DNA-triggered innate immune responses are propagated by gap junction communication

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.
DNA-triggered innate immune responses are propagated by gap junction communication

Suraj J. Patelb,c,1, Kevin R. Kingb,c,t, Monica Casalil,c, and Martin L. Yarmusha,b,c,2

1Department of Surgery, Center for Engineering in Medicine, Massachusetts General Hospital, Boston, MA 02114; 2Division of Health, Sciences, and Technology, Massachusetts Institute of Technology, Harvard Medical School, Cambridge, MA 02139; and 3Shriners Hospital for Children, Boston, MA 02114

Edited by Tadatsugu Taniguchi, University of Tokyo, Tokyo, Japan, and approved May 20, 2009 (received for review September 22, 2008)

Cells respond to infection by sensing pathogens and communicating danger signals to noninfected neighbors; however, little is known about this complex spatiotemporal process. Here we show that activation of the innate immune system by double-stranded DNA (dsDNA) triggers intercellular communication through a gap junction-dependent signaling pathway, recruiting colonies of cells to collectively secrete antiviral and inflammatory cytokines for the propagation of danger signals across the tissue at large. By using live-cell imaging of a stable IRF3-sensitive GFP reporter, we demonstrate that dsDNA sensing leads to multicellular colonies of IRF3-activated cells that express the majority of secreted cytokines, including IFNβ and TNFα. Inhibiting gap junctions decreases dsDNA-induced IRF3 activation, cytokine production, and the resulting tissue-wide antiviral state, indicating that this immune response propagation pathway lies upstream of the paracrine action of secreted cytokines and may represent a host-derived mechanism for evading viral antiviral strategies.

The ability of the innate immune system to propagate antiviral and inflammatory signals from the local cellular microenvironment to the tissue at large is critical for survival (1). At the onset of a viral infection, individual cells sense invading pathogens and elicit innate immune responses that spread from infected to uninfected cells, establishing an overall antiviral state (2). Secreted cytokines such as interferon β (IFNβ) and tumor necrosis factor (TNFα) are 2 key mediators of these responses (3). To evade the host immune system, viruses have evolved strategies for limiting the secretion of these cytokines (4). Nevertheless, the immune system remains capable of clearing many viral pathogens, suggesting that the host may have subsequently evolved additional mechanisms for propagating antiviral and inflammatory signals, beyond the paracrine action of cytokines (5).

The innate immune system uses pathogen-recognition receptors to sense nucleic acids during infection or tissue damage (6). Pathogen-derived nucleic acids generate potent immune responses, as they are not typically found in a host cell or in particular intracellular locations (7, 8). Several receptors have been identified for their ability to recognize viral RNA, and their mechanistic details have been well studied (8). In contrast, the sensing of viral DNA and the subsequent triggering of a host antiviral response remain poorly understood. Double-stranded DNA (dsDNA) derived from host, viral, or synthetic sources elicits a potent immune response by activating a TLR-independent cytosolic DNA sensor (9, 10), such as the recently identified DNA-dependent activator of IFN regulatory factors (DAI) (11). The TLR-independent pathway for dsDNA sensing activates TBK1 and IKKε for the phosphorylation of transcription factor IRF3, which binds to IFN-sensitive response element (ISRE) sequences, triggering the robust production of type I interferons such as IFNβ (9, 12, 13). In addition to IFNβ, a successful antiviral response requires the establishment of an inflammatory state through cytokines such as TNFα (14). The secretion of IFNβ and TNFα is thought to play an important role in propagating antiviral innate immune responses from individual infected cells to noninfected neighbors, priming them to resist the spread of infection (14). However, although this communication is commonly attributed to secreted cytokines, the spatiotemporal details remain speculative, and the possibility of contact-mediated communication unexplored.

