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Recognition of Cytosolic DNA Activates an IRF3-Dependent Innate Immune Response

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Summary

Nucleic acid recognition upon viral infection triggers type I interferon production. Viral RNA is detected by both endosomal, TLR-dependent and cytosolic, RIG-I/MDA5-dependent pathways. TLR9 is the only known sensor of foreign DNA; it is unknown whether innate immune recognition of DNA exists in the cytosol. Here we present evidence that cytosolic DNA activates a potent type I interferon response to the invasive bacterium *Listeria monocytogenes*. The noninvasive *Legionella pneumophila* triggers an identical response through its type IV secretion system. Activation of type I interferons by cytosolic DNA is TLR independent and requires IRF3 but occurs without detectable activation of NF-κB and MAP kinases. Microarray analyses reveal a unique but overlapping gene-expression program activated by cytosolic DNA compared to TLR9- and RIG-I/MDA5-dependent responses. These findings define an innate immune response to DNA linked to type I interferon production.

Introduction

The innate immune system uses pattern recognition to sense infection, and it initiates an immune response upon pathogen detection (Janeway, 1989). Recent advances have defined two broad categories of microbial stimuli. The first class includes products unique to microbes, such as lipopolysaccharide and lipotechoic acid. The second class encompasses nucleic acids derived from pathogens, particularly viruses. Receptors that detect nucleic acids generally activate type I interferons (IFN), which establish a cell-autonomous antiviral state, alert neighboring cells to the presence of infection, and enhance cytotoxic lymphocyte responses to infected cells (Levy et al., 2003). Importantly, self/nonself discrimination is straightforward for receptors that detect unique microbial products but more complex for nucleic acid sensors because their ligands are not unique to pathogens.

Two types of receptors detect and respond to nucleic acids. The first type includes several Toll-like receptors (TLRs) and samples material captured in endosomes (for review, see Kawai and Akira, 2005). TLR3 is activated by double-stranded RNA (dsRNA), while TLRs 7 and 8 recognize single-stranded RNA. TLR9, the only known primary sensor of foreign DNA, recognizes unmethylated CpG motifs. TLRs are important for detection of foreign nucleic acids, but they are only expressed in a subset of cells, while almost all nucleated cells can activate type I IFN in response to viral infection.

Introduction into the cytosol of double-stranded RNA or its synthetic analog Poly I:C activates potent type I IFN production. The RNA helicases RIG-I and MDA5 were recently found to be essential for this response (Yoneyama et al., 2004, 2005). Both are activated by dsRNA, and RIG-I is essential for the type I IFN response to diverse RNA viruses (Kato et al., 2005; Sumpter et al., 2005). RIG-I and MDA5 signal via a pathway that includes FADD, the RIP-1 kinase, Tank binding kinase 1 (TBK-1), and the adaptor protein MAVS/IPS-1/VISA/CARDIF (Balachandran et al., 2004; Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005).

These recent findings are a major advance in our understanding of cytosolic RNA recognition and innate antiviral responses. In contrast, TLR9 remains the only known sensor of foreign DNA. Whether a pathway analogous to RIG-I/MDA5 exists to signal an antiviral response to DNA is currently unknown. Such a sensor would be consistent with recent observations that HSV activates type I interferons by TLR9-dependent and -independent pathways depending on the cell type infected (Hochrein et al., 2004; Malmgaard et al., 2004).

Listeria monocytogenes is a gram-positive bacterium that grows in the cytosol of infected host cells (Portnoy, 2005). Upon entry of *Listeria* into cells by endocytosis, the pore-forming protein Listeriolysin O (LLO) ruptures the nascent phagosome, allowing escape into the cytosol. Invasive versus noninvasive *Listeria* activate distinct signaling pathways (Hauf et al., 1997), and cytosolic bacteria trigger a unique cytokine response that includes production of type I IFN and monocyte chemotactic protein (MCP-1) (O'Riordan et al., 2002; Serbina et al., 2003). While MCP-1 is essential for in vivo immunity to *Listeria* infection, type I IFN appear to be deleterious to the host (Serbina et al., 2003; Auerbuch et al., 2004; Carrero et al., 2004; O'Connell et al., 2004). Recent reports have shown that type I IFN activation by *Listeria* requires IRF3 and TBK-1 and is independent of TLRs and NOD proteins (O'Connell et al., 2005; Stockinger et al., 2004), but the ligand(s) responsible remain unknown.

Several lines of evidence suggest that the IFN-inducing ligand(s) detected upon cytosolic invasion are not unique to *Listeria*. First, the invasive enterobacterium *Shigella flexneri* activates potent IFN production in fibroblasts, but a noninvasive variant does not (Hess et al., 1987). Second, noninvasive bacteria engineered to express LLO, including *Escherichia coli* and *Bacillus subtilis*, activate an identical type I IFN response (O'Riordan et al., 2002). Third, *Trypanosoma cruzi*, a protozoan parasite, activates IFN production with similar kinetics to its escape from the vacuole (Vaena de Avalos et al., 2002). These studies suggest that a common feature might unite the IFN response to these pathogens and that products unique to bacteria are unlikely to be responsible for activating type I IFNs.

Here we show that apoptotic cells loaded with LLO recapitulate the IFN response activated by *Listeria* and

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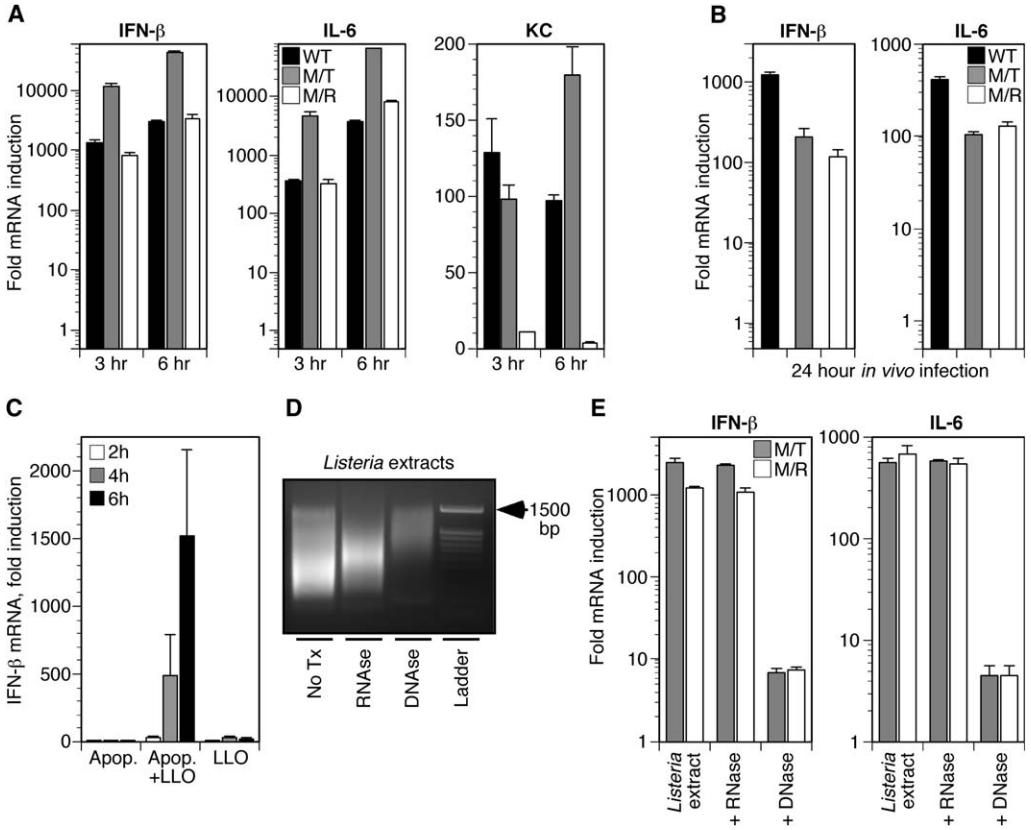

