Surprisingly, anti-HIV activity was seen in both culture supernatants from pCMV9- and pCMV9.IL27-transfected cells (Fig. 1A). In contrast, culture supernatants from mock cells had no significant impact on the antiviral activity, indicating that the transfection of noncoding pCMV9 triggers the induction of anti-HIV mediators in culture supernatants.

To identify the nature of the anti-HIV mediators associated with the empty vector transfection, we compared patterns of gene expression between untreated and pCMV9-transfected HEK293 cells, using DNA microarray analysis. DNA transfection upregulated 496 genes and downregulated 147 genes >2-fold compared with the untreated HEK293 control. An annotation analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics tool (19) illustrated that the transfection led to an upregulation of genes associated with viral infection and immune responses including some known anti-HIV proteins, IFNL-1 (20) and RANTES (21) (Supplemental Table III). A qRT-PCR assay confirmed that pCMV9 transfection induced high levels of IFNL1 and RANTES mRNA with lower levels of IFNA, IFNB, and IFNL2/3 mRNA (Fig. 1B). Quantitation of cytokine concentration using ELISA indicated that pCMV9 transfection significantly produced both IFNL-1 and RANTES; however, the induction of IFN-β was below the level of detection (<25 pg/ml) (Fig. 1C).

To characterize the DNA-mediated IFNL1 activation, cell-type specificity in the gene activation was analyzed. The activation of IFNL1 and RANTES was detected not only in HEK293, but in RD, HeLa, MDMs, and DCs (Fig. 1D). It has been reported that DNA transfection induces RANTES (4); however, the induction of IFNL-1 has not been reported.
yet. Thus, we mainly focused on the activation of *IFNL1*. To evaluate whether endogenous TLR4 or TLR9 is involved in *IFNL1* activation, we treated HEK293 cells with LPS (TLR4 ligand) or CpG motif oligonucleotides (TLR9 ligand); then we analyzed the gene activation. As a positive control, MDMs were treated with LPS or CpG for 6 or 24 h. Even though LPS and CpG induced *IFNB* mRNA within 24 h in MDM (data not shown), neither reagent had any impact on the activation of *IFNL1* in HEK293 cells (Fig. 1 *E*). Expression of TLR7, DAI, AIM-2, and LRRFIP1 mRNA was not detected after 38-cycle qRT-PCR. Western blot illustrated that neither DAI nor AIM-2 was detected (data not shown), and transfection of siRNA RNA polymerase III (POLR3F) suppressed IFNL1 activation (Supplemental Fig. 1). Thus, we concluded that none of those DNA sensors is involved in the activation of *IFNL1* in HEK293 cells. To characterize the DNA-mediated *IFNL1* activation, we assessed DNA length, dose, incubation time, sequence, and structure dependency. The *IFNL1* mRNA was induced in a size-dependent (>500 bp) and dose-dependent (>250 ng/ml) DNA (Supplemental Fig. 2A, 2B). The kinetic experiment illustrated that the gene activation could be detected within 6 h of transfection, and that activation persisted for >48 h (Supplemental Fig. 2C). *IFNL1* mRNA was induced by both supercoiled or linearized forms of pCMV9 (6.4 kbp) and pCR2.1 (3.9 kbp) plasmids, and the activation was significantly enhanced by the linearized plasmids (Fig. 1 *F*). In addition, transfection of ss-pCR2.1, PCR-amplified pCR2.1 (Un-Met-DNA), fragmented human genomic DNA (~500 bp), bacterial DNA, and infection of DNA virus (HSV-2G) (Fig. 1 *G, H*) also induced *IFNL1* activation. Taken together, these data indicated that an uncharacterized DNA sensor recognizes DNA without any restriction in structure or sequence, and induces activation of *IFNL1*. The sensor may preferentially recognize long linearized DNA. Because DNA fragment of human DNA induced *IFNL1* gene activation, apoptotic cells may also induce the gene activation.

