Cell Host & Microbe
Article

TLR-Independent Type I Interferon Induction in Response to an Extracellular Bacterial Pathogen via Intracellular Recognition of Its DNA

Marie Charrel-Dennis,[1] Eicke Latz,[1] Kristen A. Halmen,[1] Patrick Trieu-Cuot,[2] Katherine A. Fitzgerald,[1] Dennis L. Kasper,[3,4,5] and Douglas T. Golenbock[1,5,*]
[1]Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01605, USA
[2]Institut Pasteur, Unité de Biologie des Bactéries Pathogènes à Gram-positif, CNRS URA 2172, Paris, France
[3]Channing Laboratory, Department of Medicine, Brigham and Women's Hospital
[4]Department of Microbiology and Molecular Genetics
Harvard Medical School, Boston, MA 02115, USA
[5]These authors contributed equally to this work
*Correspondence: douglas.golenbock@umassmed.edu
DOI 10.1016/j.chom.2008.11.002

SUMMARY

Type I interferon (IFN) is an important host defense cytokine against intracellular pathogens, mainly viruses. In assessing IFN production in response to group B streptococcus (GBS), we find that IFN-β was produced by macrophages upon stimulation with both heat-killed and live GBS. Exposure of macrophages to heat-killed GBS activated a Toll-like receptor (TLR)-dependent pathway, whereas live GBS activated a TLR/NOD/RIG-like receptor (RLR)-independent pathway. This latter pathway required bacterial phagocytosis, proteolytic bacterial degradation, and phagolysosomal membrane destruction by GBS pore-forming toxins, leading to the release of bacterial DNA into the cytosol. GBS DNA in the cytosol induced IFN-β production via a pathway dependent on the activation of the serine-threonine kinase TBK1 and phosphorylation of the transcription factor IRF3. Thus, activation of IFN-α/-β production during infection with GBS, commonly considered an extracellular pathogen, appears to result from the interaction of GBS DNA with a putative intracellular DNA sensor or receptor.

INTRODUCTION

Interferons (IFNs) are multifunctional cytokines important in host defense, particularly against intracellular pathogens. Type I IFNs have long been acclaimed for their role in antiviral host defense; however, an expanding body of work shows that type I IFNs are likewise important in the host response to bacterial infection (Henry et al., 2007; Pitha, 2004). Their activity can influence numerous immune-effector mechanisms, exerting effects that can be either favorable or detrimental to the host upon bacterial infection (Hein et al., 2000; O'Connell et al., 2004; O'Riordan et al., 2002; Opitz et al., 2006; Stanley et al., 2007).

Conserved bacterial products have been found to activate distinct signal transduction pathways that merge with pathways activated by viruses and lead to type I IFN production. Indeed, the type I IFN response resulting from Gram-negative bacterial infections, such as those caused by *Salmonella typhimurium* and *Escherichia coli,* seems to be triggered, at least partially, by lipopolysaccharide (LPS) through the Toll-like receptor 4 (TLR4)/MD-2 signaling complex (Sing et al., 2000; Yaegashi et al., 1995). Another bacterial component that might induce type I IFN is Gram-negative flagellin, although contradictory studies have been published (Mizel et al., 2003; Opitz et al., 2006; Simon and Samuel, 2007).

The most extensive studies of the role of type I IFNs in bacterial infection have focused on the pathways activated by intracellular pathogens (O'Connell et al., 2005; Reimer et al., 2007; Stetson and Medzhitov, 2006; Stockinger et al., 2004). Several studies have suggested that IFN-inducing ligands detected upon cytosolic invasion are found in numerous intracellular pathogens. For example, the invasive *Shigella flexneri*, *Legionella pneumophila*, or *Francisella tularensis* activate a potent IFN-α/-β response in fibroblasts and macrophages, whereas their respective noninvasive strains do not (Henry et al., 2007; Hess et al., 1987, 1990; Opitz et al., 2007). Similarly, *Trypanosoma cruzi*, a protozoan parasite, activates IFN-α/-β production with kinetics similar to its escape from the lysosome (Koga et al., 2006; Vaena de Avalos et al., 2002). The TBK1/IRF3 axis appears to play a central role in the transcriptional regulation of IFN-β upon intracellular bacterial infection, but little is known regarding the bacterial ligands and receptors that function upstream of TBK1. Upon infection with the cytosolic Gram-positive bacteria *Listeria monocytogenes,* Stetson and Medzhitov have suggested that the type I IFN response might result from the binding of prokaryotic DNA to a cytosolic receptor (Stetson and Medzhitov, 2006). Moreover, it has recently been suggested that the cytosolic DNA-dependent activator of IFN-regulatory factors (IRFs), or DAI, is a bacterial DNA receptor acting as the first step in the type I IFN pathway activated by intracellular bacteria (Takaoka et al., 2007). Together, these studies indicate that a common feature may unite the IFN-α/-β responses to intracellular pathogens, and that products unique to bacteria are unlikely to be responsible for activating type I IFNs.

Until recently, extracellular Gram-positive bacteria were believed to be unable to activate type I IFN gene transcription.

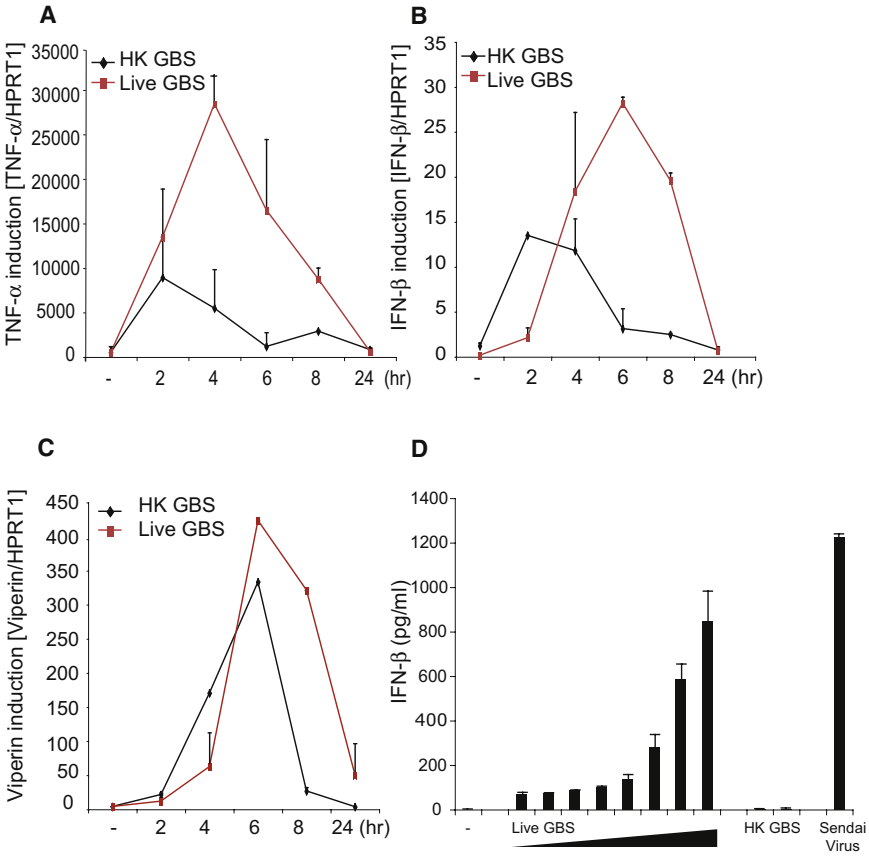


Figure 1. Live and Heat-Killed GBS Organisms Induce Type I IFNs and Proinflammatory Cytokines

(A–C) WT bone marrow-derived macrophages (BMDMs) were stimulated for the indicated amount of time with heat-killed GBS (HK GBS; 80 μg/ml) or live GBS (MOI 6:1). Levels of mRNA for *TNF-α* (A), *IFN-β* (B), and *viperin* (C) were determined by Q-PCR and normalized to *HPRT1* level of expression.

(D) WT BMDMs were stimulated for 12 hr with two-fold decreasing amounts of bacteria; the initial concentration of heat-killed GBS (HK GBS; 80 μg/ml), live GBS (MOI 10:1) and Sendai virus (150 HAU/ml). IFN-β was detected by ELISA. *, $P < 0.05$, Student's t test (all panels).

However, a study by Mancuso et al. suggested that extracellular Gram-positive bacteria such as *Streptococcus agalactiae* (group B streptococcus [GBS]) and *Streptococcus pneumoniae* were also able to induce type I IFN, and that this IFN production was critical for the clearance of infection by the host. Indeed, mice lacking either IFN-α/-β receptor (IFNAR) or IFN-β displayed an unrestrained bacteremia (Mancuso et al., 2007).

Here, we have studied and characterized the pathway leading to type I IFN production in mouse macrophages infected with the extracellular bacterium GBS, a species found in the normal flora of the gut and the female urogenital tract (Schuchat, 1999). Early perinatal infection with GBS causes pneumonia and/or septicemia—both associated with high mortality rates. In this report, we provide evidence that in addition to inducing a very potent inflammatory response, requiring TLR recognition, GBS induces a type I IFN response. This response was both TLR-dependent and TLR/NOD/retinoic-acid-inducible gene (RIG)-like receptor (RLR)-independent, depending on whether live or killed bacteria were tested. Live bacteria, in particular, seem to activate type I IFN production via TBK1-mediated phosphorylation of IRF3—a process that is associated with the release of bacterial products, especially DNA, from the phagolysosome. We observed the initiation of an autocrine loop in which GBS induces the production of important IFN-inducible genes such as that encoding viperin. Since type I IFNs play such a critical role in the outcome of serious GBS infection, these findings underscore the potentially important contributions of phagolysosomal escape in the initiation of host defenses and in the pathology that develops during GBS infection.