Cell–cell communication can be categorized by its dependency on contact. Contact-independent signaling is ideal for long-range communication whereas contact-dependent signaling is best suited for spatially localized rapid communication (15). Gap junction intercellular communication represents an important class of contact-dependent signaling. Gap junctions are assemblies of intercellular channels composed of connexin (Cx) proteins organized into 2 subsets, alpha connexins (e.g., Cx43) and beta connexins (e.g., Cx32, Cx26). Connexins from each subset oligomerize to form a hemichannel. A functional channel is formed when a hemichannel from one subset assembles with a hemichannel of the same subset from an adjacent cell (16). The resulting gap junctions directly connect the cytosol of the coupled cells, allowing the exchange of ions, nutrients, and secondary messengers for the maintenance of tissue homeostasis (17). In the context of innate immunity, gap junction communication has been shown to be regulated by pathogen-associated stimuli such as LPS and peptidoglycans, and secreted proinflammatory cytokines such as TNFα, IL-1β, and IFNγ (18, 19). However, the relative contributions of contact-dependent and contact-independent communication in the establishment of host defenses have not been explored.

Given the incomplete understanding of host innate immune response propagation, we used a stable ISRE-GFP monoclonal reporter to explore the spatiotemporal patterns of IRF3 activation in response to dsDNA stimulation and investigated the intercellular signaling pathways between infected and noninfected cells for establishing an antiviral state. We found that dsDNA stimulation induced spatially heterogeneous responses characterized by the formation of multicellular colonies of IRF3 activated cells that collectively expressed more than 95% of critical secreted cytokines, including IFNβ and TNFα. Functional gap junctions were necessary for the formation of these IRF3 active colonies and blocking gap junctions with genetic specificity limited the secretion of IFNβ and TNFα and the corresponding antiviral state. Our findings describe a previously unknown intercellular signaling pathway triggered by cytosolic dsDNA sensing and provide evidence that gap junction communication is critical for the amplification of antiviral and inflammatory responses, prior to paracrine-mediated propagation by cytokines.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

1S.J.P. and K.R.K. contributed equally to this work.

2To whom correspondence should be sent at the following address: Massachusetts General Hospital, Shriners Burns Hospital, 51 Blossom Street, Research, Boston, MA 02114. E-mail: ireis@sbi.org.

This article contains supporting information online at www.pnas.org/cgi/content/full/0809292106/DCSupplemental.
Results

**GFP Reporters Sensitive and Specific for IRF3-Activating Stimuli.** dsDNA is known to stimulate the expression of genes with ISRE consensus sequences in their promoters through activation of the IRF3 transcription factor (9, 12). To investigate the spatiotemporal evolution of dsDNA-induced gene expression, we created a stable monoclonal ISRE-GFP reporter cell line in a hepatocyte-derived H35 cell line and selected clones exhibiting low-baseline GFP expression in the absence of stimuli and high dsDNA-induced GFP expression (Fig. 1A). To characterize the reporter, we first assessed its specificity for IRF3-dependent gene expression by exposing confluent reporter monolayers to various immunostimulatory molecules and measuring ISRE reporter fluorescence by using flow cytometry (Fig. 1B). When reporters were exposed to synthetic B-form Poly(dA-dT):Poly(dA-dT) (hereafter referred to as dsDNA) or DNA encoding the red fluorescent protein (dsRED), total population fluorescence increased in a dose-dependent fashion. Similarly, when cells were stimulated with polyinosinic:polycytidylic acid [poly(I:C)], a synthetic double-stranded RNA known to activate IRF3, dose-dependent increases in total fluorescence were also observed. In contrast, IRF3-independent stimuli such as TLR9-dependent Cpg DNA, IFNβ, poly(A), ssDNA, and siRNA did not elicit measurable increases in ISRE reporter fluorescence at any of the doses examined. As anticipated, LPS, which classically signals through TLR4 and IRF3, failed to activate the ISRE reporter, consistent with the fact that nonimmune cells do not express significant levels of TLR4 (confirmed by qPCR). To verify that the ISRE reporters retained previously reported dsDNA-induced responses (9), we confirmed the expression of IFNβ and TNFα after 12 h of dsDNA stimulation (Fig. 1C). Taken together, these results demonstrate that the ISRE reporters are sensitive for both dsDNA and Poly(I:C) stimulation, and specific for IRF3-activating stimuli.

