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Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells

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Little is known of how and where bacterial recognition triggers the induction of type I interferon. Whether the type of recognition receptor used in these responses is determined by the subcellular location of bacteria is not understood. Here we show that phagosomal bacteria such as group B streptococcus, but not cytosolic bacteria, potently induced interferon in conventional dendritic cells by a mechanism that required Toll-like receptor 7, the adaptor MyD88 and the transcription factor IRF1, all of which localized together with bacterial products in degradative vacuoles bearing lysosomal markers. Thus, this cell type–specific recognition pathway links lysosomal recognition of bacterial RNA with a robust, host-protective interferon response.

The type I interferon family comprises various members, including the single interferon-β subtype (IFN-β; A001237) and many IFN-α subtypes, that activate a common and ubiquitously expressed receptor. Although initially identified on the basis of their ability to induce an 'antiviral state' in host cells[1], type I interferons have since been shown to affect almost every aspect of immunity to nonviral pathogens as well as viral pathogens[2]. These cytokines are produced early during infection and can shape 'downstream' responses by positively and negatively regulating the expression of hundreds of genes involved in secondary host defense[3]. Together with few other primary mediators, type I interferon (IFN-α/β) is produced directly in response to the recognition of conserved microbial products (for example, viral nucleic acids or bacterial lipopolysaccharide) by receptors of the innate immune system[3,4]. Each receptor senses a distinct microbial product and, after being activated, recruits a specific set of adaptors. Those in turn ultimately govern the type of host response and its functional consequences by activating different transcription factors through signaling intermediates[4,5]. Thus, the type of receptor used for microbial recognition determines the nature of the immune response, which allows host defenses to be tailored for infectious processes of different etiology and pathogenesis.

Because distinct detection systems sample different extracellular and intracellular body compartments, receptor use in innate recognition is determined not only by a pathogen's component parts but also by its location in the host[5]. Notable examples of this principle are provided by the induction of interferon by viruses. Distinct types of receptors belonging to the RNA helicase and Toll-like receptor (TLR) families sense the presence of viral nucleic acids in cytosolic and endosomal intracellular compartments, respectively[4,5]. Once stimulated by their cognate ligands, each receptor type initiates a specific signaling cascade that leads to the activation of different members of the interferon-regulatory factor (IRF) family of transcription factors and to the induction of IFN-α/β[6]. Most cell types, including fibroblasts, conventional dendritic cells (cDCs) and macrophages, rely on cytosolic receptors that activate IRF3 through signaling intermediates[7]. In contrast, plasmacytoid DC (pDCs), which specialize in abundant secretion of IFN-α, use TLR7 (A002299) and TLR9 to detect the presence of viral RNA and DNA, respectively, in endosomal vescicles[8]. Interferon induction in these cells requires the adaptor MyD88 (A003535) and the transcription factor IRF7. Notably, TLR signaling is tightly regulated by endosomal maturation events. For example, in pDCs, TLR9 signaling can produce different effects (or no effect) depending on the subset of endosomes in which ligand recognition takes place[9,10].

Defining the mechanisms whereby bacteria induce IFN-α/β is essential not only for better understanding of how pathogen location influences immune recognition but also for insights into the complex and sometimes divergent effects of such responses on the outcome of bacterial infection[11]. Indeed, it has been shown that IFN-α/β signaling unexpectedly results in much lower host resistance to the model intracellular pathogen *Listeria monocytogenes*[12–14]. In contrast, IFN-α/β is essential for host defense against several extracellular bacteria[15], including group B streptococcus (GBS), an important neonatal pathogen[16]. This protective effect is probably mediated by the ability of IFN-α/β to prime macrophages for robust proinflammatory cytokine responses to extracellular bacteria[15].

Bacterial pathogens are notable for their ability to selectively colonize a great variety of extracellular and intracellular compartments[17]. Yet it is unclear whether host cells use different recognition systems to detect bacteria residing in different niches. Both

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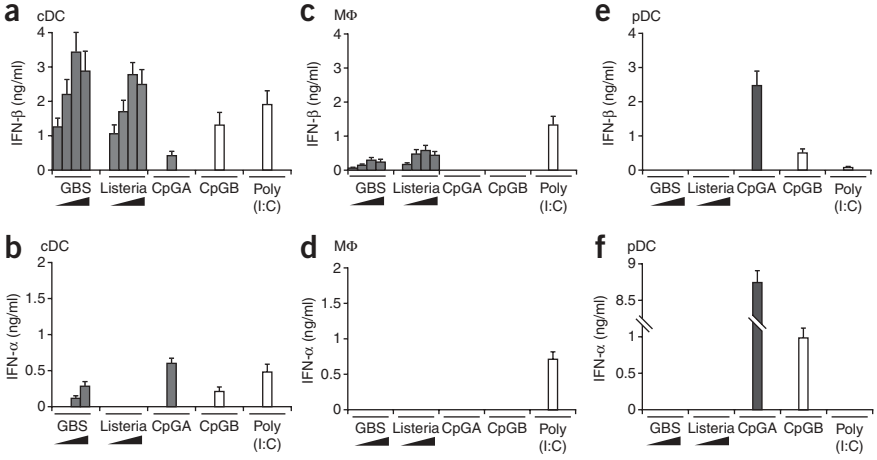

Figure 1 Production of high concentrations of IFN-β by cDCs in response to GBS or *L. monocytogenes*. Enzyme-linked immunosorbent assay (ELISA) of type I interferon in culture supernatants of bone marrow–derived, *in vitro*–differentiated cDCs (**a**,**b**), macrophages (MΦ; **c**,**d**) and pDCs (**e**,**f**) from C57BL/6 mice 24 h after infection with GBS or *L. monocytogenes* at an increasing multiplicity of infection (MOI; 5, 10, 20 or 40; wedges), measuring IFN-β (**a**,**c**,**e**) and IFN-α (**b**,**d**,**f**). Polyinosinic-polycytidylic acid (poly(I:C); 10 μg/ml), CpG A (5 μg/ml) and CpG B (1 μg/ml) serve as controls. Data (mean and s.d.) are from one experiment with five observations, each with a different mouse.

intracellular and extracellular bacteria can induce IFN-α/β through IRF3-dependent mechanisms[18–21]. Notably, this pathway is activated both in conditions in which pathogens escape from phagosomes to enter the cytosol[18,19,21] and in conditions in which bacteria remain confined in phagolysosomal compartments to be rapidly killed and degraded[20,22]. It has been proposed that the IRF3-dependent pathway is activated by an unidentified cytosolic receptor after rupture of the phagosomal membrane[18,19], injection of bacterial products into the cytosol[19] or translocation of digested bacterial components from lysosomes into the cytosol[22]. Notably, all of the studies mentioned above that failed to detect IFN-α/β induction by TLR-dependent pathways used macrophages. Can bacteria trigger TLR-dependent production of IFN-α/β in cell types other than macrophages? Because purified bacterial DNA and synthetic prokaryotic-type DNA oligonucleotides can potently induce the release of IFN-α from pDCs through the TLR9-MyD88-IRF7 pathway, it is sometimes assumed that bacteria can activate this same pathway. However, it is unclear whether whole bacteria, rather than purified DNA, can initiate TLR-dependent induction of interferon in pDCs or other cell types.