RESULTS

Live and Heat-Killed GBS Induce Type I IFN Production

We studied the response of C57BL/6 bone marrow-derived macrophages (BMDMs) stimulated with live and heat-killed GBS. As anticipated, both live and heat-killed forms of GBS evoked a very strong inflammatory response (Figure 1A). In addition, both live and heat-killed GBS induced type I IFN induction (Figure 1B). Induction of *IFN-β* peaked at 2 hr postinfection with heat-killed GBS and 6 hr postinfection with live GBS: the latter response was delayed compared with that induced by live intracellular bacteria such as *Listeria monocytogenes* (peak induction at 3–4 hr) (Stockinger et al., 2004; data not shown). A highly IFN-inducible gene, *viperin* (virus-inhibitory protein, endoplasmic reticulum-associated, IFN-inducible) (Severa et al., 2006), displayed a similar kinetic as *IFN-β* (Figure 1C). The induction of *viperin* upon GBS infection was entirely dependent on IFN-α/-β and did not occur in macrophages deficient in the type I IFN-α/-β receptor (Figure S1). At the protein level, live GBS infection induced similar amounts of IFN-β to that observed upon Sendai virus infection, whereas macrophages stimulated with heat-killed GBS produced no detectable IFN-β (Figure 1D).

Live and Heat-Killed GBS Activate Distinct Pathways Leading to Type I IFN Production

GBS is a potent activator of the innate immune system. Along with other investigators, we have reported that GBS presents multiple TLR ligands to innate immune cells (Cusumano et al., 2004; Henneke et al., 2001; Mancuso et al., 2004). Thus, we anticipated that the induction of type I IFN would be dependent on TLRs, which appear to recognize ligands that are located either extracellularly (e.g., LPS [Poltorak et al., 1998]) or within a lysosomal compartment (e.g., CpG DNA [Hemmi et al., 2000]). The TLR family of receptors uses four adaptor molecules: Mal/TIRAP,

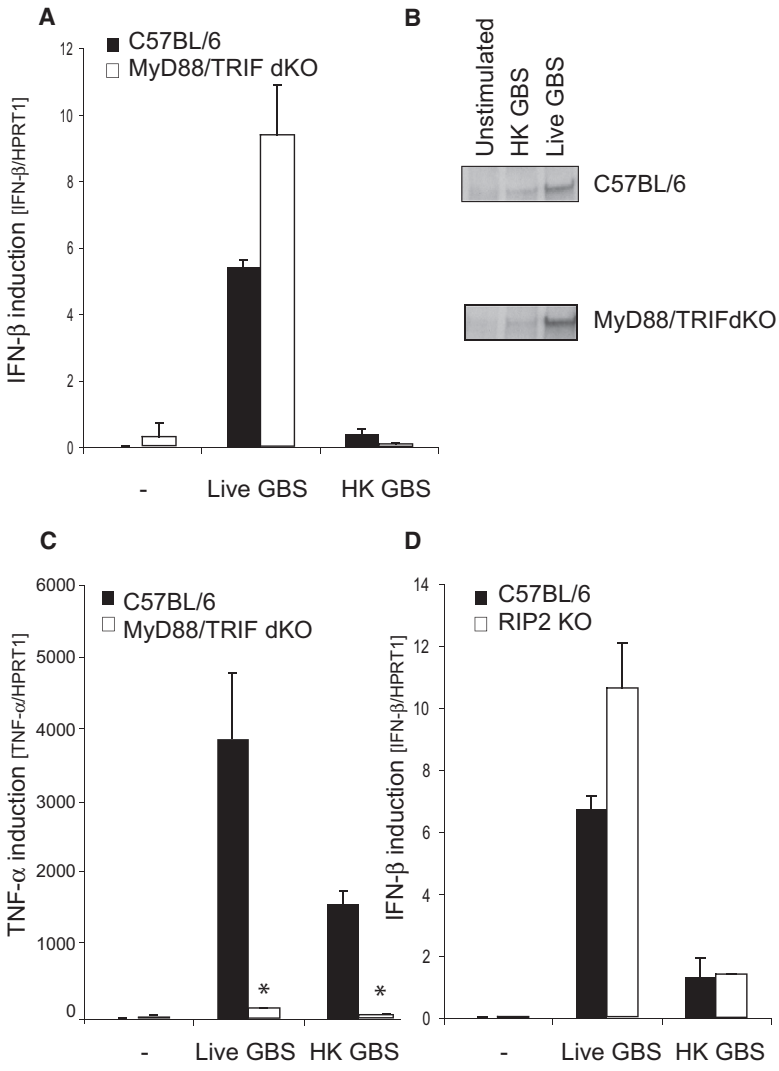


Figure 2. Live and Heat-Killed GBS Bacteria Activate Distinct Pathways Leading to Type I IFNs Production

(A–C) WT and MyD88/TRIF-deficient BMDM were stimulated as in Figure 1 for 6 hr and the amount of *IFN-β* (A) and *TNF-α* (C) mRNA determined by Q-PCR and were normalized to *HPRT1* level of expression. (B) Phosphorylation of the transcription factor STAT2 in WT or MyD88/TRIF-deficient BMDMs was compared after 2 hr infection with live GBS (MOI 10:1). *, $P < 0.05$, Student's t test.

(D) WT and RIP2-deficient BMDMs were stimulated as mentioned above and the amount of *IFN-β* mRNA determined.

MyD88, Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM) (Akira and Sato, 2003). For most of the TLRs, MyD88 is the only critical adaptor; however, TLR3 signaling relies on the TRIF adaptor exclusively (Yamamoto et al., 2003). Furthermore, where more than one adaptor is involved, Mal/TIRAP and TRAM function as bridging adapters upstream of MyD88 and TRIF, respectively (Kagan and Medzhitov, 2006; Rowe et al., 2006). Hence, MyD88 and TRIF double-knockout mice are thought to be unable to signal via TLRs. To analyze the contributions of TLRs to the induction of the type I IFN response to GBS, we used MyD88/TRIF double-knockout macrophages. The heat-killed form of GBS required MyD88 and/or TRIF for induction of type I IFN (Figure 2A). In striking contrast, TLRs were not required for induction of the type I IFN response during live GBS infection. Indeed, upon stimulation with live GBS, the induction of the IFN response was higher in macrophages lacking both MyD88 and TRIF compared to WT cells (Figure 2A). This increase in *IFN-β* induction in the MyD88/TRIF double-knockout cells suggests a role for a negative regulator of the type I IFN response downstream of MyD88 or TRIF. Phosphorylation of STAT2, a necessary step in the formation of the ISGF-3 complex downstream of the IFN-α/-β receptor, also followed a similar profile in MyD88/TRIF double-deficient macrophages (Figure 2B). In contrast, the induction of proinflammatory cytokines such as tumor necrosis factor α (TNF-α) was entirely dependent on MyD88/TRIF in studies using both killed and live bacteria (Figure 2C).

The NOD-like receptors (NLRs) are critical sensors of a variety of bacterial and nonbacterial mediators of inflammation, consisting of the NOD proteins and a large group of NALP proteins that regulate the proteolytic cleavage of IL1β (Kanneganti et al., 2007). NOD1 and NOD2 are well-established receptors for bacterial peptidoglycan (Chamaillard et al., 2003; Girardin et al., 2003; Inohara et al., 2005). NOD receptors can participate in the recognition of heat-killed bacteria leading to the induction of type I IFN (Herskovits et al., 2007). However, in receptor-interacting protein 2 (RIP2) knockout macrophages, which lack NOD1 and NOD2 signaling, both heat-killed and live GBS activated a potent *IFN-β* response (Figure 2D). We conclude from these experiments that GBS has the capacity to induce type I IFNs via a TLR-dependent and a TLR/NOD-independent manner.