When we examined the distribution of ISRE reporter fluorescence after dsDNA stimulation by flow cytometry, we found that increasing doses of dsDNA resulted in increasing numbers of activated cells rather than increasing levels of activation in all cells, indicating that the cellular response was heterogeneous (Fig. 1D and E). Therefore, we used fluorescence microscopy to examine the distribution of dsDNA-induced GFP expression and discovered a striking spatial pattern. dsDNA stimulation of confluent reporter monolayers exhibited well-delineated clusters of GFP-positive cells or “colonies” in an otherwise dark background of nonactivated cells. Increasing doses of dsDNA led to increasing numbers of colonies with eventual bridging of adjacent colonies (Fig. 1F and G). To gain further insight into colony formation, we examined the temporal evolution of ISRE-activated colonies by monitoring GFP-reporter induction with time-lapse fluorescence microscopy and quantifying colony area by using custom automated image analysis software (Fig. 1H and I) (see Movie S1 online). Colony activation began 8–12 h after dsDNA stimulation and grew to a steady-state size with clearly demarcated colony borders characterized by highly induced reporter cells inside and uninduced cells outside. These findings confirm that dsDNA stimulation leads to spatially heterogeneous patterns of ISRE-activated colonies in an otherwise uninduced confluent monolayer of cells. Interestingly, Poly(I:C) stimulation also led to ISRE reporter activation; however, the response was spatially homogeneous across the reporter monolayer and did not result in colony formation.

Because dsDNA requires polyelectrolyte complexing for immunogenicity and high molecular weight complexes have short diffusion distances, we reasoned that dsDNA complexes land in discrete...
locations within the cultured monolayer, stimulate individual dsDNA-sensing cells, and ISRE colony formation arises by a secondary intercellular communication signal. To clarify which cells were directly and indirectly activated by dsDNA, ISRE reporters were stimulated with dsRED. dsRED stimulation of ISRE-GFP reporters resulted in multicellular ISRE reporter cell colonies surrounding individual dsRED DNA-sensing cells (Fig. 1K and Fig. S1). By using the custom automated image analysis software discussed above, we determined that the average colony was comprised of 23.5 ISRE-activated cells, of which 2.3 cells were dsRED DNA-sensing cells (see Fig. S1D).

**Spatiotemporal IRF3-Mediated Gene Expression.** We next sought to determine whether the colonies of GFP+ ISRE reporters had functional significance by examining their gene expression profiles and comparing them with their GFP− neighbors. Confluent monolayers of reporters were stimulated with dsDNA for 12 h, sorted into GFP+ and GFP− populations by FACS, and examined for expression of IRF3-mediated genes by qPCR (Fig. 2A). GFP+ cells represented less than 8% of the total population, yet expressed more than 95% of secreted cytokines and chemokines including IFNβ, TNFα, and IP10 (Fig. 2B). In contrast, expression of IFNβ-inducible genes, with direct antiviral properties such as PKR and OAS1, did not differ significantly between the 2 populations (Fig. 2C). Given the commonly held view that antiviral genes are expressed in response to the paracrine action of IFNβ, these results suggest that dsDNA-sensing in individual cells leads to IRF3 activation in colonies of cells that collectively secrete cytokines, such as IFNβ and TNFα, to establish an antiviral state across the broader population.