**FIGURE 2.** Ku70 is a cytosolic DNA sensor positively regulating *IFNL1* activation. *A*, Cytosolic proteins from untreated HEK293 cells were incubated with DNA-conjugated beads in the absence or presence of DNA competitor (Supplemental Fig. 3). Proteins bound to the beads were separated on SDS-PAGE under reducing conditions, followed by Coomasie blue staining. *B* and *C*, Western blot analysis using anti-Ku70 (B) or anti-Ku80 (C) Ab demonstrated intended proteins. Because of a cross-reactivity in the anti-Ku70 Ab, it detected Ku70, as well as Ku80. *D–E*, HEK293 cells were transfected with si-Control, si-Ku70, or si-Ku80, and the expression level of *KU70* or *KU80* mRNA (*D*) and protein (*E*) was analyzed by qRT-PCR and Western blot, respectively. The expression level of mRNA was compared with that in the cells transfected with si-Control. Relative amounts of Ku70 and Ku80 protein levels were densitometrically analyzed using the National Institutes of Health image, and normalized against β-actin. *F*, HEK293 cells were transfected with si-Control, si-Ku70, or si-Ku80 followed by DNA transfection. Expression levels of *IFNL1*, *KU70*, and *KU80* mRNA were determined by qRT-PCR. The level of mRNA was compared with that in the cells transfected with si-Control. *G* and *H*, HEK293T cells were cotransfected with 100 ng full-length *IFNL1*-luciferase reporter plasmid and 10 ng *Renilla* luciferase plasmid with pKu70, FLAG-tagged anti-sense Ku70 (pAS.Ku70), pKu80, or HA-tagged Ku80 (pAS.Ku80) for 24 h, then stimulated for 18 h by transfection with 500 ng PCR2.1. *I*, Spleen cells from WT or knockout Ku70−/− mice were transfected with linearized PCR2.1 using Nucleofactor Transfection kit; then expression level of mRNA was analyzed by qRT-PCR. *J*, si-Control or si-Ku70–transfected HEK293 cells were infected with HSV-2G; then gene expression was analyzed. Data were shown as the mean ± SD (*n* = 3). **p < 0.01.

**FIGURE 3.** PRDI and ISRE elements of the *IFNL1* promoter are important for the DNA-mediated *IFNL1* activation, and *IFNL1* activation is associated with the activation of IRF-1 and IRF-7. *A* and *B*, Schematic representation of the *IFNL1* promoter region and different mutant constructs on the *IFNL1* promoter region. This diagram does not indicate the exact position of the elements. HEK293T cells were transfected with a series of variants of IFN-λ1–luciferase reporter and pTK-Renilla for 24 h, and then stimulated with transfection of pCR2.1; the luciferase activities were normalized with *Renilla* activities, and data are presented as fold inductions from promoter activity from basal promoter activation without pCR2.1 transfection. Data are shown as the mean ± SD. *n* = 3. **p < 0.01. *C* and *D*, Nuclear extracts from mock- or DNA-transfected HEK293 cells were allowed to bind to oligonucleotides (PRDI or ISRE or NF-κB elements from the *IFNL1* promoter) conjugated to beads. Proteins bound to the beads were separated on SDS-PAGE, followed by Western blot analysis with specific Abs.
by Western blot (Fig. 2B, 2C). To determine the roles of Ku70 and Ku80 in the activation of IFNL1, we applied siRNA-Ku70 (si-Ku70) or siRNA-Ku80 (si-Ku80) in HEK293 and MDMs. Transfection with si-Ku70 into HEK293 cells led to a 70% reduction in KU70 mRNA and no change in KU80 mRNA compared with siRNA-control (si-Ctrl)–transfected cells, whereas transfection with si-Ku80 led to a 75% decrease in KU80 mRNA and no change in KU70 mRNA (Fig. 2D). Western blot using cytosol fraction from the siRNA-transfected cells resulted in si-Ku70 decreasing Ku70 protein by 54% (Fig. 2B). Consistent with other reports illustrating that each subunit of the Ku protein stabilizes the other (22), the si-Ku70 transfections also decreased the protein level of Ku80 by 65% (Fig. 2B). In MDMs, si-Ku70 transfection, but not si-Ku80, significantly decreased the DNA-mediated IFNL1 activation in HEK293 cells and MDMs (Fig. 2F, Supplemental Fig. 5B). The transfection of si-Ku70, but not si-Ku80, significantly decreased the DNA-mediated IFNL1 activation in HEK293 cells and MDMs (Fig. 2F, Supplemental Fig. 5B). HSVG-2G and the ssDNA-mediated IFNL1 activation was also significantly suppressed by si-Ku70 (Fig. 2J, Supplemental Fig. 6), indicating that only Ku70 is the positive regulator of IFNL1 activation. FLAG-tagged Ku70 (pKu70) or hemagglutinin (HA)-tagged Ku80 (pKu80) expression vectors were transfected and IFNL1 promoter activity was analyzed using a reporter assay, to further delineate the roles of Ku70 and Ku80. As a control, expression vector encoding FLAG-tagged anti-sense Ku70 (pAS.Ku70) or HA-tagged Ku80 (pAS.Ku80) was applied. Transfection of pKu70 and pKu80 overexpressed Ku70 and Ku80 proteins, respectively (Supplemental Fig. 7). Overexpression of Ku70, but not Ku80, increased IFNL1 promoter activity in a dose-dependent manner (Fig. 2G, 2H). To precisely determine the role of Ku70, DNA was transfeeted into Ku70−/− mice spleen cells. DNA transfection induced transcripts of Ifnl1 in WT but not in Ku70−/−, whereas the transfection enhanced the expression of Rantes (Fig. 2F). Taken together, Ku70 is a novel cytosolic DNA sensor protein and positively regulates IFNL1 activation by invaded cytosolic DNA. Because si-Ku70 inhibited the expression of RATNES mRNA in HEK293 cells (Supplemental Fig. 8), Ku70 may differentially regulate RANTES gene in between human and mouse.