Uptake and Degradation of Live GBS Are Required for the Induction of the Type I IFN Response

All previously described bacteria activating the type I IFN pathway independently of TLRs are found in the cytosol (e.g., *Listeria monocytogenes*) or require a secretion system (e.g., *Legionella pneumophila*) (Opitz et al., 2006; Stetson and Medzhitov, 2006). Indeed, all the receptors identified as critical in the recognition of intracellular pathogens have been localized in the cytoplasm. GBS, an extracellular organism, is known to invade some specific cell types, including human brain microvascular endothelial cells and respiratory epithelial cells (Baron et al., 2004; Gibson et al., 1993, 1995; Rubens et al., 1992; Shin et al., 2006). Moreover, additional studies have documented the ability of GBS to enter macrophages by phagocytosis and to survive inside the phagocytic vacuole for up to 48 hr (Cornacchione et al., 1998; Valenti-Weigand et al., 1996). Nevertheless, the cytosolic invasion of macrophages by GBS has not been described

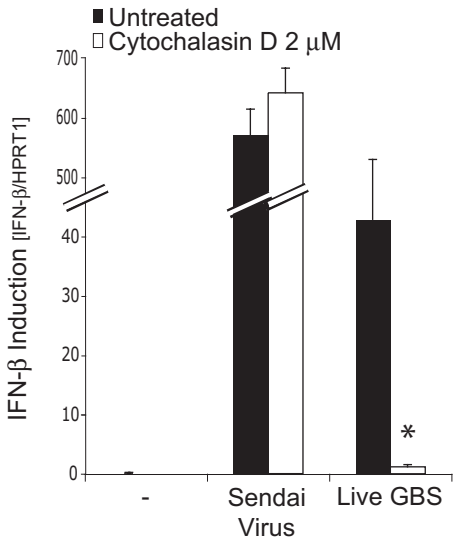


Figure 3. Phagocytosis of Live GBS Is Required for the Release of the IFN-Inducing Material

WT BMDMs were pretreated for 30 min with cytochalasin D (2 μM) before being infected with live GBS (MOI 6:1) for 6 hr or Sendai virus (150 HAU/ml) for 4 hr and *IFN-β* mRNA measured as in Figure 1. *, $P < 0.05$, Student's t test.

previously, and no GBS secretion system that allows the organism to release material into the host cytosol has been identified.

We investigated whether live GBS activated a membrane-bound receptor, or whether bacterial uptake was required for activation of the type I IFN pathway. Pretreatment of WT macrophages with cytochalasin D (Figure 3), an inhibitor of actin polymerization, inhibited activation of the pathway leading to *IFN-β* production. An important observation was that cytochalasin D had no effect on IFN induction by Sendai virus, which signals via the RIG-I pathway. These results suggested that the uptake of GBS is required for the binding of GBS-derived molecules to an intracellular receptor. Electron and confocal microscopy allowed us to visualize the entry of GBS through the phagocytic pathway (Figure 4). The great majority of the GBS cells appeared to be surrounded by a membrane (Figure 4A) colocalizing with the phagosomal marker Lamp1 (Figure 4D, top). Nevertheless, a small percentage of bacteria (less than 1%) were found consistently without a surrounding membrane (Figures 4B and 4C) and were not colocalized with Lamp1 (Figure 4D). The intracellular localization of these bacterial cells was seen by staining the mammalian membrane with fluorescent cholera toxin b.

Puzzled by this finding, we wondered whether known pore-forming toxins of GBS, namely hemolysin and CAMP factor, were responsible for the escape of GBS or GBS cell fragments from the phagolysosome, as has been shown for lysteriolysin from *L. monocytogenes* (Berche et al., 1988). Thus, we investigated the ability of GBS knockouts in hemolysin, CAMP factor, or double-knockout strains to trigger the IFN responses. We found that hemolysin plays a critical role in the induction of type I IFN (Figure 4E). Similarly, the absence of the CAMP factor toxin was associated with a reduction in the type I IFN response (Figure 4E). This result suggested that hemolysin and CAMP factor form pores in the phagosomal membrane that allow the escape of GBS or of GBS material into the host cytosol, leading to the upregulation of the type I IFN. These two toxins did not appear to play a role in the upregulation of the proinflammatory response, since the production of *TNF-α* was unaffected by GBS lacking hemolysin and/or CAMP factor with WT GBS (Figure S2).

Pretreatment of cells with bafilomycin A1 (Figure 5A), a potent and specific inhibitor of the vacuolar H^+ ATPase (V-ATPase), or with chymostatin (Figure 5B), a strong inhibitor of lysosomal serine and cysteine proteinases, abolished type I IFN induction by live GBS in WT macrophages. Neither bafilomycin A1 nor chymostatin pretreatment had any effect on Sendai virus responses. Bafilomycin A1 modifies the phagosomal pH and alters the formation of phagolysosomes, while chymostatin treatment results in the inhibition of chymotrypsin, chymases, and lysosomal enzymes such as cathepsin B, H, and L. The ability of both drugs to abolish IFN induction highlighted the essential role of bacterial degradation in phagolysosomes as a key event in the induction of the type I IFN response.

In conclusion, these experiments suggest that the type I IFN response observed during live GBS infection requires the phagocytosis of GBS followed by the alteration of the phagolysosomal membrane by pore-forming toxins leading to the release into the cytosol of bacterial degradation products. The identification of whole GBS outside of the phagolysosome is a physical manifestation of the loss of integrity of this compartment during infection.

Characterization of the Pathway Activated During Live GBS Infection

To date, the pathways that regulate the type I IFN response independently of TLRs employ the TBK1-IRF3 axis. We therefore examined the role of TBK1 and IRF3 in live GBS-induced IFN responses. Although TBK1 deficiency is embryonic lethal, this lethality is reversed when TBK1-deficient mice are crossed with TNFR1-deficient mice. We next compared TNFR1 and TBK1/TNFR1-deficient macrophages for GBS-induced IFN responses. TBK1/TNFR1-deficient macrophages had a strongly reduced IFN response upon challenge with GBS (Figure 6A). Macrophages deficient in the related kinase IKK-ε showed a weak but consistent decrease in *IFN-β* induction (Figure S3). This partial effect is probably due to the redundancy of IKK-ε and TBK1 kinases in the regulation of type I IFN production in macrophages. Consistent with a role for the IKK-related kinases in GBS-induced IFN responses, IRF3-deficient macrophages had a marked defect in *IFN-β* induction in response to GBS (Figure 6B, left panel). Likewise, phosphorylation of STAT2 was strongly reduced in IRF3-deficient macrophages compared with WT macrophages upon GBS infection (Figure 6B, right panel). Taken together, these data suggest that the pathway triggered by GBS infection involves TBK1 and IRF3.

Upstream of the TBK1/IRF3 axis, several classes of pattern recognition receptors (PRRs) have been identified in addition to the TLRs. These include the RLRs and a cytosolic sensor of DNA: DAI. RLRs such as RIG-I (Yoneyama et al., 2004) and melanoma differentiation-associated gene-5 (MDA5) (Kato et al., 2006), discriminate between different classes of RNA viruses, while DAI detects synthetic DNA delivered to the cytosol with

lipofectamine (Takaoka et al., 2007). All of these TLR-independent pathways converge at the IKK-related kinases, TBK1 and IKK-ε, which phosphorylate and activate IRF3 and IRF7. In the case of the RLRs, a mitochondrial localized adaptor molecule, MAVS (mitochondrial antiviral signaling, also called IPS-1, CARDif, or VISA), relays signals from the RLRs to TBK1. MAVS-deficient macrophages fail to produce IFN in response to ligands or viruses that signal via RIG-I and MDA5 (Kawai et al., 2005). MAVS-deficient macrophages responded normally to GBS but were severely compromised in their ability to respond to Sendai virus (Figure 6C). These observations suggest that live GBS is not recognized by either RIG-I or MDA-5.

To determine whether DAI is involved, we generated short hairpin RNA (shRNA) targeting constructs that blocked expression of *DAI* in macrophages and murine embryonic fibroblasts (MEF) (Figure 6D). In the absence of DAI expression, GBS triggered the IFN-β response normally. Taniguchi et al. have shown recently that overexpression of DAI can enhance dsDNA-induced IFN responses. Overexpression of DAI in immortalized murine macrophages or in MEFs did not result in increased production of type I IFN in response to GBS infection (Figure S4). Collectively, these data suggest that GBS-induced IFN does not involve either the RLRs or DAI.

Cytosolic DNA Is an IFN-Activating Ligand of GBS

Studies with *L. monocytogenes* have shown that delivery of bacterial DNA to the cytoplasm activates the type I IFN pathway (Stetson and Medzhitov, 2006). We performed similar experiments with GBS. Extracts from sonicated GBS were prepared and tested for their ability to trigger IFN responses. Consistent with our findings with live GBS, extracts of GBS transfected into cells induced IFN production via a pathway that was dependent on TBK1 and IRF3 but independent of TLRs (Figure 7A). Treatment of GBS extract with DNase I—but not RNase A—abolished this stimulatory activity, a result suggesting that DNA is likely to be the ligand responsible for triggering the IFN response (Figure 7B). RNase H is a ribonuclease that cleaves the 3′-O-P-bond of RNA in an RNA/DNA duplex to produce 3′-hydroxyl- and 5′-phosphate-terminated products, releasing newly accessible DNA molecules. RNase H treatment enhanced IFN response to GBS, further implicating GBS DNA as the ligand responsible for the observed IFN response. From these experiments, we can conclude that the only IFN-inducing ligand found in the intracellular content of GBS activating a cytoplasmic receptor is GBS DNA. Furthermore, we tagged GBS DNA using the cell-permeant Syto 60 red fluorescent nucleic acid stain in live GBS and followed the localization of GBS DNA upon phagocytosis and degradation of the bacteria by confocal microscopy. Using this approach, we observed GBS DNA outside of Lamp1-positive compartments in macrophages that underwent phagocytosis of bacteria (Figure 7Ca). In contrast, we could not observe GBS DNA outside of the Lamp1-positive compartments in macrophages infected with the hemolysin-deficient strain (Figure 7Cb), or within macrophages infected with WT GBS and pretreated with bafilomycin A1 (Figure 7Cc) or chymostatin (Figure 7Cd).