We found here that cDCs, but not pDCs or macrophages, were able to produce large amounts of IFN-β after bacterial degradation in phagolysosomes and that such responses required TLR7, MyD88 and IRF1. Our data emphasize a unifying theme in innate immunity whereby the host uses the same subfamily of receptors (in this case, the subfamily including TLR7, TLR8 and TLR9) to mount robust IFN-α/β responses to viruses and bacteria, albeit in different cell types. Moreover, our findings suggest that the endophagosomal system of cDCs may be specialized to internalize and process bacteria for presentation of their nucleic acids to TLR7, TLR8 and TLR9.

RESULTS

IFN-β production in different cell types

To ascertain whether different cell types differ in their ability to respond to bacterial stimuli by producing type I interferon, we measured IFN-α and IFN-β in culture supernatants of bone marrow–derived, *in vitro*–differentiated cDCs, macrophages and pDCs at 24 h after the addition of various doses of live bacteria. In initial studies we focused on GBS, a model phagosomal pathogen, and on *L. monocytogenes*, which, unlike GBS, resides in the cytosol of host cells. Both GBS and *L. monocytogenes* produced dose-dependent IFN-β responses in cDCs and macrophages (**Fig. 1**). However, cDCs produced several-fold more IFN-β than did macrophages (**Fig. 1**). In contrast, pDCs were totally unable to produce IFN-α/β after bacterial stimulation, although, as expected, they released large amounts of IFN-α in response to a synthetic A-type CpG DNA oligonucleotide[23] (CpG A; **Fig. 1**). We also found no production of IFN-α/β in pDCs and considerable production of IFN-α/β in cDCs after stimulation with additional pathogenic and nonpathogenic bacterial species (**Fig. 2a**), although the various tested species differed considerably in their relative potency as interferon inducers. Moreover, killed bacteria also induced IFN-β production in cDCs (**Fig. 2b**).

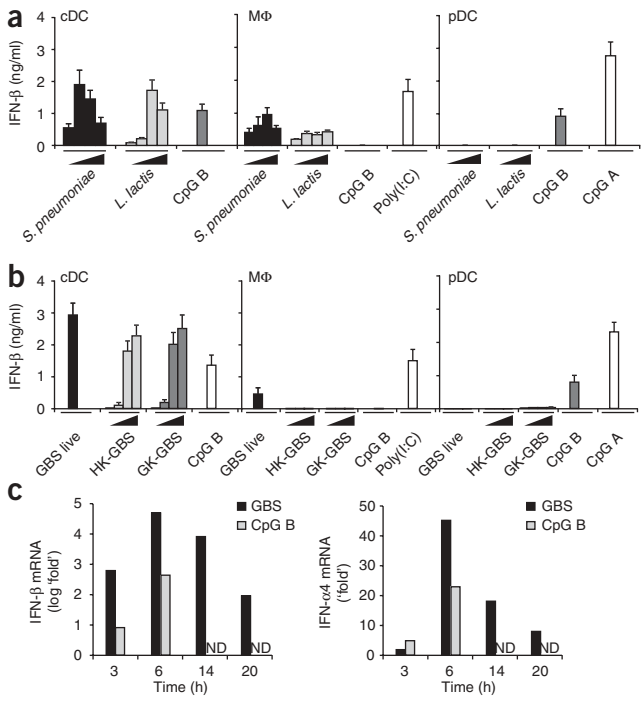

Figure 2 Type I interferon responses to various bacterial stimuli. (**a**) ELISA of IFN-β in supernatants of bone marrow–derived, *in vitro*–differentiated cDCs, macrophages and pDCs obtained from C57BL/6 mice 24 h after infection with *Streptococcus pneumoniae* or *Lactococcus lactis* at an increasing MOI (5, 10, 20 or 40; wedges). CpG A (5 μg/ml), CpG B (1 μg/ml) and poly(I:C) (10 μg/ml) serve as controls. (**b**) ELISA of IFN-β as described in **a** at 24 h after treatment with live GBS (MOI, 20) or with heat-killed GBS (HK-GBS) or gentamycin-killed GBS (GK-GBS; 0.05, 0.5, 5 or 50 μg/ml; wedges). (**c**) RT-PCR analysis of the expression of IFN-β and IFN-α4 mRNA in cDCs differentiated with granulocyte-macrophage colony-stimulating factor and exposed for various times (horizontal axes) to live GBS at an MOI of 20, presented as the 'log fold increase' (left) or 'fold increase' (right) in expression relative that of uninfected cells. CpG B (1 μg/ml) serves as a control. ND, not detectable. Data (mean and s.d.) are from one experiment with five observations, each with a different mouse (**a**,**b**) or are representative of three independent experiments (**c**).

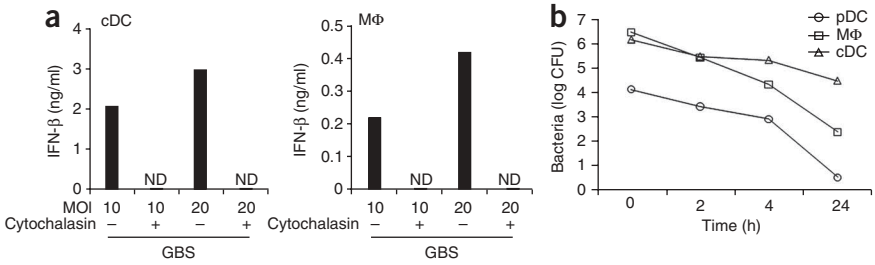

Figure 3 Phagocytosis is required for IFN-β production. (**a**) ELISA of IFN-β in supernatants of bone marrow–derived, *in vitro*–differentiated cDCs and macrophages pretreated with cytochalasin D (+; 5 μg/ml) or vehicle (–; dimethyl sulfoxide) for 45 min before infection with GBS (MOI, below graphs), assessed 24 after infection. Cytochalasin D treatment completely prevented phagocytosis, as confirmed by microscopy. Data are representative of three independent experiments. (**b**) Intracellular bacteria assessed in lysates of bone marrow–derived, *in vitro*–differentiated pDCs, macrophages and cDCs obtained from C57BL/6 mice, infected with GBS at an MOI of 20, incubated for 25 min at 37 °C, washed extensively to remove extracellular bacteria and cultured for at least 1 h (time 0) in antibiotic-containing medium. CFU, colony-forming units. Data are representative of three independent experiments.