IRF-1 and IRF-7 are associated with DNA-mediated IFN-α1 activation

To investigate mechanism of the DNA-mediated IFNL1 activation, we constructed mutants on the IFNL1 promoter region lacking positive-regulatory domain I (PRDI), IFN-stimulated response element (ISRE), NF-κB1, NF-κB2, or different combinations (Fig. 3A, 3B). The construct lacking PRDI or ISRE domain predominantly reduced the Ku70-mediated IFNL1 promoter activation, whereas the construct lacking NF-κB sites were able to induce the Ku70-mediated IFNL1 promoter activation (Fig. 3B), indicating that the PRDI and ISRE binding sites play key roles in the DNA-mediated IFNL1 activation. The pull-down assay using oligonucleotide (PRDI, ISRE, or NF-κB element)-conjugated beads with nuclear extract from mock- or pCR2.1-transfected cells, followed by Western blotting, demonstrated that DNA transfection significantly induced the binding activity of IRF-1 to the PRDI element, and a subtle increase of the IRF-7 binding activity to the same element rather than IRF-3 (Fig. 3C). Analysis of the activation profile of NF-kB indicated that DNA transfection increased only p65 and p50 binding activity (Supplemental Fig. 9). The pull-down assay illustrated that p65 and p50 bound to the NF-kB1 and NF-kB2 sites are at a low but detectable level (Fig. 3D). Taken together, these results indicated that both PRDI and ISRE sites are involved in the DNA-mediated IFNL1 activation. IRF-1 and IRF-7 play key roles in DNA-dependent IFNL1 activation.

It is known that the signaling pathway and biological activity of IFN-α1 are the same as those of IFN-β; however, anti-proliferative activity by IFN-α1 is lower than that by IFN-β. Therefore, the selective induction of IFN-α1 appears to be less cytotoxic (23). The Ku70-mediated IFNL1 activation required a longer size of DNA (>500 bp DNA). Ku protein bounds to multiple sites along linear DNA on dsDNA or ssDNA (24, 25); thus, unlike IFI16 (8), binding of multiple molecules of Ku70 on DNA may need to induce the gene activation. As previously reported (9), Ku70 and Ku80 protein expressed in cytosol fraction of all cell types tested (Supplemental Fig. 10A). A comparative analysis demonstrated no correlation between Ku70 expression and IFNL1 activation (Supplemental Fig. 10B), indicating that although Ku70 plays a key role to activate IFNL1 via activated IRF-1 and IRF-7, some other factor(s) may be involved in the activation of IRFs (Supplemental Fig. 11). Further study needs to precisely determine the mechanism by which Ku70 induces IFN-α1 induction sensing dsDNA and ssDNA, and the physiological relevance in the selective induction of IFN-α1 as innate immune response.

In summary, this study has demonstrated a role for Ku70 protein in the innate immune responses to foreign DNA through induction of IFNL1 activation. The finding that an endogenously expressed cytosolic protein can immediately trigger IFN-α1, but not IFN-β, production in response to exogenous DNA describes a new pathway of host defense to viral infection and DNA vaccination.