To exclude a role for TLR9 in GBS-DNA recognition, we also tested macrophages lacking TLR9 for their ability to trigger IFN responses. Consistent with our studies in MyD88/TRIF-deficient macrophages, the activation of the type I IFN pathway by GBS DNA did not require TLR9 (Figure S5A). Furthermore, chloroquine, used at concentrations that block TLR9 signaling, had no effect on the ability of live GBS to induce IFN-β (Figure S5B).

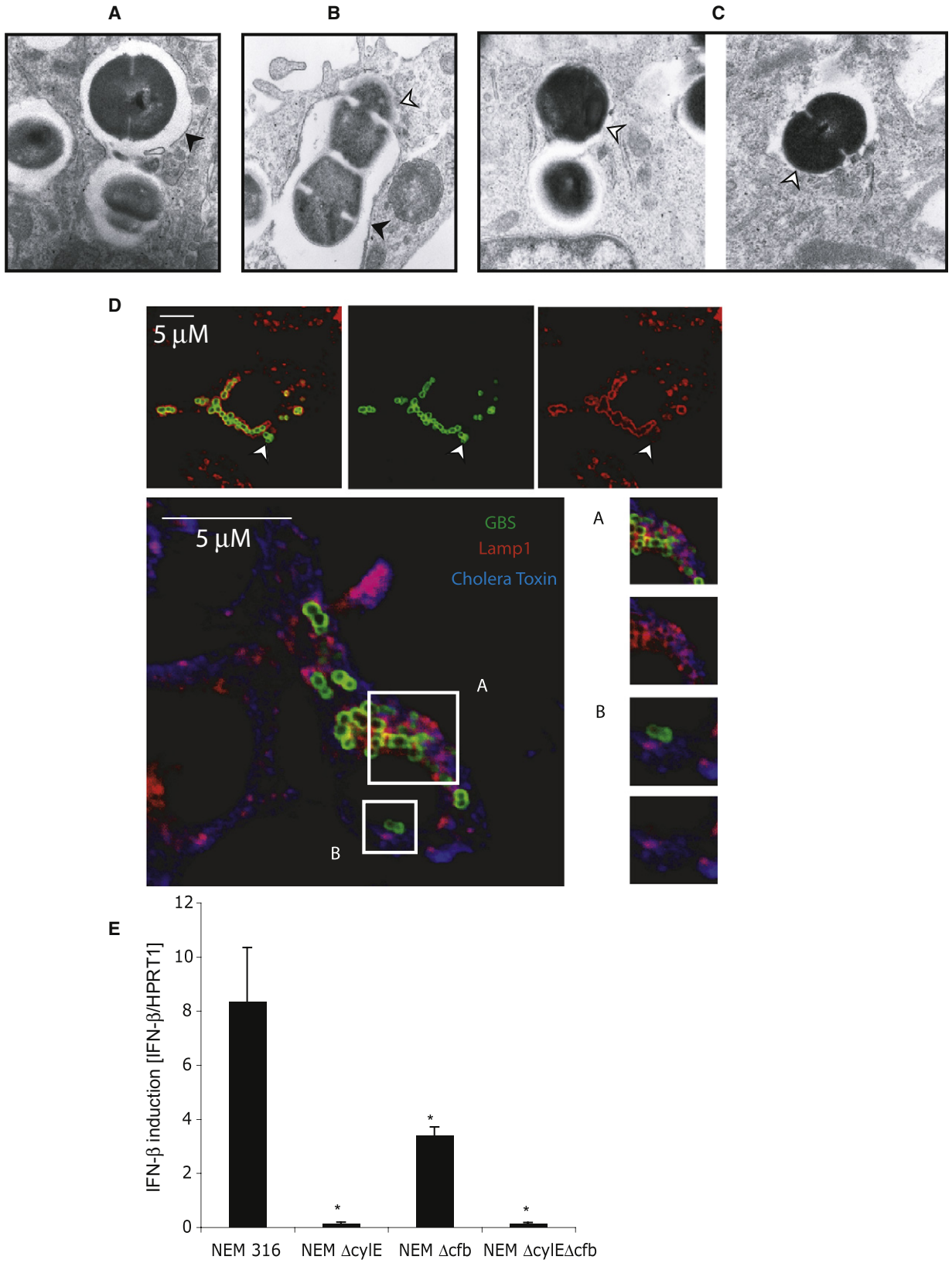
These data suggest that GBS induces IFN via TBK1/IRF3, and that triggering of this pathway in all likelihood involves a cytosolic DNA-sensing pathway that is not DAI.

DISCUSSION

The type I IFN response is critical in host defense against GBS (Mancuso et al., 2007). Mice unable to mount a type I IFN response are hypersusceptible to lethality induced by GBS, with a mortality rate surpassing that seen in the absence of IFN-γ signaling, a well-established mediator of host defenses against bacteria (Schroder et al., 2004). The inability of IFN-α/-βR-deficient mice to control GBS infection was partially accounted for by defective IFN-β signaling, however, a role for IFN-α and/or -ω subtypes is also a possibility.

Initially, TLR4 was thought to be the sole bacterial recognition receptor leading to type I IFN induction. Gram-positive bacteria were initially believed to be sensed entirely by TLR2 (Takeuchi et al., 1999; Yoshimura et al., 1999), which does not induce an IFN response. Several families of germline-encoded PRRs distributed in different subcellular compartments have since been identified, including the complement system in the extracellular environment, the TLRs and dectin-1 on the plasma membrane, TLRs in the endosomal compartment, and finally RLRs, NLRs, and DAI in the cytosol. In our study, we have examined the role of many of these sensors in regulating the IFN-β response to GBS. We have shown that, upon GBS infection, the induction of the type I IFN-β response proceeds via a pathway dependent on TBK1 and IRF3, a pathway activated by many pathogens. However, unlike many other pathogens, the GBS-induced type I IFN response appears to be independent of all currently known mediators of the IFN pathway. This TLR/NOD/RLR-independent pathway is, to the best of our knowledge, unique for a bacterium that does not have an intracellular lifestyle like *Listeria monocytogenes*, although further studies may establish that other bacteria are capable of activating this response through similar mechanisms. GBS is able to survive inside some types of macrophages for extended periods, but this has never been associated with enhanced virulence, an ability to replicate intracellularly, or escape from the phagosomal compartment. Nevertheless, our study demonstrates that live GBS can be found in the cytosol and provides evidence that various bacterial products, such as DNA, escape the phagolysosomal compartment. The translocation of whole bacteria and bacterial products appears to be due to the degradation of the phagosomal membrane by secreted GBS toxins, which form pores in the membrane, thereby enabling the release of microbial components outside of the phagosomal compartment.

The multitude of PRRs in the cytosol highlights how active this environment is in terms of immune surveillance, not only for viruses, but also for other pathogens. Our findings are consistent with the hypothesis that an as yet unidentified PRR exists in the cytosol, poised to respond to GBS infection. We believe that GBS DNA is responsible for triggering this IFN response, similar to that postulated for sensing of *Listeria monocytogenes* (Leber


A
B
C
D
5 μM
5 μM
GBS
Lamp1
Cholera Toxin
A
B
A
B
E
IFN-β induction [IFN-β/HPRT1]
12
10
8
6
4
2
0
NEM 316
NEM ΔcylE
NEM Δcfb
NEM ΔcylEΔcfb

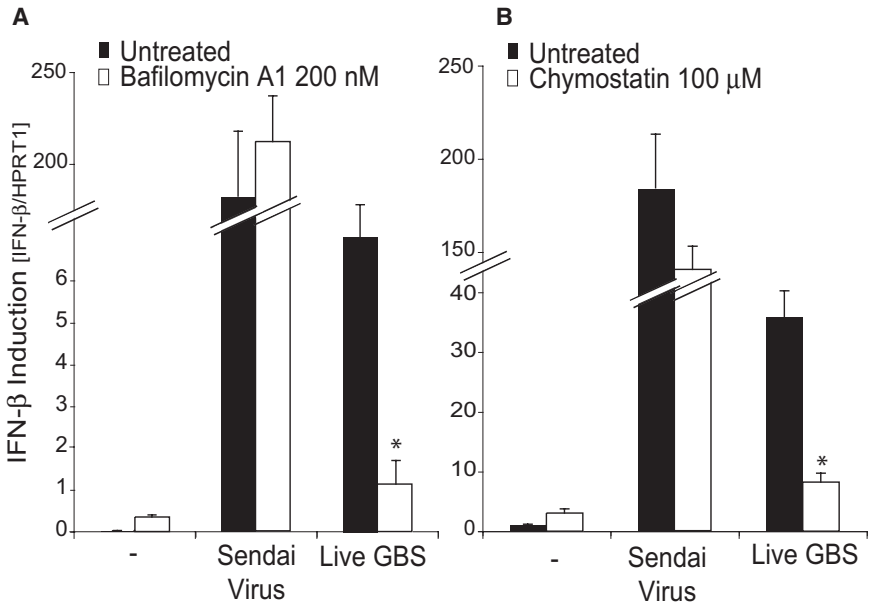


Figure 5. Degradation of the Bacteria Is Required for the Release of the IFN-Inducing Ligand

(A and B) WT BMDMs were pretreated for 30 min with bafilomycin A1 (200 nM) (A) or chymostatin (100 μM) (B) before being infected with live GBS (MOI 6:1) for 6 hr or Sendai virus (150 HAU/ml) for 4 hr. The amount of *IFN-β* mRNA was determined by Q-PCR. *, $P < 0.05$, Student's t test.

et al., 2008; Stetson and Medzhitov, 2006). Several PRRs have been identified as sensors for both microbial as well as self DNA. These include TLR9 in the endosome and DAI or NALP3 (Muruve et al., 2008) in the cytosol. Both TLR9 and DAI have been implicated in IFN responses to other pathogens; however, our studies clearly demonstrate that neither TLR9 nor DAI are responsible for IFN induction in response to GBS.