Next we measured the expression of IFN-β and IFN-α4 mRNA at various times after infection with live GBS (**Fig. 2c**). These data indicated that substantial IFN-β production in cDCs was induced and sustained by much higher expression of IFN-β mRNA, which persisted for up to 20 h after infection. Collectively, this first set of data indicated that cDCs respond to stimulation with whole bacteria by robust production of IFN-β, whereas in the same circumstances, pDCs were totally unresponsive and macrophages produced only small amounts of IFN-β. Moreover, in each cell type, interferon responses to whole bacteria could not be predicted on the basis of responses to synthetic oligonucleotides mimicking prokaryotic-type DNA (such as CpG A and CpG B).

Signaling requirements for interferon induction

We investigated whether bacterial internalization was required for interferon induction. This seemed to be the case, as cytochalasin D, an agent that disrupts microfilaments and prevents phagocytosis, totally abrogated the release of IFN-β by cDCs and macrophages after stimulation with GBS (**Fig. 3a**). We also found that pDCs were considerably less efficient at internalizing bacteria than were macrophages or cDCs (**Fig. 3b**). Thus, less phagocytosis could at least partially explain the inability of pDCs to produce IFN-α/β in response to bacterial stimulation, although the possibility of participation of other factors in this phenomenon cannot be excluded.

To identify the receptors and signal transduction pathways involved in bacteria-induced interferon responses, we used cells lacking TLRs, TLR adaptor proteins or IRF transcription factors. In macrophages, IFN-β induction by either GBS or *L. monocytogenes* was totally abrogated in the absence of IRF3 but was unaffected by the absence of TLR3, TLR4, TLR7, TLR9, TRIF, TRAM, MAL, MyD88, IRF1 or IRF7 (**Fig. 4a** and **Supplementary Fig. 1** online). In contrast, in cDCs, the GBS-induced release of IFN-β was dependent mainly on TLR7 and was absolutely dependent on MyD88 and IRF1 (**Fig. 4b–d** and **Supplementary Fig. 2** online). Residual interferon production in TLR7-deficient cDCs was probably sustained by TLR9, as moderately but consistently lower quantities of IFN-β were produced by *Tlr9*$^{-/-}$ cells. Interferon production was also significantly lower in the absence of IRF7 (**Fig. 4d**), which suggested a cooperative effect of this transcription factor with IRF1.

In contrast to results obtained with GBS, *L. monocytogenes*–stimulated IFN-β production was IRF3 dependent but occurred independently of MyD88 and IRF1 in cDCs (**Fig. 4c,d**). To rule out the possibility that these features were related to properties of *L. monocytogenes* other than its ability to invade the cytosol, we tested a mutant strain that remains sequestered in the phagosome because it lacks the necessary virulence factors[24]. IFN-β induction by this 'phagosomal' mutant was dependent mainly on MyD88 and IRF1 (**Fig. 5a**), in contrast to results obtained with the 'cytosolic' parental

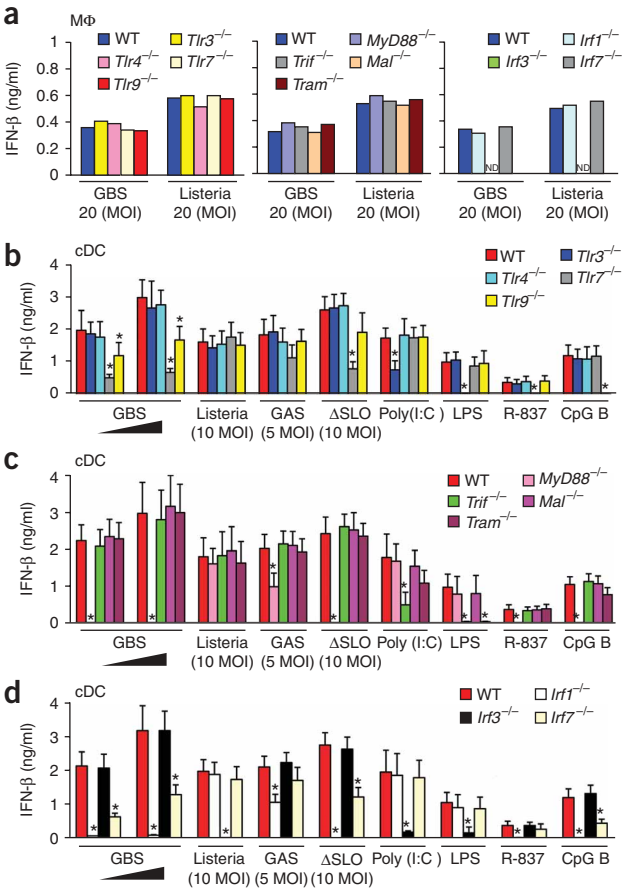

Figure 4 Signaling requirements for bacteria-induced production of IFN-β. ELISA of IFN-β in supernatants of bone marrow–derived, *in vitro*–differentiated macrophages (**a**) and cDCs (**b–d**) lacking various TLRs, TLR adaptors or IRFs (keys), measured 24 h after infection with bacteria (MOI, below graphs). For cDCs (**b–d**), wedges indicate GBS at an MOI of 10 or 20, and poly(I:C) (10 μg/ml), *Escherichia coli* lipopolysaccharide (LPS; 10 ng/ml), imiquimod (R-837 (5 μg/ml) and CpG B (1 μg/ml) serve as controls. ΔSLO, GAS mutant with a deletion in the gene encoding streptolysin O. *, $P < 0.05$, relative to wild-type (WT) cDCs (one-way analysis of variance and the Student-Newman-Keuls test). Data are representative of at least three independent experiments (macrophages) or are from one experiment with five observations, each with a different mouse (cDCs; mean and s.d.).

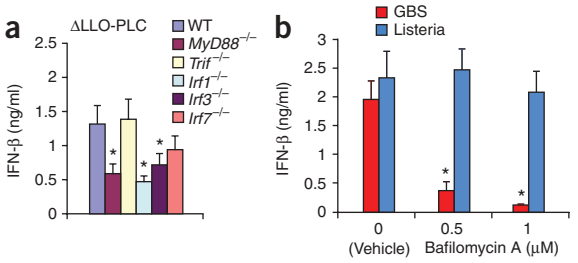

Figure 5 IFN-β induction by phagosomal bacteria. (**a**) ELISA of IFN-β in supernatants of bone marrow–derived, *in vitro*–differentiated cDCs lacking various TLRs, TLR adaptors or IRFs (key), assessed 24 h after infection with *L. monocytogenes* DP-L2319 (mutant defective in listeriolysin O and phospholypase C (ΔLLO-PLC)) at an MOI of 100. *, $P < 0.05$, relative to wild-type cDCs (one-way analysis of variance and the Student-Newman-Keuls test). Data (mean and s.d.) are from one experiment with five observations, each with a different mouse. (**b**) ELISA of IFN-β in supernatants of cDCs differentiated with granulocyte-macrophage colony-stimulating factor and treated with vehicle (dimethylsulfoxide) or 0.5 or 1 μM bafilomycin A at 30 min after infection with GBS or *L. monocytogenes* at an MOI of 20:1, assessed 24 h after infection. *, $P < 0.05$, relative to vehicle-treated cDCs (one-way analysis of variance and the Student-Newman-Keuls test). Data (mean and s.d.) are from one experiment with five observations, each with a different mouse.