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Disclosures

The authors have no financial conflicts of interest.

References

Supporting Information

Supplemental Fig.1. RNA polymerase III (POLR3F) is not involved in IFN-λ1 and RANTES gene activation. HEK293 cells were transfected with 5 nM si-Ctrl or siRNA-POLR3F (si-POLR3F) followed by DNA transfection. The expression level of IFNL1 or RANTES, POLR3F and IL6 mRNA was analyzed by qRT-PCR. The expression level of mRNA was compared with that in the cells transfected with si-Ctrl.

Supplemental Fig. 2. The DNA mediated activation of IFNL1 is DNA-size, DNA dose and incubation time dependent. A, A series of DNA fragments was prepared using pCR2.1 with a combination of restriction-enzymes digestion as follows. 1.6 Kbp (5’ overhangs) and 2.4 Kbp (5’ overhangs) fragments were obtained by a combination of NcoI and HindIII, while 1 Kbp (5’ overhangs) and 3 Kbp fragments were produced by BglII and HindIII. Restriction digestion of pCR2.1 using DraIII and HindIII resulted in 500 bp (5’ overhang and 3’ overhang) and 3.5 Kbp (5’ overhang and 3’ overhang) fragments. Each DNA fragment was purified from 1 % agarose gel using QIAquick Gel Extraction kit (Qiagen) according to the manufacture’s protocol. A total of 0.23 pmol of the DNA fragment or linearized pCMV9 DNA (6.5Kbp) was applied for DNA transfection. B, Different amounts of linearized pCR2.1 (3900 bp), 500 bp and 50 bp DNA were transfected into HEK293 cells for 24 h. C, HEK293 cells were transfected with DNA and cultured for a total 48 h. Activation of IFNL1 was quantitated by qRT-PCR. Data were presented as relative expression units compared with mocked
transfection after normalization to \( \text{GAPDH} \). Data are mean ± s.d. (n=3), \( *p<0.05 \), \( **p<0.01 \).

Supplemental Fig. 3. A diagram for the DNA pull-down assay followed by Mass Spectrometry. The cytosolic fraction was extracted from HEK293 cells as followed: 8 x 10^6 HEK293 cells were suspended in the cold hypotonic buffer (Active Motif) containing a protease inhibitor cocktail for mammalian cells (Sigma-Aldrich) and phosphatase inhibitor cocktail (Thermo Fisher Scientific). NP-40 (Roche) as added at final 0.2 % and the cell suspension was vortexed for 10 seconds at highest speed, followed by centrifugation at 14,000 x g for 30 seconds at 4 °C. Cell-free supernatant was collected as the cytosol fraction. Protein concentration was determined using the BCA protein assay (Thermo Fisher Scientific). Mass Spectrometry Analysis was performed as previously described (17). Proteins were examined by SDS-PAGE and Simply Blue staining (Invitrogen). Protein gel bands were digested with trypsin and the peptides were extracted. The samples were desalted with C18 Zip Tips (Millipore) prior to analysis by nano-capillary reversed-phase liquid chromatography using an Agilent 1200 LC system (Agilent Technologies, Inc.) coupled online to a linear ion trap (IT) mass spectrometer (LTQ XP, Thermo Finnigan). Reversed-phase separations were performed using 75 μm i.d. × 360 μm o.d. × 10 cm long capillary columns (Polymicro Technologies, Inc.) that were slurry packed in-house with 5 μm Jupiter C-18 stationary phase (Phenomenex). After sample injection, a 20 min wash with 98 % buffer A (0.1 % v/v formic acid in water) was applied and peptides were eluted using a linear gradient of 2 % solvent B (0.1 % v/v formic acid in acetonitrile) to 42 % solvent B over 40 min with a constant flow rate
of 0.2 μl/min. The IT-MS was operated in a data-dependent mode where each full MS scan was followed by seven tandem MS scans in which the seven most abundant peptide molecular ions were dynamically selected for collision-induced dissociation (CID) using a normalized collision energy of 35%. The temperature of heated capillary and electrospray voltage (applied on column base) was 225 °C and 1.75 kV, respectively. The CID spectra were searched against the UniProt Homo Sapiens Protein database using BioWork (Thermo Fisher).