DNA from GBS found in the cytosol could originate as a result of bacteria being degraded in the phagolysosome. Since the sensor for *Listeria monocytogenes* also remains unknown at this point, it is possible that the same molecule(s) could sense both organisms. Should this be the case, it would be of interest to understand why the IFN response is protective in the case of GBS, yet detrimental in the case of Listeria, despite the similarities in the sensing and signaling pathways engaged by both organisms. One partial explanation might be the difference in the amplitude of the type I IFN response upon infection with extracellular or intracellular organisms. Indeed, recent work from Leber and colleagues demonstrated that in the case of Listeria infection, NOD2 signaling appears to cooperate with the cytosolic DNA-sensing pathway for optimal triggering of the cytosolic IFN responses (Leber et al., 2008). In our study, however, we found no evidence that NOD2 plays such a role, since macrophages lacking RIP2, the downstream transducer of the NOD2 pathway, were not compromised in their ability to trigger IFN production upon GBS infection. As a consequence, the amplitude of the GBS response was lower than that triggered by Listeria.

GBS is a life-threatening infection in newborns and a common cause of disease in pregnant women, the elderly, and the immunocompromised. Given the importance of the type I IFN response in protecting the host against GBS, it is clear that understanding the molecular mechanisms responsible for triggering the IFN-β as well as IFN-α and -ω responses has both clinical and therapeutic potential, particularly in terms of vaccine development.

EXPERIMENTAL PROCEDURES

Reagents

Unless otherwise stated, reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Statistical Analysis

All experiments were performed at least three times. One representative experiment is shown with each data point derived from a triplicate determinant. Error bars represent SD from a representative experiment. *, $p < 0.05$, Student's t test.

Bacterial Strains

Streptococcus agalactiae strain 515 (type Ia) and NEM 316 (type III) were obtained from the blood of a neonate with sepsis as described previously (Eads et al., 1982; Glaser et al., 2002). The acapsular form of strain 515 (515Δ*cspE*) was used all throughout these studies unless otherwise stated (Cieslewicz et al., 2001). In-frame deletion of *cfb* in NEM316 and NEM316Δ*cylE* were constructed by using splicing-by-overlap-extension PCR with primers O1 (GTATGGGAATTCGGCATAATGGAACGATTT), O2 (GGCACGCCCGGGTGCT GCCGCTGCACCAGCCACTAGAGTTCC), O3 (GCGGCAGCACCCGGGCGTG CCCAAGCAGCACTTCAAACAGCA), and O4 (ACTTCTGGATCCCCTCTCCAC GCTTCTGT). To carry out chromosomal gene inactivation, PCR fragments

Figure 4. A Small Proportion of Live GBS Bacteria Escapes the Phagosome and Reaches the Cytosol upon Phagosomal Membranes Degradation

(A–C) Electron microscopy of WT BMDMs infected for 2 hr with GBS. Most GBS organisms were found in the phagosome (A), but a few were observed escaping the phagosome (B), and some were ultimately identified in the cytosol (C). The black arrow shows the phagosomal membrane, whereas the white arrow highlights the absence of phagosomal membranes. The magnification is 39,000×.

(D) Confocal microscopy of WT BMDMs infected with live GBS (MOI 10:1) for 2 hr. The phagosomal membrane is stained with Lamp1 (red) and the surface of the bacteria with an anti-Group B antigen antibody (green). The white arrow shows bacteria located outside the Lamp1-stained membrane. In the lower panel, the plasma and phagosomal membranes were also stained with cholera toxin b (blue). Phagocytosed bacteria were found in the phagosomal compartment (A) and outside of this compartment but within the confines of the plasma membrane (B).

(E) WT C57BL/6 BMDMs were stimulated for 6 hr with live GBS: NEM 316 (MOI 7:1), NEM Δ*cylE* (MOI 7:1), NEM Δ*cfb* (MOI 6:1), and NEM Δ*cylE*Δ*cfb* (MOI 7:1). Levels of mRNA for *IFN-β* were determined as mentioned in Figure 1. *, $P < 0.05$, Student's t test.

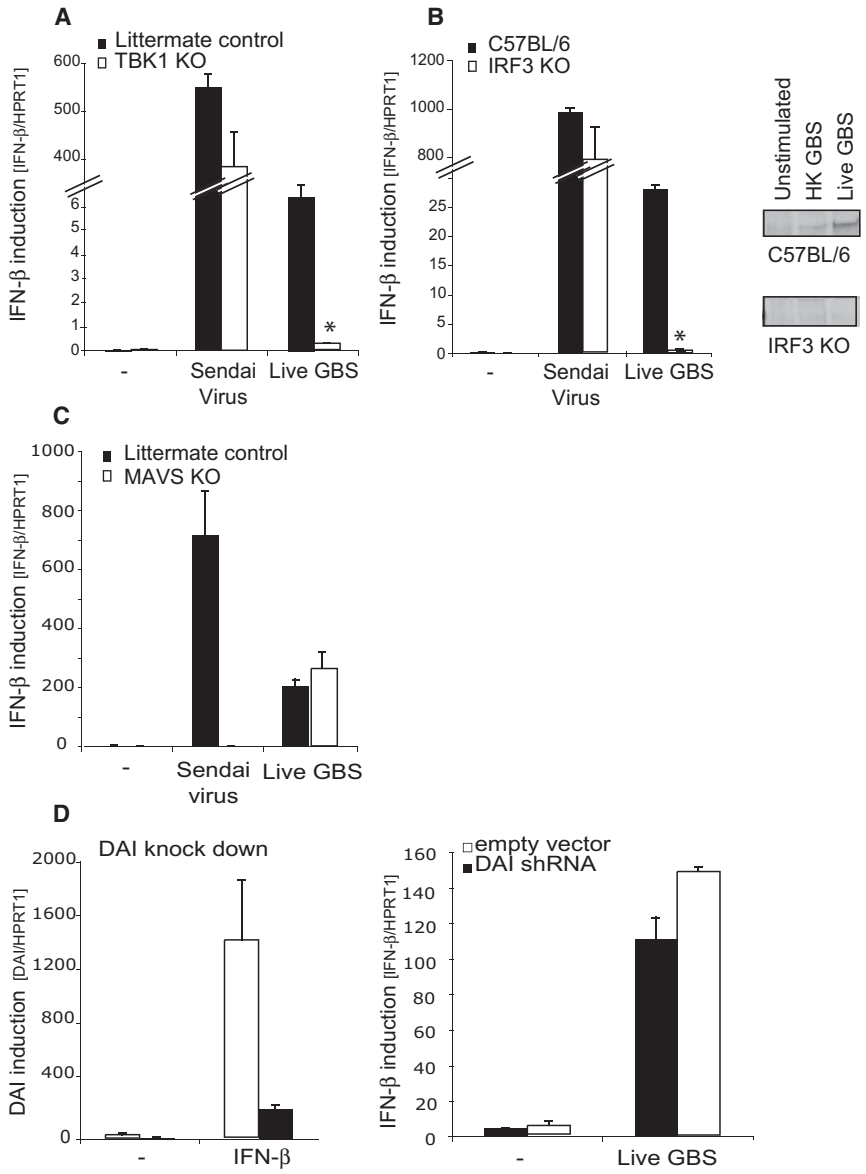


Figure 6. TBK1 and IRF3 Are Critical Components of the Pathway Activated by Live GBS

(A and B) TNFR1$^{-/-}$- and TNFR1/TBK1- deficient BMDMs (A) and C57BL/6 and IRF3$^{-/-}$ BMDMs (B) were stimulated with live GBS (MOI 6:1) for 6 hr or Sendai virus (150 HAU/ml) for 4 hr, and *IFN-β* mRNA was measured as mentioned in Figure 1. In (B), phosphorylation of the transcription factor STAT2 was examined in WT and IRF3-deficient macrophages infected with live GBS.

(C and D) In (C), C57BL/6 and MAVS-deficient BMDMs were infected with Sendai virus or GBS as mentioned above, and IFN-β was measured by Q-PCR. In (D), C57BL/6 BMDMs were infected with a retroviral vector encoding shRNA for *DAI* and *DAI* levels measured by Q-PCR before and after IFN-β treatment (250 U/ml) (left). On the right, cells infected with *DAI* shRNA under the same conditions were exposed to PBS or live GBS (MOI 1:6) for 6 hr, and *IFN-β* levels were measured by Q-PCR. *, $P < 0.05$, Student's t test.