Figure 1. Cytosolic DNA Is an Interferon-Activating Ligand of *Listeria monocytogenes*

(A) Bone marrow macrophages of the indicated genotypes were infected with *Listeria monocytogenes* at a moi of 2. Cytokine mRNA induction was measured by quantitative RT-PCR and normalized to HPRT expression for each sample. Relative induction was determined by comparison to uninfected macrophages. M/T, MyD88/Trif DKO; M/R, MyD88/RIP2 DKO. Means and standard deviations are shown and are representative of three experiments.

(B) Mice were infected i.v. with 5000 live *Listeria monocytogenes*. Splenocytes were harvested 24 hr later and the indicated cytokines were measured by quantitative RT-PCR. Means and standard deviations for three mice per group are shown.

(C) Apoptotic thymocytes were prepared from MyD88/Trif DKO mice, loaded with recombinant LLO, and fed to MyD88/Trif DKO peritoneal-elicited macrophages. IFN-β mRNA abundance was measured by quantitative RT-PCR. Means and standard deviations are shown and include three independent experiments.

(D) *L. monocytogenes* extracts were treated with RNase or DNase I and separated on an agarose gel with ethidium bromide to visualize nucleic acids.

(E) Bone marrow-derived macrophages of the indicated genotypes were transfected with the *Listeria* extracts. RNA was harvested 3 hr after transfection and the abundance of IFN-β and IL-6 mRNA was measured by quantitative RT-PCR.

that DNA is a major IFN-inducing ligand in *Listeria* extracts. We demonstrate that *Legionella pneumophila*, a vacuolar pathogen, activates an identical response through its type IV secretion system. By using purified DNA, we define the key features of a novel cytosolic innate immune recognition system and compare this pathway to other known nucleic acid sensors. Remarkably, intracellular DNA triggers a unique antiviral response without detectable activation of NF-κB or MAP kinases, suggesting that the signaling pathways activated by cytosolic DNA and RNA are distinct.

Results

Cytosolic DNA Is an Interferon-Activating Ligand of *Listeria monocytogenes*

Listeria monocytogenes contains multiple ligands for TLRs and possibly NOD proteins (Portnoy, 2005). To examine cytokine responses to *Listeria* infection in the absence of these pathways, we used MyD88/Trif double knockout (DKO) macrophages, which lack all TLR signaling (Yamamoto et al., 2003), and MyD88/RIP2 DKO macrophages, which lack TLR- and NOD1/NOD2-mediated signaling (Chin et al., 2002; Kobayashi et al., 2002). In agreement with previous studies, we found that macrophages infected with *L. monocytogenes* activated a potent IFN-β response that was intact in cells doubly deficient for MyD88/Trif or MyD88/RIP2 (Figure 1A). Robust IL-6 production was also MyD88/Trif/RIP2 independent (Figure 1A). Interestingly, induction of KC (CXCL1), a chemokine homologous to human IL-8, was intact in MyD88/Trif DKO macrophages but severely impaired in MyD88/RIP2 DKO cells (Figure 1A). We confirmed these observations in vivo and found that IFN-β and IL-6 induction in spleens 24 hr after intravenous *Listeria* infection remained intact in MyD88/Trif DKO and MyD88/RIP2 DKO mice (Figure 1B). However, KC production by splenocytes appeared to be MyD88

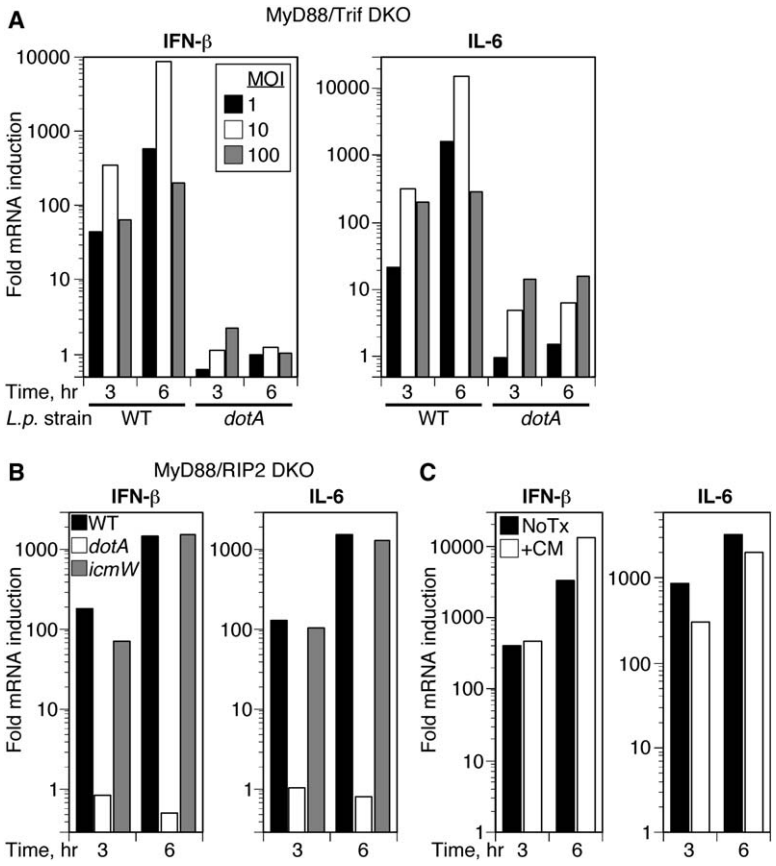

Figure 2. *Legionella pneumophila* Activates a TLR- and RIP-2-Independent Interferon Response via Type IV Secretion

(A) MyD88/Trif DKO macrophages were infected with opsonized wild-type or *dotA* mutant *L. pneumophila* at the indicated moi. Results are representative of four independent experiments.

(B) MyD88/RIP2 DKO macrophages were infected with wild-type *L. pneumophila* or *dotA* or *icmW* mutants at a moi of 10.

(C) MyD88/Trif DKO macrophages were pretreated for 20 min with chloramphenicol (+CM) or vehicle (NoTx) and then infected with wild-type *L. pneumophila* at a moi of 10.

dependent at this early time point, perhaps reflecting a contribution from other cell types (data not shown). Together with previous studies, these data demonstrate that live *Listeria* activate at least three signaling pathways in macrophages: one that requires TLR/MyD88 signaling (McCaffrey et al., 2004), one that requires RIP2, and one that is independent of both TLRs and RIP2.