**IRF3 Activation by Contact-Dependent Intercellular Communication.** We hypothesized that IRF3-activated colony formation required secondary intercellular communication from dsDNA-sensing cells. To test this hypothesis, we developed a transplant coculture system using dsDNA-stimulated nonreporter cells as donors and unstimulated ISRE reporters as recipients. Donor cells were stimulated with dsDNA for 6 h, trypsinized, thoroughly washed, and transplanted onto recipient ISRE reporters that had never been exposed to dsDNA (Fig. 3A). After 18 h of coculture, ISRE reporters were activated in small colonies surrounding donor cells (Fig. 3B and C). In contrast, no IRF3 activity was observed in the reporters when mock dsDNA stimulated nonreporter cells were transplanted (Fig. 3C). Interestingly, not all dsDNA-stimulated cells were able to activate IRF3 in neighboring reporters. dsDNA-stimulated human cervical cancer cells (HeLa) and mouse neuroblastoma cells (N2A) were unable to activate IRF3 in the reporters (Fig. 3B and C), demonstrating that the phenomenon is cell-type specific and not an artifact of nonspecific dsDNA carry-over from the donor cells. Donor cells were also stimulated with dsRED DNA, to identify them within the coculture, and to confirm that they make contact and adhere to the reporter cells (see Fig. S4). To investigate whether direct cell contact was necessary for this dsDNA-induced secondary intercellular communication, we cultured dsDNA-stimulated donors and ISRE reporter recipients on opposite surfaces of a microfluidic parallel plate bioreactor (separation gap of ~50 μm; see Fig. S5). Negligible reporter induction was observed, suggesting that dsDNA-induced intercellular communication is contact dependent. Taken together, these data suggest that dsDNA stimulation induces contact-dependent intercellular communication from dsDNA-sensing cells to their unstimulated neighbors, propagating an IRF3-activating signal and amplifying IRF3-dependent gene expression.

To further clarify the pathways connecting donor dsDNA-sensing and recipient IRF3 activation, we used genetic knockout mouse embryonic fibroblasts (MEFs) to determine the necessity of critical proteins. Wild-type MEF donors stimulated with dsDNA activated ISRE in reporter recipients (Fig. 3D and E). In addition, MEFs deficient in both TBK1 and IKKε (Tbk1−/−;Ikkbε−/−), kinases necessary for IRF3 activation, also activated ISRE in reporter cells (Fig. 3D and E). Similarly, dsDNA-stimulated MEFs...
Deficient in both IKKα and IKKβ (IKKα/−/−IKKβ/−/−), kinases essential for NFκB activation, also activated ISRE in reporter cells (Fig. 3 D and E). MEFs deficient in both MyD88 and TRIF (MyD88−/−/−TRIF−/−) were also able to activate ISRE reporter recipients. Together, these data suggest that dsDNA-induced intercellular communication is TLR-independent and occurs upstream of both IRF3 and NFκB activation in dsDNA sensing cells.

**Gap Junctions Are Necessary for Amplified IRF3 Activation.** Gap junction communication enables the rapid, localized exchange of information between cells linked through connexin protein channels (16). To determine the necessity of gap junctions in the dsDNA-induced intercellular communication, we pretreated ISRE reporters with 18β-glycyrrhetinic acid (18βGA), a molecular inhibitor of gap-junctions (20), before dsDNA stimulation. Compared with vehicle controls, pretreatment with 18βGA dramatically reduced colony size, as shown in the images (Fig. 4A). Images were quantified by creating isointensity contour maps and calculating the average size per colony for each colony present in the images (Fig. 4B). The average size of dsDNA-induced ISRE-activated colonies was reduced by more than 10-fold with 18βGA treatment compared with no 18βGA treatment. More than 75% of all colonies formed in the presence of 18βGA contained fewer than 3 cells, whereas more than 90% of all colonies formed in the absence 18βGA contained more than 3 cells (Fig. 4C). In addition, the overall number of ISRE-activated GFP-positive cells was decreased from 38% to 5%, as a result of gap junction blockage (Fig. 4D). These results were further validated by Cx32 knockdown analysis using Cx32-targeted siRNA (see Fig. S3). Compared with control siRNA, Cx32 siRNA knockdown resulted in a significant decrease in both the size of dsDNA-induced ISRE active colonies and in the overall number of dsDNA-induced GFP-positive reporters. More than 70% of all colonies formed in the presence of Cx32 knockdown contained fewer than 3 cells, whereas more than 85% of all colonies formed in the presence of control knockdown contained more than 3 cells (see Fig. S3).