(O1-O2::O3-O4) were cloned into the thermosensitive shuttle plasmid pG+host1, and chromosomal gene exchanges were carried out as described previously (Dramsi et al., 2006). All strains were grown on 5% sheep blood agar plates overnight at 37°C (Remel; Lenexa, KS). Bacterial colonies were removed from the plates after overnight culture, used to inoculate Todd Hewitt Broth (THB) prepared from endotoxin-free water, and grown overnight without agitation at 37°C. The next morning, the culture was diluted in THB and grown to mid-logarithmic phase (OD_{650} = 0.5). After two washes in PBS, GBS was used to stimulate macrophages. Heat-killed GBS was prepared from 515Δ*cspE* strain, which was grown to an OD_{600} of 0.5; the bacteria were spun down, resuspended in PBS, and heated at 80°C for 1 hr. The bacteria were then lyophilized and weighed.

GBS DNA Staining

Bacteria were prepared as described above, grown to mid-logarithmic phase (OD_{650} = 0.5), and stained for 30 min at room temperature with Syto 60 red fluorescent nucleic acid stain (15 μM in PBS) (Invitrogen; Carlsbad, CA). Macrophages seeded at a confluency of 50%–70% were fed Syto60 incorporated bacteria at a ratio of 20:1. Two hours after infection, the macrophages were washed extensively and then fixed in 4% paraformadehyde.

Mice

C57BL/6 mice (5- to 6-weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). MyD88$^{-/-}$, TRIF$^{-/-}$, and TLR9$^{-/-}$ mice were provided by S. Akira (Osaka University; Osaka, Japan) and backcrossed onto a C57BL/6 background for at least 12 generations. MyD88/TRIF$^{-/-}$ double-knockout mice were bred from MyD88$^{-/-}$ and TRIF$^{-/-}$ mice. IRF3$^{-/-}$ mice were a gift from T. Taniguchi (University of Tokyo; Tokyo, Japan). IFN-α/-βR$^{-/-}$ mice on a C57BL/6 background were a gift from J. Sprent (Scripps Research Institute; San Diego, CA). IKK-ε$^{-/-}$ mice were generated at Millennium Pharmaceutical (Cambridge, MA). TBK1$^{-/-}$ mice were a gift from W.C. Yeh (University of Toronto; Toronto, ON, Canada) and were bred with TNFR1$^{-/-}$ mice at the University of Massachusetts Medical School. RIP2$^{-/-}$ mice were from V. Dixit (Genentech; San Fransisco, CA). MAVS$^{-/-}$ mice were a kind gift of Z.J. Chen (UT Southwestern Medical School; Dallas, TX). All experiments were conducted with Institutional Animal Care and Use Committee approval.

Cell Culture

BMDMs were generated as described previously (Fitzgerald et al., 2003), and their differentiation state was confirmed by staining for F4/80 (Caltag; Burlingame, CA) and CD11b (PharMingen; Mississauga, ON, Canada). All cells were cultured at 37°C in 5% CO_2 in air in DMEM (CellGro; Herndon, VA) containing L-glutamine (CellGro; Herndon, VA), supplemented with 10% (vol/vol) FBS (HyClone Laboratories; Logan, UT) and ciprofloxacin (10 μg/ml) (Bayer; West Haven, CT). PBS was obtained from CellGro. When stimulated with live GBS, the macrophages were maintained in antibiotic-free DMEM for the first hour of infection. Ciprofloxacin was later added to the macrophages infected with GBS.

ELISA

Cell culture supernatants were assayed for IFN-β by ELISA (PBL IFN source; Piscataway, NJ) according to the manufacturer's instructions.

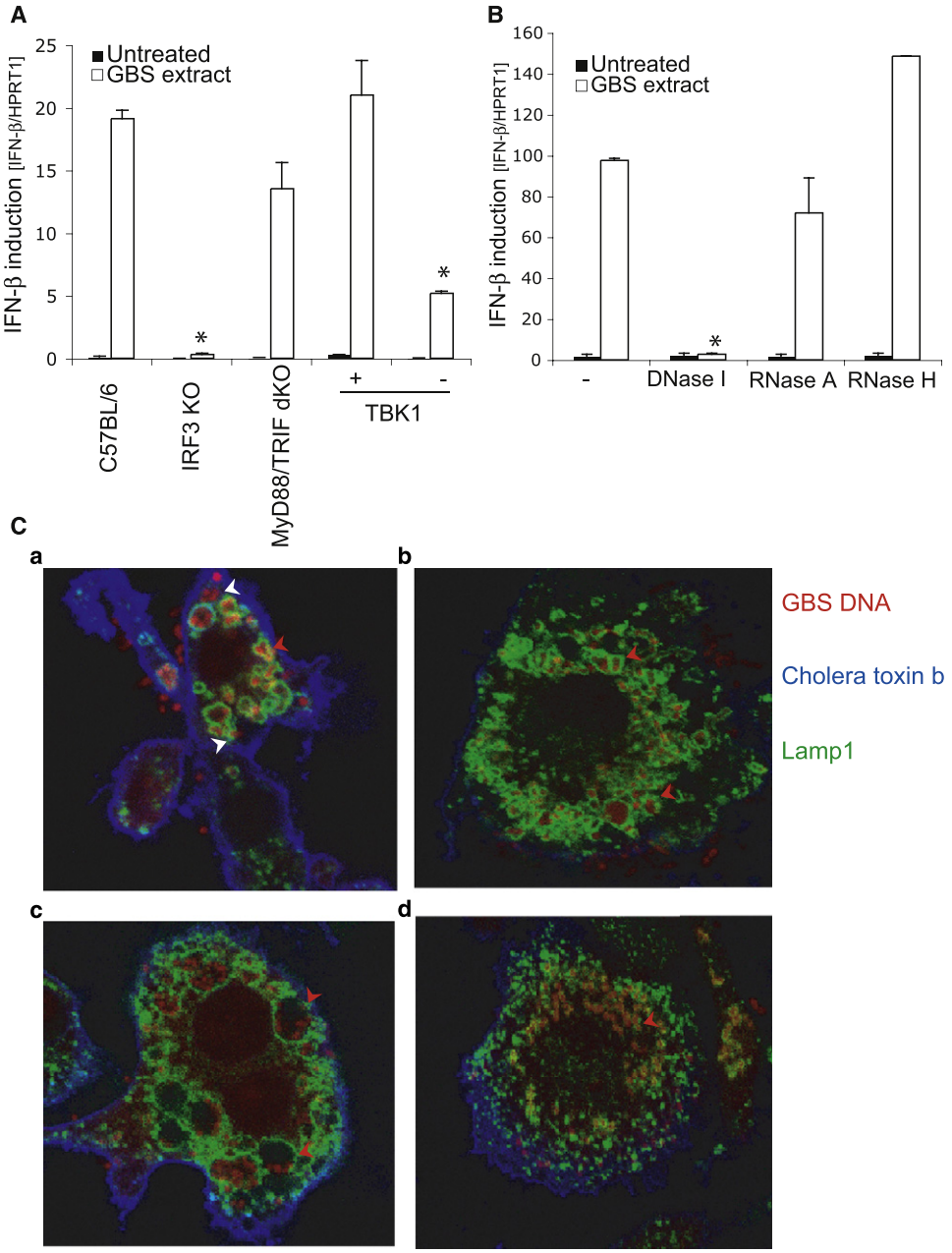


Figure 7. Induction of Type I IFNs by GBS DNA Has the Same Pathway Requirements as Induction by Live GBS

(A) GBS extract was transfected with lipofectamine into WT, IRF3-deficient, or TBK1-deficient BMDMs. *, $P < 0.05$, Student's t test.

(B) GBS extract was pretreated with vehicle, DNase I, RNase A, or RNase H and transfected into the cytosol of WT BMDMs, and *IFN-β* measured by Q-PCR. *, $P < 0.05$, Student's t test.

(C) Confocal microscopy of WT macrophages fed DNA-tagged live GBS (MOI 20:1) for 2 hr. GBS DNA was stained with syto60 dye (red), the phagosomal membrane with Lamp1 (green), and the plasma membrane with Cholera toxin b (blue). Untreated macrophages were fed WT (a) or hemolysin-deficient (b) NEM strain. Macrophages were treated with bafilomycin A1 (200 nM) (c) or chymostatin (100 μM) (d). The red arrows show GBS DNA located in a Lamp1-stained compartment, whereas white arrows highlight GBS DNA located outside of this compartment.

Quantitative Real-Time PCR

RNA from murine BMDMs was extracted with an RNeasy kit (QIAGEN, Inc.; Valencia, CA) according to the manufacturer's instructions. cDNA was synthesized with the iScript cDNA Synthesis Kit (Biorad; Hercules, CA), and quantitative RT-PCR analysis was performed on a DNA Engine Opticon 2 cycler (MJ Research; Watertown, MA) with the iQ SYBR Green Supermix (Biorad; Hercules, CA). The specificity of amplification was assessed for each sample by melting curve analysis. Relative quantification was performed by mean of standard curve analysis. The murine quantifications data are presented as a ratio to the hypoxanthine phosphoribosyltransferase 1 (HPRT1) level. The standard errors (95% confidence limits) were calculated with Student's t test. All gene expression data are presented as a ratio of gene copy number per 100 copies of *HPRT1* (as indicated) ± SD. The following primers for detection of *IFN-β*, *TNF-α*, *viperin*, and *HPRT1* mRNAs were designed: vig1, 5-AACCCCCGTGAG

TGTCAACTA (forward) and 5-AACCAGCCTGTTTGAGCAGAA (reverse); HPRT1, 5-CTGGTGAAAAGGACCTCTCG (forward) and 5-TGAAGTACTCATT ATAGTCAAGGGCA (reverse); TNF-α, 5-CAGTTCTATGGCCCAGACCCT (forward) and 5-CGGACTCCGCAAAGTCTAAG (reverse); IFN-β, 5-ATAAGCAGC TCCAGCTCCAA (forward) and 5-CTGTCTGCTGGTGGAGTTCA (reverse). The primers for detection of mouse *DAI* mRNA has been described elsewhere (Takaoka et al., 2007).