We next tested whether material from mammalian cells would initiate a similar response if endowed with the ability to enter the cytosol. We prepared dexamethasone-treated apoptotic thymocytes, loaded them with recombinant LLO, and fed them to peritoneal macrophages. Both the thymocytes and macrophages were derived from MyD88/Trif DKO mice to eliminate any TLR signaling activated by LLO (Park et al., 2004). We observed an induction of IFN-β transcription when macrophages were fed LLO-loaded apoptotic cells, but negligible induction with apoptotic cells alone or a similar amount of recombinant LLO alone (Figure 1C). Therefore, ligand(s) that activate IFN-β upon cytosolic entry are also present in mammalian cells.

We tested whether nucleic acids could account for the potent IFN-β response to *Listeria* infection. Extracts prepared from sonicated *Listeria* recapitulated the MyD88/Trif/RIP2-independent induction of both IFN-β and IL-6 when transfected into macrophages (Figure 1E). With extracts alone, we were unable to consistently reproduce the RIP2-dependent KC induction observed during live *Listeria* infection, possibly because the ligand(s) that activate RIP2-mediated signaling were lost during extract preparation or are not transfectable (data not shown). Pretreatment of *Listeria* extracts with DNase impaired their ability to activate both IFN-β and IL-6 by more than 98%, while RNase pretreatment had no effect (Figures 1D and 1E). Identical results were obtained with *E. coli* extracts (data not shown). These data demonstrate that DNA in *Listeria* extracts is a major activating ligand of IFN-β and IL-6 and that a cytosolic sensor of DNA may account for the potent TLR- and RIP2-independent IFN response to *Listeria*.

LLO-mediated delivery of bacteria or apoptotic cells into the cytosol activates the response described above. Whether a vacuole-confined pathogen can trigger a similar pathway has not been fully explored. We examined the innate immune response to *Legionella pneumophila*, a pathogen of phagocytic protozoa and the causative agent of Legionnaire's disease (McDade et al., 1977). *L. pneumophila* injects proteins into the host cell cytosol that disrupt phagosome-lysosome transport to establish a replicative organelle. These effectors are transferred via the Dot/Icm type IV secretion apparatus, which is ancestrally related to bacterial conjugation systems (Vogel and Isberg, 1999). Importantly, the Dot/Icm system retains the ability to transfer DNA (Segal et al., 1998; Vogel et al., 1998).

We infected MyD88/Trif DKO macrophages with several doses of opsonized *L. pneumophila* and observed potent IFN-β and IL-6 transcription that was detectable 3 hr after infection and dramatically increased by 6 hr (Figure 2A). Mutant bacteria lacking the *dotA* gene, which encodes a polytopic inner membrane protein essential for translocation of all Dot/Icm substrates (Roy

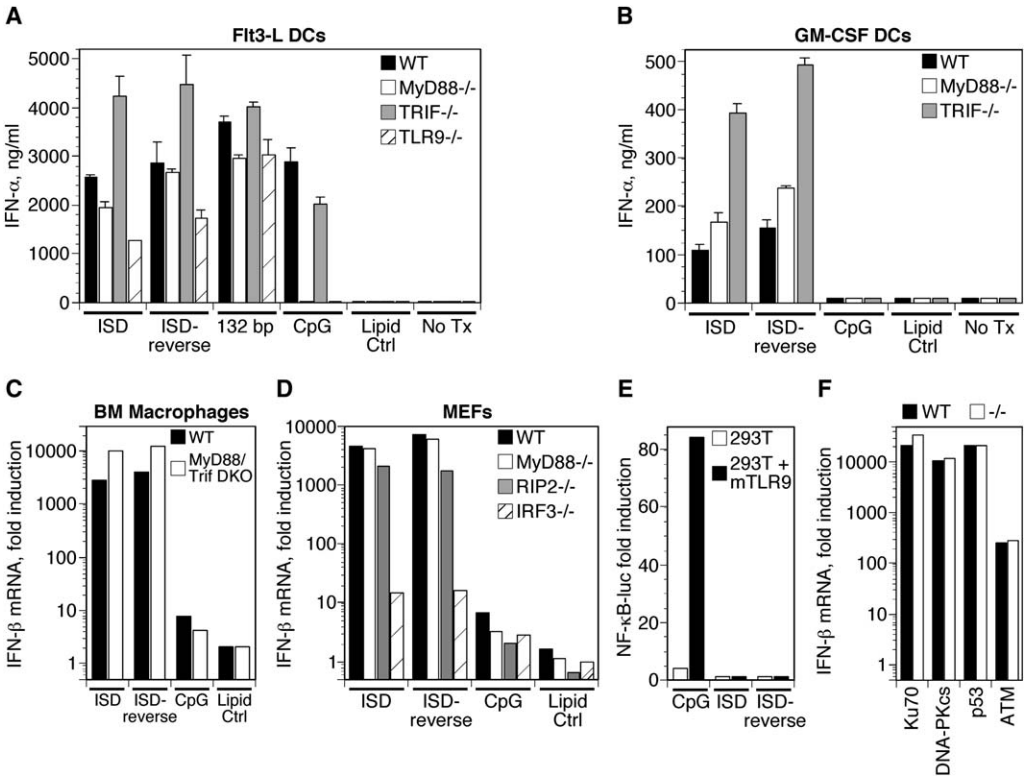

Figure 3. Transfected DNA Activates a Potent TLR- and RIP2-Independent Type I Interferon Response in a Broad Range of Cell Types

(A) Plasmacytoid dendritic cells were treated with the indicated stimuli for 18 hr and IFN-α in culture supernatants was measured by ELISA. Untreated cells, No Tx.

(B) Conventional dendritic cells were stimulated for 18 hr before measurement of IFN-α by ELISA.

(C) Bone marrow-derived macrophages were stimulated for 3 hr. IFN-β mRNA abundance was determined by quantitative RT-PCR.

(D) Primary MEFs of the indicated genotypes were stimulated for 3 hr and relative IFN-β mRNA levels were determined.

(E) HEK293T cells were transfected with a NF-κB-luciferase reporter plasmid alone or with a vector encoding murine TLR9. Cells were stimulated 24 hr posttransfection, and luciferase activity in lysates was measured 8 hr poststimulation.