strain. However, we nevertheless detected some involvement of IRF3. To gain further insight into the relationship between intracellular location and pattern-recognition pathways, we used group A streptococcus (GAS), a pathogen that transiently invades the cytosol by virtue of streptolysin O but, in contrast to *L. monocytogenes*, is unable to replicate in the cytosol and is retrapped in autophagosomes[25]. GAS-stimulated production of IFN-β was partially dependent on MyD88 and IRF1 but became totally so in the absence of streptolysin O (**Fig. 4c,d**). Therefore, there was a strong association between use of the MyD88- and IRF1-dependent pathway and bacterial location in phagosomes. In agreement with that, phagosomal acidification was required for the induction of IFN-β by GBS, as indicated by the inhibitory effects of bafilomycin A, a vacuolar ATPase proton pump inhibitor; in contrast, this drug had no effect on *L. monocytogenes*–induced production of IFN-β (**Fig. 5b**). These data collectively suggested that cDCs use IRF1 or IRF3 to mount robust interferon responses to bacteria residing in phagosomal or cytosolic compartments, respectively. In contrast, weak IFN-β induction in macrophages occurs independently of the organism's intracellular location.

Localization of GBS antigens together with signaling molecules

A notable feature of TLR-dependent induction of interferon in pDCs is its tight regulation by endosomal maturation events[9,10]. To gain insight into the point along the phagolysosomal maturation pathway at which bacterial recognition occurred in cDCs, we studied the spatial relationships among GBS, signaling molecules and endosomal markers with structured illumination fluorescence microscopy. We found GBS antigens in two distinct cDCs intracellular compartments: phagosomes that contained single GBS cells that stained positive for bacterial DNA and sequentially acquired early and late endosomal markers but were negative for the lysosomal marker cathepsin D; and cathepsin D-positive, DNA-negative phagolysosomes filled with amorphous GBS material, intensely stained by GBS-specific antibodies (**Fig. 6**).

Both of the structures described above (the DNA^+ phagosomes and the DNA^- phagolysosomes containing partially digested GBS material) had high expression of TLR7 (**Fig. 7** and **Supplementary Movies 1** and **2** online). In contrast, MyD88 and IRF1 were not present in

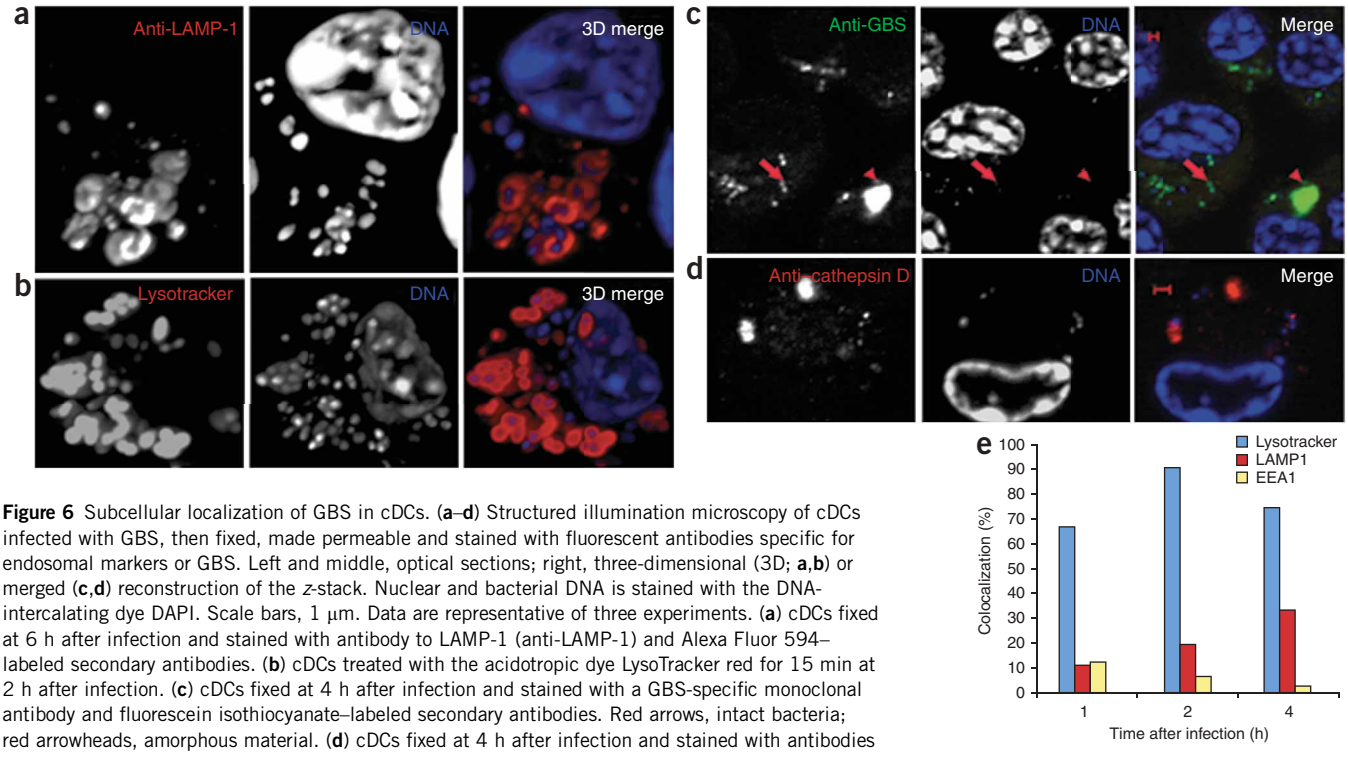

Figure 6 Subcellular localization of GBS in cDCs. (**a**–**d**) Structured illumination microscopy of cDCs infected with GBS, then fixed, made permeable and stained with fluorescent antibodies specific for endosomal markers or GBS. Left and middle, optical sections; right, three-dimensional (3D; **a**,**b**) or merged (**c**,**d**) reconstruction of the *z*-stack. Nuclear and bacterial DNA is stained with the DNA-intercalating dye DAPI. Scale bars, 1 μm. Data are representative of three experiments. (**a**) cDCs fixed at 6 h after infection and stained with antibody to LAMP-1 (anti-LAMP-1) and Alexa Fluor 594–labeled secondary antibodies. (**b**) cDCs treated with the acidotropic dye LysoTracker red for 15 min at 2 h after infection. (**c**) cDCs fixed at 4 h after infection and stained with a GBS-specific monoclonal antibody and fluorescein isothiocyanate–labeled secondary antibodies. Red arrows, intact bacteria; red arrowheads, amorphous material. (**d**) cDCs fixed at 4 h after infection and stained with antibodies specific for cathepsin D and with Alexa Fluor 594–labeled secondary antibodies. Original magnification (**a**,**b**), ×1,000; scale bars (**c**,**d**), 1 μm. (**e**) GBS in compartments positive for LysoTracker, LAMP-1 or EEA1 at various times after infection (horizontal axis), presented relative to total GBS numbers identified by DNA staining. Data are representative of three independent experiments with at least 300 cDCs.