Cell Extracts and Western Blotting

The nuclear cell extraction method has been described elsewhere (Severa et al., 2006). The nuclear extracts were stored at −20°C before being separated by 7.5% SDS-PAGE gel (NuStep; French Forests, Australia) and blotted onto nitrocellulose membrane (Amersham Bioscience; Bucks, UK). Blots were incubated with rabbit polyclonal antibody anti-phosphorylated STAT2 (Millipore; Lake Placid, NY), followed by a secondary Ab conjugated with horseradish peroxidase. The blots were developed with an ECL system (Amersham Biosciences; Little Chalfont, Bucks, UK).

Cell Infections and Treatments

Macrophages were infected with GBS at a multiplicity of infection (MOI) as stated. Sendai virus (150 HAU/ml), Cantrell strain was from Charles River Laboratories (Boston, MA). GBS extract (80 μg/ml) was prepared as previously described (Stetson and Medzhitov, 2006). The treatments with DNase I (QIAGEN; Valencia, CA), RNase A (Ambion; Austin, TX), or RNase H (Invitrogen; Carlsbad, CA) were performed according to the manufacturers instructions. GBS DNA was delivered to the cell with Lipofectamine 2000 (Invitrogen; Carlsbad, CA) at a ratio of 3 μl Lipofectamine to 1 μg DNA in OptiMEM (Invitrogen; Carlsbad, CA).

Electron Microscopy

Macrophages were infected with GBS at a MOI of 10:1 and incubated for 2 hr. The cells were then washed extensively in PBS before being fixed in a solution of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Cells were then embedded and sectioned for transmission electron microscopy.

Confocal Microscopy

Confocal reflection microscopy was combined with fluorescence microscopy on a Leica SP2 AOBS confocal laser scanning microscope. Reflection was captured by placing the detector channel directly over the wavelength of the selected laser channel for reflection light capture, and the AOBS was set to allow 5%–15% of laser light into the collection channel. Fluorescence was simultaneously captured by standard confocal imaging techniques.

Overexpression and Knock Down of DAI Receptor

To generate the DAI retroviral expression vector, DAI cDNA was excised from pCAGGS-HA-*DAI* (a kind gift from T. Taniguchi [University of Tokyo; Tokyo, Japan]) and cloned into the SalI and NotI sites of GFP expression retrovirus vector, based on the CLNCX vector (Reeves et al., 1996). The retrovirus encoding for *DAI* as well as the empty vector were packaged in 293T cells and added to the target cells 48 hr prior to stimulation, following a previously described protocol (http://www.broad.mit.edu/genome_bio/trc/publicProtocols.html). The quality of the infection was assessed using the GFP tag.

The shRNA vectors were purchased from MISSION shRNA clones (Sigma Aldrich; St. Louis, MO) and used to generate lentiviral transduction particles in packaging cell lines, following protocols obtained from the RNAi consortium (http://www.broad.mit.edu/genome_bio/trc/rnai.html). IFN-β was from Biogen, Inc. (Cambridge, MA) and was used to induce upregulation of the *DAI* gene to check for the quality of the shRNA.

SUPPLEMENTAL DATA

Supplemental Data include five figures and can be found online at http://www.cell.com/cellhostandmicrobe/supplemental/S1931-3128(08)00369-7.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants 2AI052455 (to D.T.G. and D.L.K.) and AI067497 (to K.A.F.). We thank Martina Severa, Nadege Goutagny, and Veit Hornung for helpful discussion and advice. We thank Elise Caliot and Sophie Brinster for their help with the construction of the *cfb* mutants.

Received: March 10, 2008
Revised: June 26, 2008
Accepted: November 13, 2008
Published: December 10, 2008

REFERENCES

Akira, S., and Sato, S. (2003). Toll-like receptors and their signaling mechanisms. Scand. J. Infect. Dis. *35*, 555–562.

Baron, M.J., Bolduc, G.R., Goldberg, M.B., Auperin, T.C., and Madoff, L.C. (2004). Alpha C protein of group B Streptococcus binds host cell surface glycosaminoglycan and enters cells by an actin-dependent mechanism. J. Biol. Chem. *279*, 24714–24723.

Berche, P., Gaillard, J.L., and Richard, S. (1988). Invasiveness and intracellular growth of Listeria monocytogenes. Infection *16* (*Suppl 2*), S145–S148.

Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Qiu, S., Saab, L., Ogura, Y., Kawasaki, A., Fukase, K., Kusumoto, S., et al. (2003). An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat. Immunol. *4*, 702–707.

Cieslewicz, M.J., Kasper, D.L., Wang, Y., and Wessels, M.R. (2001). Functional analysis in type Ia group B streptococcus of a cluster of genes involved in extracellular polysaccharide production by diverse species of streptococci. J. Biol. Chem. *276*, 139–146.

Cornacchione, P., Scaringi, L., Fettucciari, K., Rosati, E., Sabatini, R., Orefici, G., von Hunolstein, C., Modesti, A., Modica, A., Minelli, F., and Marconi, P. (1998). Group B streptococci persist inside macrophages. Immunology *93*, 86–95.

Cusumano, V., Midiri, A., Cusumano, V.V., Bellantoni, A., De Sossi, G., Teti, G., Beninati, C., and Mancuso, G. (2004). Interleukin-18 is an essential element in host resistance to experimental group B streptococcal disease in neonates. Infect. Immun. *72*, 295–300.

Dramsi, S., Caliot, E., Bonne, I., Guadagnini, S., Prevost, M.C., Kojadinovic, M., Lalioui, L., Poyart, C., and Trieu-Cuot, P. (2006). Assembly and role of pili in group B streptococci. Mol. Microbiol. *60*, 1401–1413.

Eads, M.E., Levy, N.J., Kasper, D.L., Baker, C.J., and Nicholson-Weller, A. (1982). Antibody-independent activation of C1 by type Ia group B streptococci. J. Infect. Dis. *146*, 665–672.

Fitzgerald, K.A., Rowe, D.C., Barnes, B.J., Caffrey, D.R., Visintin, A., Latz, E., Monks, B., Pitha, P.M., and Golenbock, D.T. (2003). LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J. Exp. Med. *198*, 1043–1055.

Gibson, R.L., Lee, M.K., Soderland, C., Chi, E.Y., and Rubens, C.E. (1993). Group B streptococci invade endothelial cells: type III capsular polysaccharide attenuates invasion. Infect. Immun. *61*, 478–485.

Gibson, R.L., Soderland, C., Henderson, W.R., Jr., Chi, E.Y., and Rubens, C.E. (1995). Group B streptococci (GBS) injure lung endothelium in vitro: GBS invasion and GBS-induced eicosanoid production is greater with microvascular than with pulmonary artery cells. Infect. Immun. *63*, 271–279.

Girardin, S.E., Boneca, I.G., Carneiro, L.A., Antignac, A., Jehanno, M., Viala, J., Tedin, K., Taha, M.K., Labigne, A., Zahringer, U., et al. (2003). Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science *300*, 1584–1587.

Glaser, P., Rusniok, C., Buchrieser, C., Chevalier, F., Frangeul, L., Msadek, T., Zouine, M., Couve, E., Lalioui, L., Poyart, C., et al. (2002). Genome sequence of Streptococcus agalactiae, a pathogen causing invasive neonatal disease. Mol. Microbiol. *45*, 1499–1513.

Hein, J., Kempf, V.A., Diebold, J., Bucheler, N., Preger, S., Horak, I., Sing, A., Kramer, U., and Autenrieth, I.B. (2000). Interferon consensus sequence binding protein confers resistance against Yersinia enterocolitica. Infect. Immun. *68*, 1408–1417.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000). A Toll-like receptor recognizes bacterial DNA. Nature *408*, 740–745.

Henneke, P., Takeuchi, O., van Strijp, J.A., Guttormsen, H.K., Smith, J.A., Schromm, A.B., Espevik, T.A., Akira, S., Nizet, V., Kasper, D.L., and Golenbock, D.T. (2001). Novel engagement of CD14 and multiple toll-like receptors by group B streptococci. J. Immunol. *167*, 7069–7076.

Henry, T., Brotcke, A., Weiss, D.S., Thompson, L.J., and Monack, D.M. (2007). Type I interferon signaling is required for activation of the inflammasome during Francisella infection. J. Exp. Med. *204*, 987–994.

Herskovits, A.A., Auerbuch, V., and Portnoy, D.A. (2007). Bacterial ligands generated in a phagosome are targets of the cytosolic innate immune system. PLoS Pathog. *3*, e51.

Hess, C.B., Niesel, D.W., Cho, Y.J., and Klimpel, G.R. (1987). Bacterial invasion of fibroblasts induces interferon production. J. Immunol. *138*, 3949–3953.