The utility of chemical gap junction inhibitors such as 18βGA is limited because of their nonspecific side effects and unknown mechanism of action (21). Therefore, to definitively demonstrate the necessity of gap junction communication for the propagation of IRF3 activity, we used the transplant coculture system with genetically modified cell lines. HeLa and N2A cell lines have been historically shown to be gap junction- and connexin-deficient (21, 22). We obtained modified monoclonal HeLa and N2A cells stably transfected with individual connexin transgenes (Cx26, Cx32, or Cx43), thereby reconstituting connexin expression and gap junction communication (21, 22). PCR analysis was performed to verify appropriate connexin expression in all HeLa and N2A cells. PCR analysis showed that ISRE reporters expressed only Cx32 of the β-subset and therefore only form functional gap junction channels with other cells expressing β connexins 26 or 32. When WT and Cx32-expressing HeLa and N2A cells were stimulated with dsDNA, minimal ISRE reporter activation was measured (HeLa WT, 0.5%; N2A WT, 1.0%; HeLa Cx32, 2%; N2A Cx32, 1.5%), suggesting a lack of intercellular communication (Fig. 4E–H). However, when Cx26- and Cx32-expressing cells were stimulated with dsDNA, significant ISRE reporter activation was observed (Fig. 4E and F), with 63% of reporters activated by Cx32-expressing HeLa cells and 23% by Cx32-expressing N2A cells (Fig. 4G and H). These data suggest that functional gap junction channels are necessary for dsDNA-induced intercellular communication and for amplifying IRF3 activation. To further generalize the utility of gap junctions for...
human-specific PCR primers and ELISAs. When dsDNA-stimulated cells were then analyzed for cytokine expression and secretion using WT) or expressed Cx32 gap junctions (HeLa Cx32). Recipient HeLa ture system described above. Rat ISRE reporter cells, which express responses by using genetically modified cells and the transplant cocul-

Propagation of Antiviral and Inflammatory Responses by Gap Junctions. To investigate the physiological significance of gap junction-mediated amplification of IRF3 activity, we disrupted gap junctions and examined the ability of cells to mount innate immune responses. When ISRE reporters were pretreated with 18βGA and stimulated with dsDNA, they expressed significantly lower levels of critical antiviral cytokines in comparison to vehicle pretreatment. After 8 h of dsDNA stimulation, 18βGA pretreated cells expressed 6-fold less IFNβ than vehicle-treated cells (Fig. 5A). Expression of the IFNβ-stimulated antiviral protein PKR was also significantly reduced by 2-fold (P < 0.05) in the 18βGA-treated cells, compared with vehicle treatment (Fig. 5A). Similarly, the expression of the proinflammatory cytokine, TNFα, was reduced by 3.5-fold with gap junction inhibition (Fig. 5A).

We further verified the role of gap junctions in innate immune responses by using genetically modified cells and the transplant coculture system described above. Rat ISRE reporter cells, which express only Cx32, were stimulated with dsDNA and then cocultured with unstimulated human HeLa cells that either lacked gap junctions (HeLa WT) or expressed Cx32 gap junctions (HeLa Cx32). Recipient HeLa cells were then analyzed for cytokine expression and secretion using human-specific PCR primers and ELISAs. When dsDNA-stimulated reporter cells were cocultured with HeLa Cx32 cells, HeLa IFNβ and TNFα expression was increased by 6- and 4-fold, respectively, compared to coculture with gap junction-deficient HeLa WT cells (Fig. 5B). Additionally, coculture of dsDNA-stimulated reporters with HeLa Cx32 cells triggered a 5-fold increase in production of human IFNβ compared to coculture with gap junction-deficient HeLa WT cells (Fig. 5C). Taken together, these data suggest that gap junctions amplify dsDNA-induced expression and secretion of IFNβ and TNFα, and that without functional gap junctions, cytokine secretion is impaired, consequently reducing the antiviral state in the overall population.