(F) Cells of the indicated genotype and matched wild-type controls were stimulated with ISD for 3 hr, and IFN-β mRNA induction was measured by quantitative RT-PCR. The Ku70-, DNA-PKcs-, and p53-deficient cells (and controls) were bone marrow macrophages, and the $Atm^{-/-}$ cells were early passage MEFs. All data are representative of at least three independent experiments.

and Isberg, 1997), failed to activate either cytokine (Figure 2A). Identical results were obtained with MyD88/RIP2 DKO cells (Figure 2B). *L. pneumophila* deficient for the *icmW* gene, which retain type IV secretion but are unable to evade transport to lysosomes due to a defect in transfer of a subset of Dot/Icm protein substrates (Zuckman et al., 1999), activated a potent cytokine response comparable to wild-type bacteria (Figure 2B). Macrophages infected in the presence of chloramphenicol, which inhibits bacterial protein synthesis but not type IV secretion, activated similarly robust IFN-β and IL-6 transcription, suggesting that neither newly formed proteins nor other indirect consequences of bacterial growth can account for activation of these cytokines (Figure 2C). *L. pneumophila* therefore triggers, via Dot/Icm-dependent transfer of a preformed substrate into the host cell cytosol, a TLR- and RIP2-independent response identical to that activated by *Listeria*. Induction of these same cytokines by *Listeria* extracts is sensitive to DNase pretreatment (Figure 1E). Together with the well-established capability of type IV secretion systems to transfer DNA (Christie et al., 2005), these data lead us to suggest that cytosolic DNA might also activate the interferon response to *Legionella* and that this innate immune recognition pathway may detect a broader range of pathogens than just cytosol-invasive bacteria.

Detection of Intracellular DNA Is Sequence Independent and Requires a Native Sugar-Phosphate Backbone

Because the TLR9-mediated response to DNA is strictly CpG sequence specific (Hemmi et al., 2000; Krieg et al., 1995), we reasoned that DNA devoid of these motifs would allow us to explore TLR9-independent responses to DNA. Double-stranded 45 base pair oligonucleotides lacking contiguous CpG sequences failed to activate a detectable cytokine response when added to the cell-culture medium at concentrations of up to 100 μg/ml (data not shown). However, we observed a potent induction of type I IFN when the DNA was transfected into several different cell types, including plasmacytoid dendritic cells (Flt3 ligand DCs, Figure 3A), "conventional" dendritic cells (GM-CSF DCs, Figure 3B), macrophages (Figure 3C), and embryonic fibroblasts (MEFs, Figure 3D), as measured by both ELISA for IFN-α (Figures 3A and 3B) and quantitative RT-PCR for IFN-β (Figures 3C and 3D). We refer hereafter to these DNA oligos as interferon stimulatory DNA (ISD) for simplicity. Type I IFN

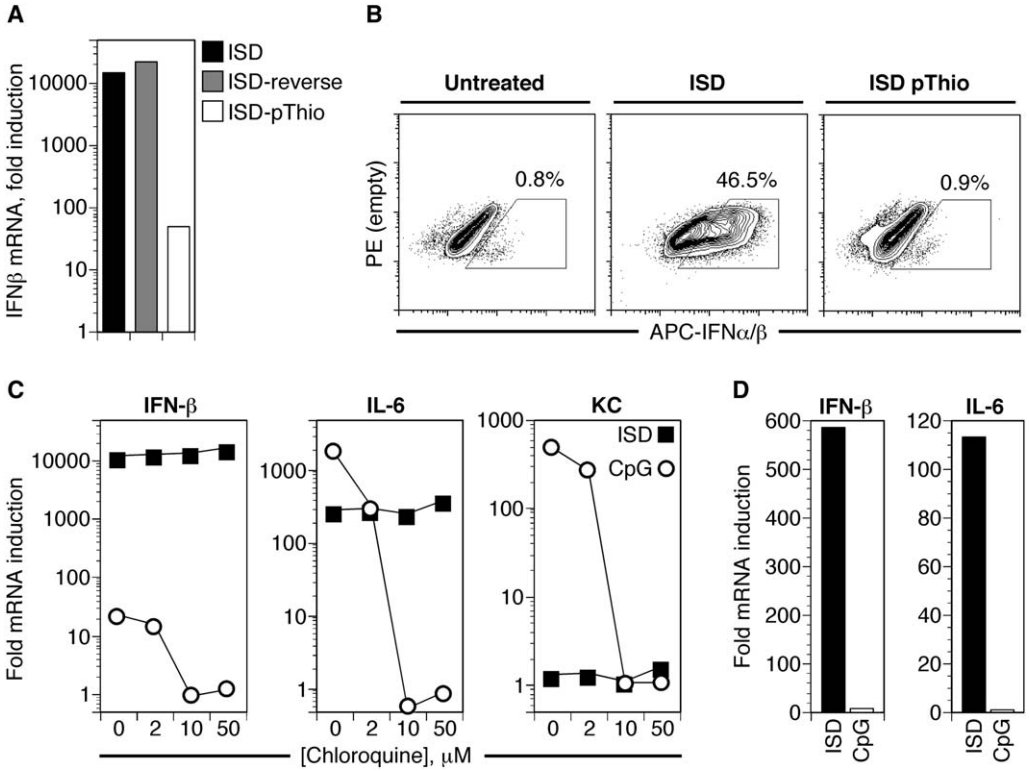

Figure 4. Intracellular DNA Recognition Requires a Native Sugar-Phosphate Backbone

(A) Macrophages were transfected with the indicated DNA ligands for 3 hr, and IFN-β induction was measured by quantitative RT-PCR.

(B) Macrophages were transfected with the indicated oligonucleotides. Brefeldin A was added 2 hr after treatment to trap cytokines. Cells were harvested 4 hr posttreatment, fixed, permeabilized, and stained with antibodies to murine IFN-α and IFN-β.

(C) Bone marrow macrophages were pretreated for 1 hr with the indicated concentrations of chloroquine before transfection of ISD or stimulation with CpG DNA for 3 hr. Cytokine induction was measured by quantitative RT-PCR.

(D) Macrophages were pretreated for 1 hr with 50 μM chloroquine before addition of calcium phosphate-precipitated ISD or CpG DNA. Cytokine induction was measured 3 hr poststimulation. All data are representative of at least three independent experiments.

induction by ISD was intact in cells lacking MyD88, Trif, or RIP2 (Figures 3A–3D). However, IFN-β activation in IRF3-deficient MEFs after ISD treatment was reduced by more than 99% compared to wild-type controls (Figure 3D), consistent with the key role for IRF3 in virus-induced type I IFN production (Sato et al., 2000). The IFN-α/β response to ISD was intact in TLR9-deficient cells (Figure 3A), and ISD did not activate murine TLR9 expressed in HEK-293T cells (Figure 3E). In contrast, CpG oligonucleotides induced type I IFN production in a strictly TLR9/MyD88-dependent manner only in pDCs (Figures 3A–3D). The potency of ISD was sequence independent, because ISD with a reversed nucleotide sequence and an unrelated 132 bp DNA fragment had identical activity (Figures 3A–3D). However, DNA oligonucleotides smaller than 25 bp failed to activate type I IFN production, suggesting a minimum size required for their recognition (data not shown). ISD failed to activate an IFN-β reporter plasmid in 293T cells expressing RIG-I or MDA5 (data not shown). These data show that purified DNA recapitulates the IFN-inducing activity of *Listeria* and *Legionella* and that a broad range of primary cell types mounts an IRF3-dependent innate immune response to intracellular DNA.