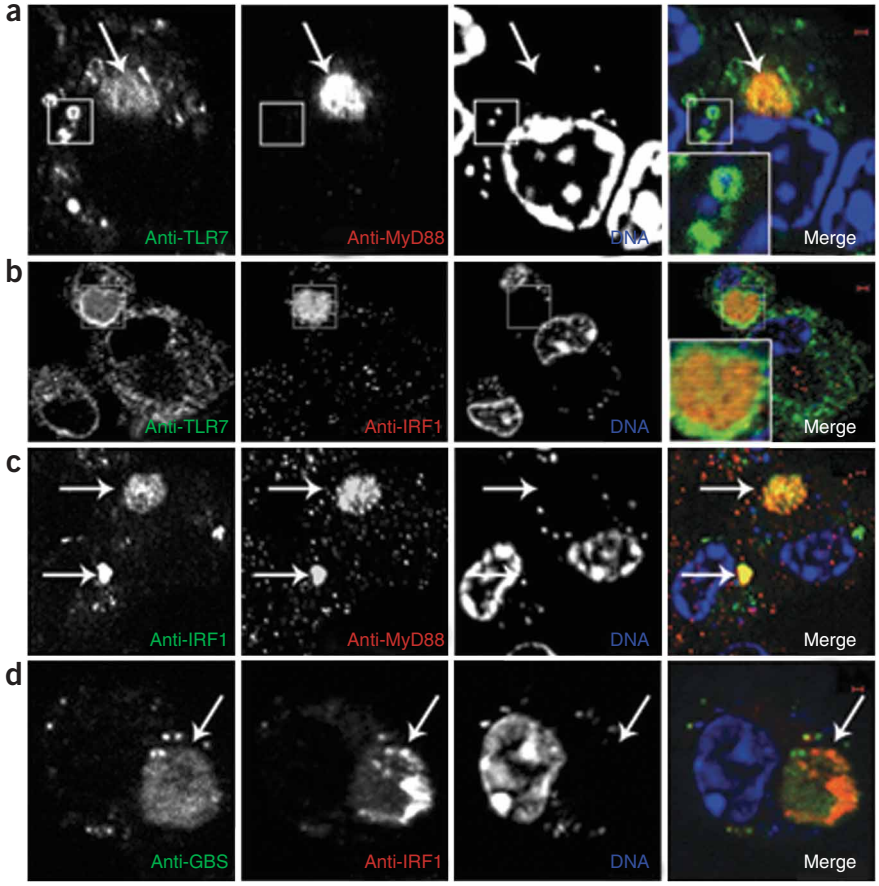

Figure 7 Subcellular localization of TLR7, MyD88 and IRF1 in GBS-infected cDCs. Structured illumination microscopy (optical sections) of cDCs infected for 4 h with GBS, then fixed, made permeable and stained with fluorescent antibodies. Nuclear and bacterial DNA is stained with DAPI. Scale bars, 1 μm. The *z*-stacks corresponding to **a**–**c** are also in **Supplementary Movies 1**–**3**. Data are representative of three experiments. (**a**) Staining with anti-TLR7 and anti-MyD88 plus fluorescein isothiocyanate– and Texas red–labeled secondary antibodies, respectively. Arrows indicate localization of TLR7 together with MyD88 in a DNA^- area. Boxes outline $TLR7^+$ vacuoles containing bacterial DNA (higher magnification, inset, far right). (**b**) Staining with anti-TLR7 and anti-IRF1 plus fluorescein isothiocyanate– and Alexa Fluor 594–labeled secondary antibodies, respectively. Boxes outline localization of TLR7 together with IRF1 in a DNA^- area (higher magnification, inset, far right). (**c**) Staining with anti-IRF1 and anti-MyD88 plus Alexa Fluor 488– and Alexa Fluor 594–labeled secondary antibodies, respectively. Arrows indicate localization of IRF1 together with MyD88 in two DNA^- areas. (**d**) Staining with anti-GBS and anti-IRF1 plus fluorescein isothiocyanate– and Alexa Fluor 594-labeled secondary antibodies, respectively. Arrows indicate extensive localization of GBS antigen together with IRF1 in a large DNA^- structure.

phagosomes containing intact bacterial DNA. Instead, both of these molecules accumulated in phagolysosomes containing amorphous GBS material, where they extensively localized together with TLR7 (**Fig. 7** and **Supplementary Movies 1–4** online). The vacuoles showing such colocalization always stained with GBS-specific antibodies (**Fig. 7d** and **Supplementary Fig. 3a** online) and were not present in uninfected cells (data not shown). These structures ranged in size from 1 μm to 6 μm, and we identified them as phagolysosomes on the basis of their positivity for the late endosomal-lysosomal marker LAMP-1 and the lysosomal marker cathepsin D (**Supplementary Fig. 3**). Moreover, they were negative for the early endosomal marker EEA1 (data not shown). These data are collectively compatible with a model in which in cDCs, TLR7 activation and the subsequent recruitment of MyD88 and IRF1 occur very distally in the vacuolar pathway, in degradative compartments formed after the fusion of bacteria-containing phagosomes with lysosomes.

TLR7 and IRF1 are needed for defense against GBS

IFN-α/β is crucial in host defenses against GBS and other extracellular bacteria, as shown by the hypersusceptibility of IFN-α/β receptor–deficient mice to infection by these pathogens[15]. The inability of such mice to control GBS disease could be accounted for mostly by defective IFN-β signaling, as shown with IFN-β-defective mice[15]. Therefore, we investigated the function in host defenses of the TLR7-dependent IFN-β-induction pathway described here. We studied the susceptibility to GBS infection of mice lacking TLR7, IRF1, IRF3 or IRF7 with a neonatal sepsis model characterized before[26]. After challenge with a low dose of GBS inoculum, 47% of the TLR7-deficient mice died and 65% of the IRF1-deficient mice died, whereas all the wild-type control mice survived (**Fig. 8a**). In contrast, IRF3-deficient pups were as susceptible to GBS infection as were wild-type control mice, and IRF7-deficient pups were only moderately more susceptible to GBS infection than were wild-type controls (**Fig. 8b**). These data suggested that the TLR7- and IRF1-dependent vacuolar pathway is physiologically relevant *in vivo*.