Supplemental Fig. 4. Peptide sequences of Band I and Band II. Peptide sequences of Band I and II were obtained from LC-MS after in-gel tryptic digests for sequencing and database. Band I and II were identified as Ku80 and Ku70, respectively.

Supplemental Fig. 5. RNA interference of Ku70 inhibits the activation of IFNL1 in MDM. A. Monocytes were transfected with si-Ctrl, si-Ku70 or si-Ku80 and then differentiated into MDM as described in the Materials and Methods. KU70 and KU80 mRNA level before DNA transfection was determined by qRT-PCR. Gene expression is presented as relative expression units compared with that in the cells transfected with si-Ctrl. B. The siRNA-transfected MDMs were transfected with DNA, and then cultured for 48 h. The expression level of IFNL1, KU70 and KU80 mRNA was analyzed by qRT-PCR. Gene expression is presented as relative expression units compared with that in the cells transfected with si-Ctrl. Data are shown as the mean ± s.d. (n = 3). *p<0.05, **p<0.01
Supplemental Fig. 6. RNA interference of Ku70 inhibits the ssDNA-mediated \textit{IFNL1} activation.

HEK293 cells were transfected with 5 nM si-Ctrl or si-Ku70 followed by transfection with 1 \( \mu \)g ssDNA or dsDNA (linearized pCR2.1). The expression level of \textit{IFNL1} and \textit{KU70} mRNA was analyzed by qRT-PCR. Gene expression is presented as relative expression units compared with that in the si-Ctrl transfected cell.

Supplemental Fig. 7. Confirmation of expression of Flag-tagged Ku70 and HA-tagged Ku80 proteins. HEK293 cells were transfected with 1 \( \mu \)g pKu70-FLAG, pASKu70-FLAG, pKu80-HA or pASKu80-HA for 48 h, and then whole cell lysate was extracted using RIPA buffer as previously described (18). Expression of Flag-tagged Ku70 and HA-tagged Ku80 proteins were validated by Western blot using anti-FLAG antibody (Sigma-Aldrich) and anti-HA antibody (Abcam). For verification of the same protein amounts were loaded, the blots were re-probed with anti-\( \beta \)-Actin antibody.

Supplemental Fig. 8. RNA interference of Ku70 inhibits the DNA-mediated \textit{RANTES} activation, but no impact on \textit{IL6} activation. HEK293 cells were transfected with 5 nM si-Ctrl or si-Ku70 followed by DNA transfection. The expression of \textit{RANTES} and \textit{IL6} mRNA was analyzed by qRT-PCR. The expression level of mRNA was compared with that in si-Ctrl transfected cells.

Supplemental Fig. S9. DNA transfection induces activation of p50 and p65. HEK293 cells were mock- or DNA-transfected for 18h, and then nuclear fraction was extracted
using the nuclear extraction kit (Active Motif). NFκB activation profile was determined using TransAM NFκB family kit (Active Motif) with a total 20 μg of protein. NFκB activity was determined by optical density at 450 nm following the manufacturer’s protocol.

Supplemental Fig. 10. Comparative analysis of induction of *IFNL1* and *RANTES* activation and expression of Ku70 and Ku80 protein.  
*A*, Cytosol fraction was extracted from untreated HEK293, RD, HeLa, MDM and DC as described in the Materials and Methods, and Western blot was performed using anti-Ku70 and anti-Ku80 antibody. To normalize relative expression level of proteins, the blot was re-probed with Anti-β-Actin antibody.  
*B*-E, Relative amounts of the protein level of Ku70 and Ku80 in Supplemental Fig. S10*A* were densitometrically analyzed using the NIH image and normalized against protein level of β-Actin. To define a correlation between relative expression of Ku protein and relative activation of *IFNL* or *RANTES*, a scatter plot analysis was performed using data from Fig 1*D*. The analysis demonstrated that activation of neither *IFNL1* nor *RANTES* had no linear correlation with the expression of Ku70 (*B*, *D*) and Ku80 (*C*, *E*) among cell types tested.

Supplemental Fig. 11. A Proposed model by which DNA induces *IFNL1* activation. Invaded cytoplasmic DNA is recognized by Ku70 and then activates IRF-1 and 7 through uncharacterized mediator(s). The activated IRF-1 and -7 play a key role to induce *IFNL1* activation, while IRF-3 and NFκB may not play an important role if any in the DNA-mediated *IFNL1* activation.
Supplemental Table 1. A list of siRNA used in knockdown experiments.