We considered whether components of the DNA damage repair pathway might be involved in the detection of ISD, especially since the DNA-dependent protein kinase (DNA-PK) can directly phosphorylate IRF3 (Karpova et al., 2002). However, cells lacking Ku70, the catalytic subunit of DNA-PK (DNA-PKcs), p53, or the Ataxia-Telangiectasia mutated kinase (ATM) mounted an identical IFN-β response when compared to their wild-type controls (Figure 3F). Moreover, IFN-β activation by ISD was normal in cells treated with the PI-3 kinase family inhibitors wortmannin (20 μM) or caffeine (5 mM), suggesting that DNA-PK, ATM, the ATR kinase, and PI-3K itself do not participate in ISD-activated signaling (data not shown).

Although the response to phosphodiester ISD transfection was sequence independent, CpG oligonucleotides synthesized with nuclease-resistant phosphorothioate bonds failed to activate the same pathway (Figure 3). We found that while phosphodiester ISD potently activated type I IFN as measured by quantitative RT-PCR and intracellular cytokine staining, transfected p-thio oligonucleotides of identical sequence stimulated negligible IFN-β transcription and no production of type I IFN proteins (Figures 4A and 4B), suggesting that a native sugar-phosphate DNA backbone is essential for ISD activity.

TLR9 activation by CpG DNA occurs in endosomes and can be inhibited by preventing endosomal acidification with the buffering agent chloroquine (Hacker et al., 1998). As expected, chloroquine pretreatment

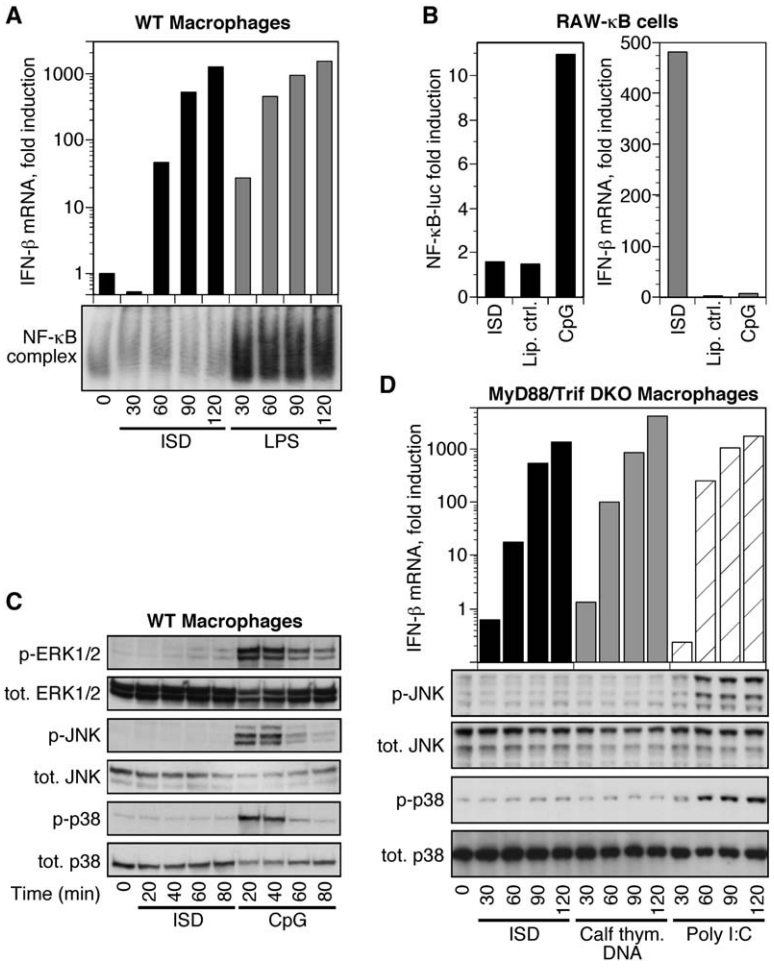

Figure 5. Biochemical Analysis of the Signaling Response to ISD

(A) Nuclear extracts from macrophages stimulated for the indicated times were used in a gel shift assay with a probe containing a consensus NF-κB binding sequence. A representative autoradiograph is shown in the bottom panel, and IFN-β mRNA induction from a parallel set of identically treated samples is depicted in the top panel.

(B) RAW264.7 macrophages stably expressing a NF-κB-luciferase reporter were stimulated for 6 hr with ISD or CpG DNA. Duplicate samples were processed for analysis of luciferase activity (left) or IFN-β mRNA induction (right). Data are representative of three independent experiments.

(C) Whole-cell lysates of macrophages treated for the indicated times were probed by Western blot with the indicated antibodies. Data are representative of three independent experiments. Similar results were obtained with cDCs and MEFs (data not shown).

(D) MyD88/Trif DKO macrophages were transfected with the indicated ligands. Parallel samples were prepared for quantitative RT-PCR analysis of IFN-β induction (top) and Western blot analysis of MAP kinase activation (bottom).

completely inhibited the response to CpG DNA; however, ISD activity was unimpaired (Figure 4C). ISD activated robust IFN-β and IL-6 transcription but not KC (Figure 4C), which is identical to the TLR- and RIP2-independent pathway activated by live *Listeria* and *Legionella* (Figures 1A and 2A). We next transfected ISD or CpG DNA into chloroquine-pretreated macrophages by calcium phosphate precipitation. ISD activated robust IFN-β and IL-6 transcription while CpG DNA was inactive when transfected by this method (Figure 4D). Together with the data from Figures 1 and 2, these findings indicate that ISD recognition occurs in the cytosol.

NF-κB and MAP-Kinase-Independent Activation of IFN-β by Intracellular DNA

We examined several well-characterized signaling responses to inflammatory stimuli and nucleic acid recognition, particularly those activated by TLRs and RIG-I. We first compared NF-κB activation in macrophages transfected with ISD or treated with LPS, which activates TLR4/Trif-dependent IFN-β production (Yamamoto et al., 2003). Both treatments activated robust IFN-β transcription, with ISD-stimulated IFN-β mRNA induction delayed by 30 min compared to LPS (Figure 5A, top). LPS stimulation led to rapid and sustained nuclear NF-κB binding activity assessed by EMSA, while ISD treatment failed to stimulate detectable NF-κB binding (Figure 5A, bottom). We next examined NF-κB activity in RAW 264.7 murine macrophages stably expressing a luciferase reporter driven by two consensus NF-κB binding sites. ISD transfection of RAW-κB cells failed to activate the NF-κB-luciferase reporter but stimulated transcription of the endogenous IFN-β gene (Figure 5B). In contrast, CpG stimulation of these cells activated the NF-κB reporter but not IFN-β (Figure 5B). Taken together, these data suggest that ISD potently activate type I IFN without NF-κB.

We next compared MAP kinase activation in response to ISD transfection versus CpG DNA treatment. As expected, treatment of wild-type macrophages with CpG DNA induced a rapid and robust activation of ERK, JNK, and p38 (Figure 5C). In contrast, ISD treatment did not activate any of these MAP kinases (Figure 5C). Identical results were observed in cDCs and MEFs (data not shown).