To confirm the conclusion above, we used an additional model of GBS disease involving intraperitoneal challenge of adult mice. In these experiments, we compared the susceptibility of TLR7-deficient mice to infection with the susceptibility of IFN-β-defective mice, which are known to be hypersusceptible to GBS[15]. After a sublethal bacterial dose, 38% of the TLR7-defective adult mice died and 56% of the IFN-β-defective adult mice died, whereas all wild-type control mice survived (**Supplementary Fig. 4a** online), which confirmed the results

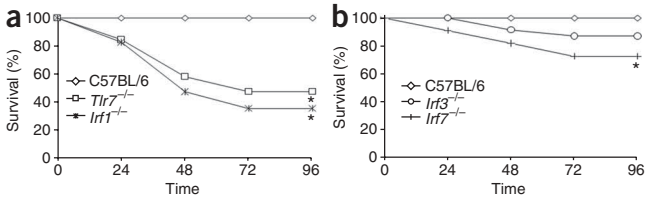

Figure 8 Neonatal mice lacking TLR7 or IRF1 are highly susceptible to GBS infection. Survival of neonatal mice (24–48 h old) inoculated subcutaneously with approximately 10 colony-forming units of GBS (a dose sublethal to wild-type pups) and monitored for signs of death or irreversible disease. Number of mice: wild-type (C57BL/6), $n = 24$ (**a**) or $n = 20$ (**b**); TLR7-deficient, $n = 19$; IRF1-deficient, $n = 17$; IRF3-deficient, $n = 23$; IRF7-deficient, $n = 22$. *, $P < 0.05$, relative to wild-type mice (Kaplan-Meier survival plot analysis). Data represent results pooled from three independent experiments with similar results.

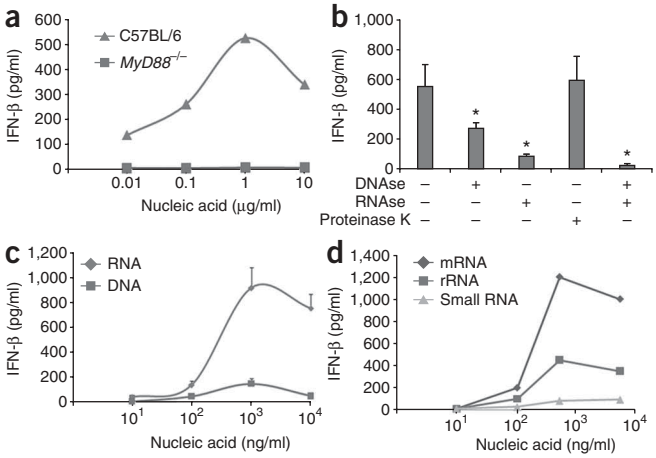

Figure 9 IFN-β responses to GBS extracts and RNA preparations. IFN-β concentrations in supernatants of bone marrow–derived cDCs differentiated with granulocyte-macrophage colony-stimulating factor and stimulated with GBS extracts (**a**), GBS extracts treated with DNAse, RNAse or proteinase K (**b**), purified GBS RNA or DNA (**c**) or GBS mRNA, rRNA or small RNA (**d**). Data (mean and s.d.) are from one experiment with three observations, each with a different mouse.

obtained with the neonatal model. Next we measured bacteria in the organs of mice infected in a similar way[15]. None of the wild-type mice but most of the TLR7- or IFN-β-deficient mice had detectable bacteria in the blood and in the kidneys (**Supplementary Fig. 4b**,**c**). These data demonstrate that TLR7 deficiency is associated with less ability to control *in vivo* growth of bacteria and to prevent their systemic spreading beyond a local site of infection. This defect was similar in extent to that of IFN-β-deficient mice.

Bacterial RNA is an interferon-inducing agent

As TLR7 was required for the considerable induction of IFN-β by GBS and for host protection against this pathogen, we sought to gain insight into the nature of the TLR7-activating ligand of GBS. Because single-stranded RNA of viral or synthetic origin can induce IFN-α/β in pDCs through TLR7 (refs. 27,28), we assessed whether bacterial RNA could account for the potent GBS-induced TLR7-dependent IFN-β response of cDCs. In preliminary experiments, GBS lysates in complex with the cationic lipid carrier DOTAP recapitulated, in a dose-dependent way, the MyD88-dependent induction of IFN-β noted with live GBS (**Fig. 9a**). Pretreatment of these GBS extracts with RNAse almost completely abrogated induction of IFN-β, whereas DNAse treatment was comparatively less effective and proteinase K treatment was totally ineffective (**Fig. 9b**). Moreover, combined treatment with RNAse and DNAse almost completely suppressed the IFN-α/β-inducing ability of the extracts. As these data suggested that RNA was a chief IFN-β-activating stimulus in GBS lysates, in further experiments we analyzed purified GBS RNA for its ability to induce IFN-β relative to that of GBS DNA. RNA was much more effective than DNA at inducing the release of IFN-β over a wide dose range (**Fig. 9c**).

To identify the main RNA type responsible for IFN-β induction, we next tested purified GBS mRNA, rRNA and small RNA (composed mostly of tRNA and 5S rRNA) for their ability to induce IFN-β and found that mRNA was the most potent IFN-β inducer (**Fig. 9d**). Finally, we examined the function of TLR7 in interferon induction by GBS RNA with cells from mice lacking TLR7. This receptor was required for responses to either total RNA or mRNA, as in response to either stimulus, IFN-β concentrations in the supernatants of TLR7-deficient cDCs were over 85% lower than those of wild-type cells (data not shown). These data indicate that GBS RNA, particularly mRNA, is a potent immunostimulatory agent that can account for substantial, TLR7-dependent induction of IFN-β by whole bacteria in cDCs.

DISCUSSION

The past several years have witnessed considerable progress in the genetic identification of the signaling pathways involved in interferon induction by conserved viral and bacterial products. There is now a need to integrate this information in the context of events taking place at the subcellular level after interaction of host cells with whole organisms. Thus, we were particularly interested in studying the mechanisms of IFN-α/β induction by bacteria sequestered in the classical phagosomal pathway, compared with those of pathogens that escape from phagosomes into the cytosol. The identification of such mechanisms may have practical implications because of the notable pathogen-specific effects of interferon signaling on the outcome of bacterial infection[11–15].

Here we have identified a TLR7-MyD88-IRF1-dependent pathway that led to IFN-β production and was activated exclusively by phagosomal bacteria. Indeed, bacterial internalization was found here to be absolutely required for interferon production through this pathway, in agreement with the possibility that all pathways leading to induction of this cytokine family originate from an intracellular location[29]. We found that pDCs were totally unable to mount interferon responses to the phagosomal bacteria tested here, probably because of a much lower ability to internalize these organisms.

Moreover, we found that cDCs produced more IFN-β than did macrophages after bacterial stimulation. This observation is what originally prompted us to study the requirements for such responses in both cell types, as most studies thus far have dealt exclusively with macrophages. In macrophages, interferon responses occurred independently of the prevalent intracellular location of the stimulating pathogen and uniformly required IRF3 but not IRF1 or MyD88. In contrast, cDCs were able to use at least two distinct pathways to mount robust interferon responses to bacteria residing in cytosolic and phagosomal compartments. Responses to the former group of pathogens required IRF3, whereas responses to the latter group totally depended on IRF1 and MyD88 and partially depended on IRF7.