Discussion

Investigations of innate immune response propagation have typically focused on the paracrine action of secreted cytokines (23). Here we provide evidence that gap junctions amplify innate immune responses triggered by cytosolic dsDNA. Using a stable monoclonal GFP reporter cell line that is sensitive and specific for IRF3-mediated gene expression, we visualized the spatiotemporal evolution of IRF3 activity in response to dsDNA stimulation. Sorting these cells by GFP activity, we demonstrated that dsDNA stimulation of confluent cell monolayers leads to the formation of 2 distinct cell populations, each with a unique gene expression program. The IRF3-active subpopulation was spatially arranged in multicellular colonies that collectively served as the dominant source of diffusible cytokines for the establishment of an overall antiviral state. These colonies were formed by gap junction-dependent communication between dsDNA-stimulated cells and their unstimulated neighbors. In the absence of gap junctions, IRF3-activated colonies and total cytokine secretion were significantly diminished, as was the resulting antiviral state. These findings place contact-dependent communication upstream of secreted cytokines, at the earliest stages of dsDNA-induced antiviral and inflammatory responses, and they offer gap junction communication as a mechanism for amplifying dsDNA-mediated innate immunity.

Gap junctions are networks of intercellular communication channels that allow local cell populations to rapidly share and spread signals (16). This work has identified a gap junction-dependent “recruitment” process whereby infected cells engage surrounding noninfected cells to secrete cytokines. The molecular details of this process, its mediator, its regulation, and the pathways that lead to its generation remain unclear, and further investigation is necessary. Many common gap junction communication mediators such as Ca2+, CAMP, and IP3 have been implicated in the activation of inflammatory transcription factors (24, 25). For example, calcium fluxes have been shown to activate transcription factors such as NFκB and AP1, resulting in the expression of proinflammatory cytokines (24). Although there are links connecting gap junction communication and inflammation, the results represent the first connection to antiviral responses.

Secreted cytokines such as IFNβ and TNFα are known to mediate the spread of antiviral and inflammatory signals for the protection of noninfected cells against subsequent attack (3). However, at the earliest stages of an infection when only a limited number of cells have been exposed to the pathogen, recruitment of neighboring noninfected cells and amplification of cytokine production is particularly important. Paracrine feed-forward loops have been proposed to increase the number of cytokine secreting cells beyond those initially infected; however, their significance is unclear (23). For example, dsDNA stimulation of type I IFN receptor-deficient macrophages induced similar IFNβ expression compared with wild-type controls, suggesting that the IFNβ paracrine loops may not be necessary for amplifying IFNβ secretion (10). In this work, we show that gap junction communication provides a mechanism for single infected cells to recruit their neighbors and amplify cytokine production prior to the activation of paracrine loops. Compared with secreted cytokine amplification, gap junction-mediated signaling is typically faster and therefore better suited for anticipating and preventing the rapid spread of an invading pathogen (16).

Although the existence of dsDNA-stimulated intercellular communication is clear, the precise signaling pathway remains unknown. Evidence from transplant coculture experiments showed that communication does not require MyD88 or TRIF, thereby eliminating the necessity of all known TLR pathways. Additionally, the communication was independent of TBK1, IKKe, IKKα, and IKKβ in the dsDNA sensing cell, demonstrating that signal gener-
proinflammatory signaling by inhibiting NF-κB and IRF3-activated signals from infected cells to their noninfected neighbors, potentially allowing the escape of host danger signals before the virus has time to disable the antiviral program in the infected cell. This type of immediate response has the advantage of rapidly mobilizing host defenses against infection, resulting in the early secretion of large amounts of cytokines for broadly inducing an antiviral state. Indeed, we found that gap junctions enable the rapid local spread of dsDNA-induced IRF3-activating signals from infected cells to their noninfected neighbors, suggesting that viruses have evolved numerous strategies for silencing the defenses of infected cells. Many viruses prevent infected cells from propagating “danger signals” by inhibiting IRF3-mediated production and secretion of antiviral cytokines such as IFN-α/β, TNF, and IL-1β (26). This work demonstrated that gap junctions enable the rapid local spread of dsDNA-induced IRF3-activating signals from infected cells to their noninfected neighbors, potentially allowing the escape of host danger signals before the virus has time to disable the antiviral program in the infected cell. This type of immediate response has the advantage of rapidly mobilizing host defenses against infection, resulting in the early secretion of large amounts of cytokines for broadly inducing an antiviral state. Indeed, we found that gap junctions produce significantly less IFN-β and TNFα, resulting in substantial decreases in expression of antiviral genes such as PKR. Furthermore, dsDNA viruses that represent important causes of human disease, herpesvirus HSV2 and human papilloma virus HPV16, were shown to express viral proteins close to gap junctions of infected cells, suggesting that viruses have identified gap junction communication as a critical mode for host defense signaling and have begun evolving strategies to inhibit these defenses (27, 28).