Finally, we used MyD88/Trif DKO macrophages to directly compare the cytosolic response to transfected DNA and dsRNA. The use of cells deficient in all TLR signaling allowed us to examine MAP kinase activation by ISD, genomic DNA isolated from calf thymus (which is contaminated with TLR ligands), and Poly I:C (which activates RIG-I and MDA5 upon transfection but also activates TLR3). Quantitative RT-PCR analysis of RNA from cells processed in parallel revealed that all three stimuli activated similarly robust transcription of the endogenous IFN-β gene that was detectable by 60 min

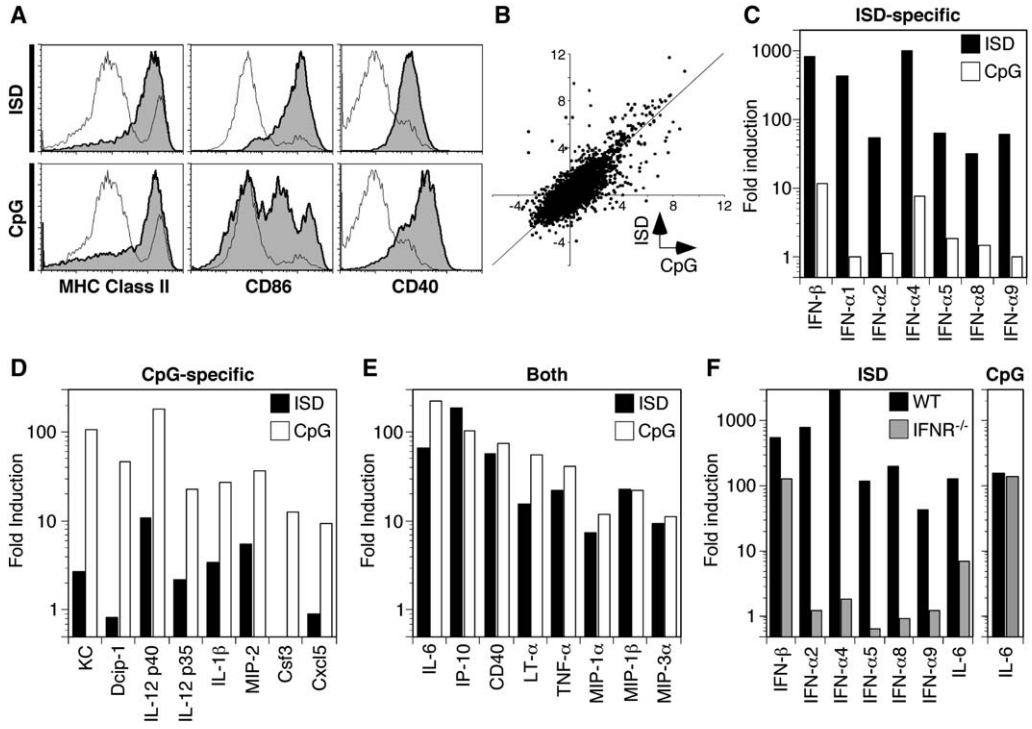

Figure 6. Microarray Analysis Comparing the ISD Pathway to the CpG/TLR9-Dependent Response

(A) cDCs were stimulated for 18 hr with ISD or CpG DNA. Surface expression levels of MHC Class II, CD86, and CD40 on gated CD11c+ cells are shown. Filled histograms depict treated cells; empty histograms represent untreated cells.

(B) cDCs were stimulated for 4 hr with ISD or CpG DNA and total RNA was prepared for microarray analysis. For all expressed genes, the change relative to untreated cells after ISD (y axis) or CpG DNA (x axis) stimulation is plotted in $\log_2$ format. Data are from one of two independent experiments.

(C–E) Bar graphs depict the average induction of the indicated genes from two independent experiments. A cutoff of a 5-fold difference in induction between ISD- and CpG DNA-treated cells was applied to determine genes specifically activated by each stimulus.

(F) Macrophages from wild-type or IFNαR$^{-/-}$ mice were treated for 4 hr with ISD (left) or CpG (right). Two independent microarray experiments gave comparable results.

posttransfection (Figure 5D, top). In contrast, only Poly I:C transfection activated JNK and p38 with similar kinetics to IFN-β induction (Figure 5D, bottom), suggesting that distinct signaling pathways are activated by cytosolic DNA versus dsRNA.

Intracellular DNA Recognition Activates a Unique Gene Expression Program

We performed a microarray analysis of cDCs stimulated for 4 hr with ISD or CpG DNA to identify the gene-expression programs activated by each response. Both ISD and CpG DNA matured DCs, as evidenced by an increase in surface expression of MHC Class II, CD86, and CD40 (Figure 6A). A two-dimensional plot of the n-fold change in expression of each gene revealed an overlapping response with evidence for unique genes activated by both ISD and CpG (Figure 6B). Importantly, several type I IFNs were specifically induced by ISD but not CpG (Figure 6C), confirming the ability of ISDs to activate a potent antiviral response. As seen in Figure 4C, CpG DNA treatment but not ISD stimulation induced robust transcription of KC (Figure 6D). CpG DNA activated expression of both subunits of IL-12, the chemokine Dcip-1, and IL-1 far more potently than ISD (Figure 6D). Both ISD and CpG DNA activated expression of IL-6, IP-10 (CXCL10), CD40, lymphotoxin-α, and tumor necrosis factor, among others (Figure 6E).

To examine primary response genes in the ISD pathway versus those that required type I IFN receptor signaling for expression, we compared the ISD response in wild-type versus IFN-α receptor (IFNαR1)-deficient macrophages. IFN-β induction in IFNαR1$^{-/-}$ cells was mildly reduced but still robust (Figure 6F). In contrast, all other type I IFNs strictly required IFNαR1 signaling for expression (Figure 6F). This observation is consistent with the recently defined role for IRF7 in IFNαR1-dependent transcriptional activation of all type I IFNs (Honda et al., 2005). Remarkably, IL-6 induction by ISD was impaired by more than 95% in IFNαR1$^{-/-}$ cells, while TLR9-mediated IL-6 activation was normal in these cells (Figure 6F). While IL-6 is an NF-κB-dependent gene in response to IL-1 receptor and TLR signaling (Yamamoto et al., 2004), these data suggest that IL-6 is an IFN-dependent gene in the ISD pathway. These findings detail a transcriptional profile of the acute response to ISD and demonstrate distinct gene expression programs activated by detection of endosomal versus cytosolic DNA.

We next compared gene expression in MyD88/Trif DKO macrophages after ISD or Poly I:C transfection. Despite the differences in ISD- and Poly I:C-activated signaling pathways (Figure 5), microarray analysis revealed a remarkably similar gene expression pattern 3 hr after transfection (Figure 7A). Type I IFNs, IL-6, and key IFN-inducible genes were comparably activated by ISD

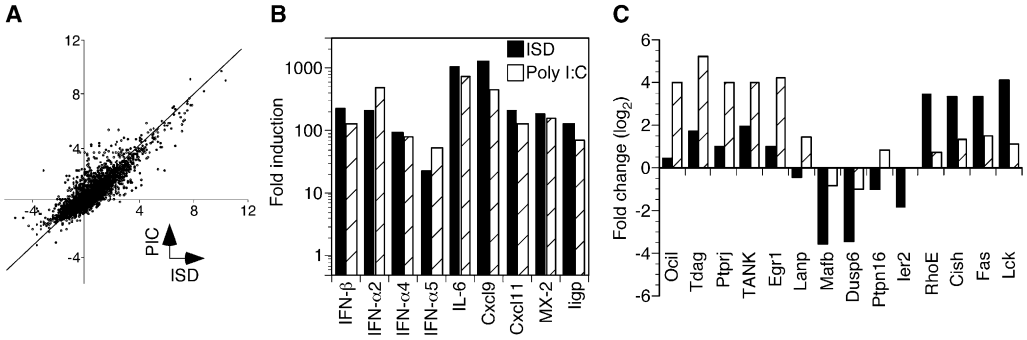

Figure 7. Intracellular DNA and dsRNA Activate Overlapping but Unique Gene Expression Programs

(A) The change relative to untreated cells of over 10,000 expressed genes 3 hr after Poly I:C (y axis) versus ISD (x axis) transfection of MyD88/Trif DKO macrophages is plotted in $\log_2$-based format similar to Figure 5B.