All siRNAs were obtained from ABI (Ambion).

Supplemental Table 2. A list of probes used in real-time RT-PCR

All probes were obtained from ABI.

Supplemental Table 3. A list of upregulated genes associated with viral infection and immune responses in DNA transfected HEK293 cell.

HEK293 cells were transfected with mock (lipid alone) or 1 μg of pCMV9 for 24 h. Differentially expressed genes were selected between mock and DNA-transfected cells, and were greater than 2-fold changes (p<0.05). Table indicates a list of genes known to be associated with soluble proteins, cytokines, chemokines, response to viral infection and immune responses, which were selected using the DAVID bioinformatic tool. Genes listed in the table were consistently induced by the DNA transfection in three independent experiments. The data discussed in this publication have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus (GEO). GEO series accession number and the URL of the database are GSE26944 and http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26944, respectively.
**Supplemental Table 1: A list of siRNA used in knockdown experiments.**

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All siRNAs were obtained from Ambion.
**Supplemental Table 2: A list of probes used in real-time RT-PCR**

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<tr>
<td>TLR9</td>
<td>Hs00928321_ml</td>
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<td>ZBP1</td>
<td>Hs00229199_ml</td>
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All probes were obtained from ABI.
Supplemental Table 3:

A list of upregulated genes associated with viral infection and immune responses in DNA transfected HEK293 cell

<table>
<thead>
<tr>
<th>Affymetrix ID</th>
<th>Gene name</th>
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<tr>
<td>8131803</td>
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<tr>
<td>7967969</td>
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<td>8028613</td>
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<tr>
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<td>CCL 5 (RANTES)</td>
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<tr>
<td>8006459</td>
<td>CCL 13 (MCP-4)</td>
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<td>8048864</td>
<td>CCL 20 (MIP-3alpha)</td>
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<td>8101126</td>
<td>Chemokine c-x-c motif ligand (CXCL) 10</td>
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<tr>
<td>7903786</td>
<td>COLONY STIMULATING FACTOR 1</td>
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<tr>
<td>8144995</td>
<td>FIBROBLAST GROWTH FACTOR 17</td>
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</table>

HEK293 cells were transfected with mock (lipid alone) or 1 μg of pCMV9 for 24 h. Differentially expressed genes were selected between mock and DNA-transfected cells, and were greater than 2-fold changes (p<0.05). Table indicates a list of genes known to be associated with soluble proteins, cytokines, chemokines, response to viral infection and immune responses, which were selected using the DAVID bioinformatic tool. Genes listed in the table were consistently induced by the DNA transfection in three independent experiments. The data discussed in this publication have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus (GEO).
GEO series accession number and the URL of the database are GSE26944 and
S. Figure 3

HEK293 cells → Trypsin treatment → Collect Cells → Hypotonic buffer → Cytosolic Fraction

Bead → Streptavidin → Biotin → dsDNA → Preclean with streptavidin beads

Washing protein/beads

Mix with or without DNA competitors → Mass Spectrometry
Band I

K.KVITMFVQR.Q  
K.IQPGSQQQADFLDALIVSM*DVIQHETIGK.K  
K.SQLDIIIHSLK.K  
R.LTIGSNLLSIR.I  
R.YGSDIVPFSDK.V  
K.DEKTDTLEDLFPTTK.I  
R.EPLPPIQQHIWNM*LNPPAEVTTK.S  
K.TEQGGAHFSVSSLAEGSVTSVGSVNPAENFR.V  
K.QLNFWEIWWQDGITLITK.E  
K.DKPSGDTAAVFEEGGDVDDLLDM

Band II

K.TEGDEEAECEEQQEENLEASGDYK.Y  
R.DSLIFLVDASK.A  
R.DLLAWFYGTEKDK.N  
R.ILELDQFK.G  
K.KPGGFDISLFLYR.D  
K.RSQIYGSR.Q  
R.NIPYYFVALVPQEELDDQK.I  
R.NLEALALDL*EPEQAVDLTPK.V  
K.KQELLLEALT.K.H
**Figure 5**

**A**

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**B**

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S.Figure 7

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S. Figure 8

Relative expression (\%)

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<tr>
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<td>100</td>
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<tr>
<td>si-Ku70</td>
<td>ND</td>
<td>100</td>
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