Hess, C.B., Niesel, D.W., Holmgren, J., Jonson, G., and Klimpel, G.R. (1990). Interferon production by Shigella flexneri-infected fibroblasts depends upon intracellular bacterial metabolism. Infect. Immun. *58*, 399–405.

Inohara, Chamaillard, McDonald, C., and Nunez, G. (2005). NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. Annu. Rev. Biochem. *74*, 355–383.

Kagan, J.C., and Medzhitov, R. (2006). Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. Cell *125*, 943–955.

Kanneganti, T.D., Lamkanfi, M., and Nunez, G. (2007). Intracellular NOD-like receptors in host defense and disease. Immunity *27*, 549–559.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K.J., et al. (2006). Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature *441*, 101–105.

Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K.J., Takeuchi, O., and Akira, S. (2005). IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat. Immunol. *6*, 981–988.

Koga, R., Hamano, S., Kuwata, H., Atarashi, K., Ogawa, M., Hisaeda, H., Yamamoto, M., Akira, S., Himeno, K., Matsumoto, M., and Takeda, K. (2006). TLR-dependent induction of IFN-beta mediates host defense against Trypanosoma cruzi. J. Immunol. *177*, 7059–7066.

Leber, J.H., Crimmins, G.T., Raghavan, S., Meyer-Morse, N.P., Cox, J.S., and Portnoy, D.A. (2008). Distinct TLR- and NLR-mediated transcriptional responses to an intracellular pathogen. PLoS Pathog. *4*, e6.

Mancuso, G., Midiri, A., Beninati, C., Biondo, C., Galbo, R., Akira, S., Henneke, P., Golenbock, D., and Teti, G. (2004). Dual role of TLR2 and myeloid differentiation factor 88 in a mouse model of invasive group B streptococcal disease. J. Immunol. *172*, 6324–6329.

Mancuso, G., Midiri, A., Biondo, C., Beninati, C., Zummo, S., Galbo, R., Tomasello, F., Gambuzza, M., Macri, G., Ruggeri, A., et al. (2007). Type I IFN signaling is crucial for host resistance against different species of pathogenic bacteria. J. Immunol. *178*, 3126–3133.

Mizel, S.B., Honko, A.N., Moors, M.A., Smith, P.S., and West, A.P. (2003). Induction of macrophage nitric oxide production by Gram-negative flagellin involves signaling via heteromeric Toll-like receptor 5/Toll-like receptor 4 complexes. J. Immunol. *170*, 6217–6223.

Muruve, D.A., Petrilli, V., Zaiss, A.K., White, L.R., Clark, S.A., Ross, P.J., Parks, R.J., and Tschopp, J. (2008). The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. Nature *452*, 103–107.

O'Connell, R.M., Saha, S.K., Vaidya, S.A., Bruhn, K.W., Miranda, G.A., Zarnegar, B., Perry, A.K., Nguyen, B.O., Lane, T.F., Taniguchi, T., et al. (2004). Type I interferon production enhances susceptibility to Listeria monocytogenes infection. J. Exp. Med. *200*, 437–445.

O'Connell, R.M., Vaidya, S.A., Perry, A.K., Saha, S.K., Dempsey, P.W., and Cheng, G. (2005). Immune activation of type I IFNs by Listeria monocytogenes occurs independently of TLR4, TLR2, and receptor interacting protein 2 but involves TNFR-associated NF kappa B kinase-binding kinase 1. J. Immunol. *174*, 1602–1607.

O'Riordan, M., Yi, C.H., Gonzales, R., Lee, K.D., and Portnoy, D.A. (2002). Innate recognition of bacteria by a macrophage cytosolic surveillance pathway. Proc. Natl. Acad. Sci. USA *99*, 13861–13866.

Opitz, B., Vinzing, M., van Laak, V., Schmeck, B., Heine, G., Gunther, S., Preissner, R., Slevogt, H., N'Guessan, P.D., Eitel, J., et al. (2006). Legionella pneumophila induces IFNbeta in lung epithelial cells via IPS-1 and IRF3, which also control bacterial replication. J. Biol. Chem. *281*, 36173–36179.

Opitz, B., Rejaibi, A., Dauber, B., Eckhard, J., Vinzing, M., Schmeck, B., Hippenstiel, S., Suttorp, N., and Wolff, T. (2007). IFNbeta induction by influenza A virus is mediated by RIG-I which is regulated by the viral NS1 protein. Cell. Microbiol. *9*, 930–938.

Pitha, P.M. (2004). Unexpected similarities in cellular responses to bacterial and viral invasion. Proc. Natl. Acad. Sci. USA *101*, 695–696.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., et al. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science *282*, 2085–2088.

Reeves, M.E., Royal, R.E., Lam, J.S., Rosenberg, S.A., and Hwu, P. (1996). Retroviral transduction of human dendritic cells with a tumor-associated antigen gene. Cancer Res. *56*, 5672–5677.

Reimer, T., Schweizer, M., and Jungi, T.W. (2007). Type I IFN induction in response to Listeria monocytogenes in human macrophages: evidence for a differential activation of IFN regulatory factor 3 (IRF3). J. Immunol. *179*, 1166–1177.

Rowe, D.C., McGettrick, A.F., Latz, E., Monks, B.G., Gay, N.J., Yamamoto, M., Akira, S., O'Neill, L.A., Fitzgerald, K.A., and Golenbock, D.T. (2006). The myristoylation of TRIF-related adaptor molecule is essential for Toll-like receptor 4 signal transduction. Proc. Natl. Acad. Sci. USA *103*, 6299–6304.

Rubens, C.E., Smith, S., Hulse, M., Chi, E.Y., and van Belle, G. (1992). Respiratory epithelial cell invasion by group B streptococci. Infect. Immun. *60*, 5157–5163.

Schroder, K., Hertzog, P.J., Ravasi, T., and Hume, D.A. (2004). Interferon-gamma: an overview of signals, mechanisms and functions. J. Leukoc. Biol. *75*, 163–189.

Schuchat, A. (1999). Group B streptococcus. Lancet *353*, 51–56.

Severa, M., Coccia, E.M., and Fitzgerald, K.A. (2006). Toll-like receptor-dependent and -independent viperin gene expression and counter-regulation by PRDI-binding factor-1/BLIMP1. J. Biol. Chem. *281*, 26188–26195.

Shin, S., Paul-Satyaseela, M., Lee, J.S., Romer, L.H., and Kim, K.S. (2006). Focal adhesion kinase is involved in type III group B streptococcal invasion of human brain microvascular endothelial cells. Microb. Pathog. *41*, 168–173.

Simon, R., and Samuel, C.E. (2007). Innate interferon response in macrophage and epithelial cells infected with wild-type compared to DNA adenine methylase and flagellin mutant Salmonella enterica serovar Typhimurium. J. Interferon Cytokine Res. *27*, 317–327.

Sing, A., Merlin, T., Knopf, H.P., Nielsen, P.J., Loppnow, H., Galanos, C., and Freudenberg, M.A. (2000). Bacterial induction of beta interferon in mice is a function of the lipopolysaccharide component. Infect. Immun. *68*, 1600–1607.

Stanley, S.A., Johndrow, J.E., Manzanillo, P., and Cox, J.S. (2007). The Type I IFN response to infection with Mycobacterium tuberculosis requires ESX-1-mediated secretion and contributes to pathogenesis. J. Immunol. *178*, 3143–3152.

Stetson, D.B., and Medzhitov, R. (2006). Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. Immunity *24*, 93–103.

Stockinger, S., Reutterer, B., Schaljo, B., Schellack, C., Brunner, S., Materna, T., Yamamoto, M., Akira, S., Taniguchi, T., Murray, P.J., et al. (2004). IFN regulatory factor 3-dependent induction of type I IFNs by intracellular bacteria is mediated by a TLR- and Nod2-independent mechanism. J. Immunol. *173*, 7416–7425.

Takaoka, A., Wang, Z., Choi, M.K., Yanai, H., Negishi, H., Ban, T., Lu, Y., Miyagishi, M., Kodama, T., Honda, K., et al. (2007). DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature *448*, 501–505.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999). Differential roles of TLR2 and TLR4 in

recognition of gram-negative and gram-positive bacterial cell wall components. Immunity *11*, 443–451.

Vaena de Avalos, S., Blader, I.J., Fisher, M., Boothroyd, J.C., and Burleigh, B.A. (2002). Immediate/early response to Trypanosoma cruzi infection involves minimal modulation of host cell transcription. J. Biol. Chem. *277*, 639–644.

Valenti-Weigand, P., Benkel, P., Rohde, M., and Chhatwal, G.S. (1996). Entry and intracellular survival of group B streptococci in J774 macrophages. Infect. Immun. *64*, 2467–2473.

Yaegashi, Y., Nielsen, P., Sing, A., Galanos, C., and Freudenberg, M.A. (1995). Interferon beta, a cofactor in the interferon gamma production induced by gram-negative bacteria in mice. J. Exp. Med. *181*, 953–960.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., and Akira, S. (2003). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science *301*, 640–643.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., and Fujita, T. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat. Immunol. *5*, 730–737.

Yoshimura, A., Lien, E., Ingalls, R.R., Tuomanen, E., Dziarski, R., and Golenbock, D. (1999). Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J. Immunol. *163*, 1–5.