(B) Induction of the indicated transcripts was compared after ISD versus Poly I:C treatment.

(C) Changes in the transcript levels for the indicated genes after ISD and Poly I:C treatment are presented in a $\log_2$-based format. All data are representative of two independent experiments.

and Poly I:C (Figure 7B). Among the relatively few transcripts differentially expressed at this early time point, we observed several interesting genes that may account for unique responses to DNA and dsRNA (Figure 7C). Consistent with our observations that the RIG-I/MDA5 pathway activates MAP kinases but the ISD response does not (Figure 5D), the MAP kinase-inducible transcription factor Early Growth Response 1 (Egr1) was strongly induced only by Poly I:C (Figure 7C). Transcripts for Mafb were dramatically reduced after ISD but not Poly I:C treatment. The lectin Ocil/Clrb, the apoptosis-associated gene Tdag, and the NF-κB-activating adaptor TANK were selectively activated by Poly I:C treatment. Transcripts for the protein phosphatase PtprJ were robustly increased only by Poly I:C, while the phosphatases Dusp6 and Ptpn16 were selectively downregulated by ISD treatment. Among the genes with expression specifically increased after ISD treatment, we found the GTPase RhoE, the cytokine-inducible SH2 protein Cish, Fas, and Lck (Figure 7C). Further work will be required to establish the functional relevance of these genes and whether these pathways diverge more dramatically at later time points. These data provide a direct comparison of genes activated by intracellular DNA and dsRNA and suggest that overlapping but unique responses are initiated depending on the type of nucleic acid detected.

Discussion

We provide evidence for a novel cytosolic innate immune response to DNA and suggest that recognition of bacterial DNA in the cytosol may be responsible for the potent induction of type I IFN upon *Listeria monocytogenes* and *Legionella pneumophila* infection. Removal of DNA from *Listeria* extracts abolishes their IFN-inducing activity, and purified DNA recapitulates all aspects of the TLR- and RIP2-independent cytokine response activated by live bacteria. We directly compare the signaling and gene expression programs activated by ISD, TLR9, and RIG-I/MDA5. These findings define a unique innate immune pathway and implicate a novel mechanism for nucleic acid recognition.

Our data implicating DNA in *Listeria*-activated IFN production may explain why diverse bacteria, eukaryotic parasites, and even apoptotic cells trigger an identical response upon entry into the cytosol. DNA may be released during *Listeria* infection as a passive consequence of LLO-mediated disruption of the vacuole. The majority of *Listeria* are killed shortly after infection of macrophages (Portnoy et al., 1989), which could provide a source of DNA for cytosolic recognition. Moreover, LLO-expressing *E. coli* are lysed upon entry yet still dissolve the vacuole and expose its contents to the cytosol, including soluble proteins and DNA once contained within the bacteria (Higgins et al., 1999). Alternatively, live *Listeria* may actively secrete DNA, which is a fundamental process in all prokaryotes. *L. monocytogenes* can conjugate with both gram-negative and gram-positive bacteria, and conjugal transfer of DNA into eukaryotic cells is known to occur (Charpentier et al., 1999; Waters, 2001). *Legionella pneumophila*, which remains confined within a host cell vacuole, triggers an identical response through its Dot/Icm type IV secretion system. We have not formally demonstrated that DNA is the activating ligand for this TLR- and RIP2-independent response. However, *L. pneumophila* injects effector proteins into recipient bacteria during Dot/Icm-dependent conjugal DNA transfer (Luo and Isberg, 2004), suggesting that the nature of the target cell does not influence substrate selection and that DNA might be translocated along with effector proteins into eukaryotic cells.

The elegant original demonstration of the cytosolic surveillance pathway activated by *Listeria* showed that IFN-β induction requires p38 and is associated with NF-κB activation (O'Riordan et al., 2002), while purified DNA activates neither of these (Figure 5). We found that p38 inhibitors delayed IFN-β induction and reduced it by approximately 70%, but significantly fewer bacteria entered inhibitor-treated cells, perhaps because p38 is important for phagocytosis of bacteria (D.B.S., unpublished data; Blander and Medzhitov, 2004). We also found that live *Listeria* activate a RIP2-dependent pathway in macrophages that is separable from the IFN-β and IL-6 response to bacterial DNA. Further characterization of the multiple signaling pathways activated by

Listeria will be required to establish the relative contributions of these individual components.

A native phosphodiester sugar-phosphate backbone is required for activation of the antiviral response by cytosolic DNA. Several studies have shown sequence-independent immunostimulatory activity specifically associated with transfected DNA (Ishii et al., 2001; Suzuki et al., 1999), and one recent report found TLR9-independent activity of phosphodiester DNA (Yasuda et al., 2005). While we used this feature to our advantage to compare the cytosolic DNA response to TLR9-dependent recognition, it may offer important clues to the nature of the sensor. Perhaps the sensor has intrinsic nuclease activity that is essential for DNA recognition. However, chimeric oligonucleotides with p-thio linkages at the ends and phosphodiester bonds in the center are fully active (D.B.S. and R.M., unpublished data). Alternatively, the phosphodiester backbone may provide a sequence-independent binding site for the sensor and allow it to distinguish between DNA and RNA.

We found that intracellular DNA potently activates the IFN-β gene without detectable activation of NF-κB and MAP kinases. Classical studies of this promoter established that robust transcriptional activation requires the coordinated assembly of several transcription factors termed the enhanceosome (Maniatis et al., 1998). These factors include IRF3/7, NF-κB p50/p65, and the MAP kinase-activated AP-1 (Atf2/c-Jun). The vast majority of studies defining the assembly of the enhanceosome were done with RNA viruses, and the recent characterization of the RIG-I-dependent response to RNA viruses validates the classical model (Kato et al., 2005). We suggest that ISD recognition might activate IFN-β transcription by using some but not all components of the enhanceosome. Numerous studies have shown that overexpression of IRF3 alone robustly activates the IFN-β promoter, presumably without any direct contribution of NF-κB or MAP kinases, and multimerization of IRF3 binding sites renders an ectopic promoter virus inducible (Fujita et al., 1987). We emphasize that live DNA viruses may activate these pathways via other ligands or effects on cells, such as recognition of viral RNA transcripts or a global stress response to viral replication. Identification of the sensor and unique elements of the ISD signaling pathway will allow dissection of the components of the response to DNA viruses and intracellular bacteria.