The activation of distinct sets of transcription factors by cytosolic and phagosomal pathogens may provide further insight into the mechanisms with which the innate immune system responds to organisms residing in different locations. Moreover, these data may be useful for better understanding of the strategies used by intracellular organisms to escape immune recognition. It is likely that by avoiding progression along the phagosomal pathway, some pathogens prevent the activation of IRF1, which promotes proinflammatory and often host-protective T helper cell type 1 responses[30]. Our data are in agreement with that possibility, as IRF1 but not IRF3 was required *in vivo* for defense against phagosomal bacteria. Notably, the IRF1-dependent pathway described here required TLR7, an antiviral receptor not associated before with protective antibacterial activities[27,28]. We found here that TLR7 was crucial in anti-GBS defenses, as shown by the greater susceptibility of TLR7-deficient mice to infection with GBS. This effect was associated with lethality approaching that noted with IFN-β-deficient mice. Our *in vitro* and *in vivo* findings are compatible with the possibility that substantial, IRF1-dependent production of type I interferon by cDCs is required for antibacterial host defenses, whereas insubstantial, IRF3-dependent production of interferon by macrophages is dispensable. However,

further studies will be needed to determine whether cDCs serve a predominant function in host defenses against GBS and IFN-β production *in vivo*. Ablation of DCs in mice results in less resistance to GAS[31] and other sepsis-causing bacteria[32], but whether this effect is associated with less IFN-β production has not been studied.

TLR9, in addition to TLR7, may participate in the activation of the IRF1-dependent phagosomal pathway, as suggested by the moderately but significantly lower IFN-β production found in response to phagosomal bacteria in the absence of TLR9. Those data are in agreement with the known ability of TLR9 agonists to induce moderate, IRF1-dependent IFN-β responses in cDCs[33,34]. Therefore, TLR7 and TLR9 may act together in bacterial detection, although the effects of TLR7 deficiency outweighed those of TLR9 deficiency in terms of IFN-β expression in our study here. Of course, it is possible that TLR9 is more important in IFN-β induction by bacterial pathogens other than GBS. It will be useful to assess the contributions of TLR7 and TLR9 in IFN-β production and host protection against infection by a wide range of organisms.

Several considerations suggest that RNA is the bacterial ligand targeted by TLR7 in cDCs. First, TLR7 is strictly RNA specific, and single-stranded RNA is its only known natural agonist[27,28]. Second, RNA is abundantly present in prokaryotic cells (usually in amounts two to ten times greater than DNA) and is likely to be present in particularly high concentrations in the phagolysosomal lumen. Finally, and perhaps most notably, the ability of GBS to induce TLR7-dependent induction of IFN-β could be recapitulated here by bacterial RNA. Moreover, RNAse treatment almost completely abrogated the induction of IFN-β by GBS lysates. Studies have demonstrated the immunostimulatory activities of whole bacterial RNA[35,36], including its ability to activate cells transfected *in vitro* with human TLR3, TLR7 or TLR8 (ref. 36). Our data indicate that bacterial RNA is highly effective (and considerably more potent than bacterial DNA) at inducing IFN-β in cDCs. Moreover, we have shown that different types of bacterial RNA have different interferon-inducing abilities, with mRNA having the greatest potency.

Collectively, our findings further emphasize a link among the infected cell type, the pathogen's intracellular location, the sensing receptors and the transcription factors involved in IFN-α/β responses. Such a link has been documented before during infection by viruses, which can activate IRF3-dependent responses from a cytosolic location in most cell types, and an IRF7-dependent pathway from the endosomes of pDCs[3,4,6]. We have shown here that parallel, cell type–specific pathways of this kind can also be activated by most bacterial pathogens, specifically by those that undergo killing and degradation in cDCs. Like viruses, these bacteria can induce low-grade interferon production in macrophages as well as a more robust response in a specialized DC type (for example, in cDCs for bacteria and in pDCs for viruses) through the activation of endosomal TLRs.

The mechanisms that underlie the unique ability of cDCs to mount robust antibacterial interferon responses are unclear at present. Our data suggest that endophagosomal trafficking in these cells, which entails efficient phagocytosis and rapid translocation of ingested material to late, mature compartments, may facilitate the processing of prokaryotic organisms and subsequent presentation of their nucleic acids to endosomal TLRs. It is reasonable to assume that only lysosome-type vacuoles are sufficiently 'degradative' to allow digestion of the thick and hardy prokaryotic envelope and subsequent exposure of bacterial nucleic acids. In agreement with that, we found that TLR7-dependent recognition was triggered at a distal point along the phagosomal maturation pathway (after phagolysosome fusion). The mechanism described here for interferon induction by bacteria in cDCs is in contrast to the TLR9-dependent induction of IFN-α by DNA oligonucleotides in pDCs, which is initiated in early but not late endosomes[9,10] (for example, in conditions associated with the uncoating of most viral genomes).

In conclusion, our data indicate the existence of a biologically relevant, nonredundant phagolysosomal bacteria-recognition system that operates in cDCs but not in macrophages or pDCs and is probably induced by bacterial RNA. This detection system is 'upstream' of IRF1, which serves a central function in innate and adaptative responses and is key to the development of T helper type 1 responses[6,30]. Thus, by disrupting phagosomal maturation and/or integrity, bacterial pathogens may avoid not only direct killing but also immune recognition in lysosomal compartments and the subsequent establishment of IRF1-dependent host-protective responses.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A001237, A002299 and A003535.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

G.M. and A.M., design and performance of *in vivo* and *in vitro* infection experiments; M.G., design and performance of microscopy studies; S.P., generation of reagents; S.A., provision of experimental models and discussions; G.M., C.Bi., G.T. and C.Be., data analysis and manuscript preparation.

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ONLINE METHODS

Bacterial strains. The following strains of bacteria were used: GBS COH1 (ref. 15); GBS H36B; *L. monocytogenes* 10403S (wild-type) and DP-L2319 (ref. 24; lysteriolysin O– and phospholypase C–defective mutant); *S. pneumoniae* D39 (ref. 15); *L. lactis* MG1363 (ref. 37); GAS 3348 (ref. 38; wild-type) and ΔSLO (streptolysin O–defective mutant obtained from GAS 3348 by published methods)[39].