Mammalian cells are exposed to cytosolic dsDNA during viral and intracellular bacterial infection, during exposure to self-DNA from dying cells, and during DNA-based gene therapy (7, 9, 11, 14). The ability to increase and decrease the innate immune response in these settings would have significant potential as a clinically relevant therapeutic. In this regard, the stable monochonal ISRE reporter represents an important experimental tool for discovering modulators of the innate immune responses to these stimuli and the intercellular communication mechanisms they use for propagation and amplification. However, modulation of gap junction communication continues to be experimentally challenging. Genetic methods are certainly the most definitive; however, the degeneracy of gap junction proteins required to form functional gap junctions is such that cells deficient in individual connexins may not show significant defects due to compensatory expression of other connexins. Future studies will be needed to evaluate the full physiologic significance of gap junction communication in vivo to discern responses to dsDNA-based stimuli.

Materials and Methods

Cells and Reagents. Hepatocyte-derived H35 cells were maintained as previously described (29). Construction of the ISRE-GFP reporter was performed as described in Materials and Methods. dsDNAs from WT and knockout mice and Cx26-, Cx32-, and Cx43-expressing HeLa and N2A cells were gifts (see Acknowledgments). Synthetic polydeoxyamino nucleotides poly(dA·dT)·poly(dA·dT) dsDNA and poly(dA·dT)·dsDNA, synthetic poly(C)-poly(gC), and pdsRED was purchased from commercial sources (see SI Materials and Methods). DNA transfections were performed by using Lipofectaamine LTX reagent (Invitrogen) at a ratio of 1:5 (volumeweight) with DNA as per manufacturer’s protocol. For experiments involving the use of 18S/ββ, cells were pretreated with 25 μM 18S/ΓA in DMEM for 1 h before dsDNA stimulation and during stimulation.

Quantitative RT-PCR and ELISA. Analysis of gene expression by quantitative RT-PCR, and protein secretion by ELISA is detailed in SI Materials and Methods. RT-PCR primer sequences are provided in Table S1.

Transplant Coculture Assay. Donor cells were transfected with 10 μg/ml of poly(A:T) dsDNA. Six hours after transfection, donor cells were trypsinized, washed three times in PBS, and counted. dsDNA-stimulated donor cells (1 × 10⁷ cells/ml) were transplanted onto a subconfluent layer of recipient ISRE reporters, maintaining a 1:10 ratio between donor and recipient cells. After 18 h of culture, recipient reporter cells were analyzed by fluorescence microscopy and FACS for ISRE activity. In some cases, IFNα and TNFα secretion by recipient cells was measured by ELISA. Gene expression analysis on recipient cells was performed by quantitative RT-PCR using recipient cell species-specific primers (provided in Table S2).

ACKNOWLEDGMENTS. We thank S. Akira (Osaka University, Osaka, Japan) for the generous gift of MyD88−/− TNF−/− and Tbk1−/−/Ikk−/− MEFs; I. Verma (The Salk Institute of Biological Studies, La Jolla, CA) for Ikk−/−/Ikk−/−/MEFs; S. L. Friedman (Duke University, Durham, NC) for HSC-T6 cells; D. Spray (Albert Einstein College of Medicine, New York) for Cx26−/−, Cx32−/−, and Cx43-expressing N2A cells; and K. Willecke (University of Bonn, Bonn, Germany) for Cx26−/−, Cx32−/−, and Cx43-expressing HeLa cells. The authors thank Ken Wieder and Arno Tilles for technical support. S. J. P. was supported by a Department of Defense Congenital disorder of metabolism research program Prostate Cancer Predoctoral Training Award. K. R. K. was supported by a Shriners Hospital for Children postdoctoral fellowship. The work was supported by U.S. National Institutes of Health Grants GM065474 and P41 EB-002503, and by a grant from the Shriners Hospital for Children.

References