We used microarray analysis to directly compare the gene expression patterns activated by ISD versus TLR9 and RIG-I/MDA5 and identified a unique but overlapping response to intracellular DNA. Together, RIG-I, MDA5, and the ISD pathway may be sufficient to recognize a majority of viruses based on the type and structure of nucleic acids exposed inside the cell. Importantly, just as RNA viruses encode proteins that antagonize the RIG-I and MDA5 pathways (Andrejeva et al., 2004; Foy et al., 2005; Meylan et al., 2005), it is almost certain that DNA viruses encode proteins that interfere with the ISD response. Examination of these proteins will illuminate the specific mechanisms of DNA virus detection.

Finally, the existence of this pathway raises an important question of self/nonself discrimination: how does a sequence-independent sensor avoid recognition of the more than 10^9 base pairs of genomic DNA in all nucleated cells? Exclusion from the nucleus is an attractive explanation, but the breakdown of the nuclear envelope during cell division suggests that other mechanisms might prevent autoreactivity. Perhaps genomic DNA, with its myriad modifications and association with chromatin components, is specifically marked as "self." This would suggest that a failure to mark the genome as "self" would result in activation of the sensor. Such self-recognition could be beneficial in sensing genomic instability in severely damaged or neoplastic cells and may contribute to the important role for type I IFNs in tumor immunosurveillance (Dunn et al., 2005). Alternatively, a loss of cell-autonomous tolerance to genomic DNA would result in IFN-dependent autoimmunity. While these proposed functions are entirely speculative, we suggest that this pathway is responsible for the TLR-independent, type I IFN-mediated pathology observed in DNase II-deficient embryos, where liver macrophages become engorged with undigestable nuclei from erythrocyte precursors (Okabe et al., 2005). Further characterization of this pathway will provide valuable insights into the mechanisms of nucleic acid recognition and host resistance to pathogens.

Experimental Procedures

Mice, Cells, and Antibodies

C57Bl/6 mice and IFNαR1$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). MyD88$^{-/-}$, Trif$^{-/-}$, and TLR9$^{-/-}$ mice were kindly provided by Dr. Shizuo Akira. RIP2$^{-/-}$ mice and MEFs have been described (Kobayashi et al., 2002). Ku70$^{-/-}$ and DNA-PK$^{-/-}$ bone marrow and Atm$^{-/-}$ MEFs were generously provided by Dr. Frederick Alt. IRF3$^{-/-}$ MEFs were kindly provided by Dr. Tadatsugu Taniguchi. All animals were maintained in accordance with guidelines of the Yale Institutional Animal Use and Care Committee. RAW264.7 cells stably expressing an NF-κB-luciferase reporter were generated by standard procedures. Antibodies to MHC Class II (I-A^b), CD86, CD40, CD11c, and p38α were from BD Biosciences (San Diego, CA). Antibodies to SAPK/JNK and p44/42 MAPK (Erk1/2) were from Cell Signaling Technologies (Beverly, MA). Antibodies to IFN-α were from HyCult Biotechnology (the Netherlands) and PBL laboratories (Piscataway, NJ), and monoclonal antibody to murine IFN-β was from Seikagaku America (East Falmouth, MA).

Nucleic Acid Ligands

Calf thymus genomic DNA was from Sigma (St. Louis, MO). Poly I:C was from Amersham (Piscataway, NJ). All oligonucleotides were synthesized at the Yale W.M. Keck facility, with p-thio linkages represented as lowercase: ISD (sense), TACAGATCTACTAGTGATC TATGACTGATCTGTACATGATCTACA; CpG, tccatgacgttcctgacgtt.

Equimolar amounts of ISD and their antisense oligos were annealed in PBS at 75ºC for 30 min before cooling to room temperature. ISD-reverse was the exact reverse sequence of that listed above.

Cell Infections and Treatments

Macrophages were infected with *Listeria monocytogenes* strain 10403S at a moi of 2 bacteria per cell. Gentamycin (50 μg/ml) was added to cells 1.5 hr postinfection to kill extracellular bacteria. For in vivo infections, mice were injected intravenously with 5000 *L. monocytogenes*. Apoptotic thymocytes from MyD88/Trif DKO mice were prepared by a 4.5 hr treatment with 10 μM dexamethasone. Washed cells were loaded with recombinant LLO (100 ng per 10^6 cells) for 15 min in ice-cold PBS, washed, and added to MyD88/Trif DKO peritoneal-derived macrophages at a ratio of 5:1. *Listeria* extracts were prepared from a pelleted 5 ml culture freeze-thawed in liquid nitrogen, resuspended in 400 μl PBS containing 1 mg/ml lysozyme (Roche), and sonicated for 2 min using a VirSonic

Digital 600 Cell Disrupter (VirTis, Gardiner, NY). Remaining debris was removed by centrifugation, and cleared extracts were adjusted to contain 2 mM $MgCl_2$, 50 mM KCl, and 20 mM Tris-Cl (pH 8). 100 μl aliquots were treated separately with DNase I (Invitrogen, Carlsbad, CA; 100 units/ml) or RNase (Qiagen, Valencia, CA; 100 μg/ml) for 45 min at 37ºC. EDTA was added to 2.5 mM and samples were heated for 10 min at 70ºC. Extracts were again cleared by a 5 min centrifugation, and 5–10 μl were complexed with Lipofectamine 2000 (Invitrogen) and transfected as described below. *Legionella pneumophila* strains and opsonizing antibody were generously provided by Dr. Craig Roy. ISD were complexed with Lipofectamine 2000 (Invitrogen) at a ratio of 1 μl lipofectamine to 1 μg DNA. Cells in 12-well plates were stimulated with 5 μg ISD. Calcium phosphate transfections were done in the presence of 50 μM chloroquine by standard techniques. CpG was added directly to the media at 3 μM. *E. coli* LPS (Sigma) was used at 100 ng/ml. For analysis of IFN-α production, supernatants were harvested 18 hr poststimulation and used for a sandwich ELISA. For quantitative RT-PCR, cells were harvested into RNA-Bee (Teltest, Friendswood, TX). RNA was reverse transcribed with Superscript II (Invitrogen), and cDNAs were used for PCR with Quantitect SYBR Green reagents (Qiagen) on a Stratagene MX3000 bioanalyzer (La Jolla, CA). The abundance of each cytokine mRNA was normalized to HPRT expression and compared to untreated cells to calculate the relative induction.

Microarray Analysis

cDCs or macrophages were stimulated for 4 hr and harvested into RNA Bee. Sample preparation and hybridization to Affymetrix Mouse genome 430 2.0 arrays were performed at the Yale W.M. Keck facility. The complete data set has been submitted to the GEO database (http://www.ncbi.nlm.nih.gov/geo; submission #GSE2197).

Western Blots and Gel Shift Assays

Whole-cell lysates from macrophages stimulated for the indicated times were separated by SDS-PAGE, transferred to PVDF membranes, and probed with antibodies specific for the indicated proteins. Gel shift analysis was performed with standard techniques.

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