Bone marrow–derived cells. Cells were obtained from the long bones of mice and were cultured in the presence of macrophage colony-stimulating factor (macrophages), granulocyte-macrophage colony-stimulating factor (cDCs) or the cytokine Flt3L (pDCs) as described[40] (all three reagents from PreproTech). The cDCs were enriched from cells differentiated with granulocyte-macrophage colony-stimulating factor by positive selection after passage through Pan DC MicroBead columns (Miltenyi Biotech); pDCs were enriched from Flt3L-differentiated cells by positive selection with the Plasmacytoid Dendritic Cell Isolation kit (Miltenyi Biotech). In some experiments, cDCs were also obtained with that kit by negative selection of Flt3L-differentiated cells. Similar results for IFN-α/β production after bacterial stimulation were obtained with cDCs differentiated with granulocyte-macrophage colony-stimulating factor and Flt3L. Macrophages differentiated with macrophage colony-stimulating factor were over 95% $CD11b^+$, over 85% $F4/80^+$ and less than 5% $CD11c^+$, by flow cytometry. Purified cDCs were over 90% $CD11c^+CD11b^+B220^-$. The purity of pDCs ($CD11c^+CD11b^-B220^+$) was also over 95%. Macrophages or cDCs lacking TLR7, TLR9, MyD88, IRF1, IRF3 or IRF7 did not differ from wild-type cells in their expression of phenotypic markers (**Supplementary Table 1** online). The following monoclonal antibodies were used for flow cytometry: anti-CD11b (M1/70.15.11.5), anti-CD11c (N418) and anti-B220 (RA3-6B2; all from Miltenyi Biotec), and anti-F4/80 (BM8; eBioscence).

Bacterial infection *in vitro*. All bacterial strains were grown to the late exponential phase in chemically defined media[15], were collected by centrifugation, were washed three times in PBS and were added to the cell monolayers at various MOI values (bacteria/cell). The number of viable bacteria used in each experiment was carefully determined by plate counting. After incubating for 25 min at 37 °C, monolayers were washed extensively for removal of extracellular bacteria and were incubated for various times in the presence of penicillin (50 UI/ml), streptomycin (50 μg/ml; GBS COH1 and *S. pneumoniae* D39), or gentamycin (100 μg/ml; *L. monocytogenes*, *L. lactis* and GAS). In some experiments, cell monolayers were treated with various concentrations of cytochalasin D (Sigma) at 45 min before infection or bafilomycin A (Sigma) at 30 min after infection. In preliminary experiments, cell viability, as determined by Trypan blue exclusion, was not affected by any of the treatments described above.

Killed bacteria. Killed bacteria were prepared by treatment with heat (45 min at 80 °C) or gentamycin (400 μg/ml for 2 h at 37 °C), followed by extensive washing with distilled water and lyophilization. The endotoxin concentration of all of the lyophilized bacterial preparations was under 0.06 EU/mg, as determined by limulus amebocyte lysate assay (PBI).

Control stimuli. The following TLR agonists from InvivoGen served as controls for *in vitro* stimulation experiments: poly(I:C), *E. coli* K12 ultrapure lipopolysaccharide, CpG A (ODN 1585), CpG B (ODN 1826) and imiquimod (R-837).

Cytokine measurement. IFN-α and IFN-β were measured by ELISA in culture supernatants with the mouse IFN-α ELISA kit and the VeriKine Mouse IFN-β ELISA kit, respectively (both from PBL Biomedical Laboratories).

Real-time PCR. RT-PCR analysis of IFN-β and IFN-α4 mRNA was done as described[15].

Structured illumination microscopy. Monolayers of cDCs cultured on glass coverslips were infected for various times with GBS and were then fixed for 15 min at 20 °C with 3.7% (vol/vol) paraformaldehyde in PBS, pH 7.4. After being washed, cells were made permeable for 10 min with PBS containing 0.25% (vol/vol) Triton X-100 and were blocked overnight at 4 °C with 10% (vol/vol) normal goat serum in PBS or 1% (vol/vol) BSA. Cells were then stained according to a double-immunofluorescence protocol. All antibodies were used at the concentrations and times suggested by the manufacturers. The following primary antibodies were used: mouse monoclonal anti-GBS (IgG1; 072; Santa Cruz Biotechnology), rabbit polyclonal anti–mouse EEA1 (ab50313; Abcam), rabbit polyclonal anti–mouse LAMP-1 (ab24170; Abcam), rabbit polyclonal anti–mouse cathepsin D (06-467; Upstate), goat polyclonal anti–mouse IRF1 (AF 4715; R&D Systems), rat monoclonal anti–mouse MyD88 (316902; R&D Systems) and chicken polyclonal IgY anti–mouse TLR7 (15-288-22789B; GenWay Biotech). Secondary antibodies and labels were as follows: polyclonal goat anti–rabbit IgG (Texas Red; ab6719; Abcam), donkey anti–chicken IgY (fluorescein isothiocyanate; 25-288-11002; GenWay Biotech), anti–mouse IgG (fluorescein isothiocyanate; sc-2010; Santa Cruz Biotechnology), chicken anti–goat IgG (Alexa Fluor 488; A21467; Invitrogen) and goat anti–rat IgG (Alexa Fluor 594; A11007; Invitrogen). In the experimental conditions used here, cDCs obtained from TLR7-, MyD88- or IRF1-deficient mice were not labeled by anti-TLR7, anti-MyD88 or anti-IRF1, respectively, which indicated a complete absence of background staining. DAPI (4,6-diamidino-2-phenylindole) was used according to the manufacturer's instructions (Molecular Probes). For LysoTracker red staining, 500 nM LysoTracker red DND-99 (Molecular Probes) was added to the monolayers 15 min before fixation. Coverslips were mounted with Vectashield mounting medium (Vector Laboratories). Stacks of optical sections were obtained with an Axio Observer fluorescence microscope equipped with a structured illumination system (Apotome) and with the Axiovision software (all from Carl Zeiss). Figures were constructed with Adobe Photoshop and Illustrator.

Mice. $Irf1^{-/-}$, $Irf3^{-/-}$ and $Irf7^{-/-}$ mice[6] were from T. Taniguchi. $Ifnb^{-/-}$ mice[15] were from T. Leanderson. Mice lacking TLRs or TLR adaptors were developed as described[4]. Wild-type C57BL/6 mice, used as controls, were from Charles River Laboratories.

Mouse GBS infection models. Mice were from colonies established at the animal facility of the Metchikoff Department (University of Messina). GBS infection models involved neonatal or adult mice. In the neonatal model, breeding was done by timed mating; pups 24–48 h old were injected subcutaneously with the highly virulent type III COH1 strain of GBS in 30 μl PBS as described[15,26]. In the adult model, 8-week-old female mice were inoculated intraperitoneally with 2×10^5 colony-forming units of GBS type Ib strain H36B. Bacteria were grown to the late-log phase in Todd-Hewitt broth (Oxoid) and were diluted to the appropriate concentration in PBS before inoculation of mice. In each experiment, the number of injected bacteria was carefully determined by colony counts on blood agar plates (BD Bioscences). Each experiment included two groups of 'genetically defective' mice and a wild-type control group. The groups did not differ in age or weight. Mice were monitored daily for 10 d after infection, but deaths never happened after 4 d. All studies were in agreement with the European Union guidelines of animal care and were approved by the relevant national committees (Istituto Superiore di Sanità).

GBS extracts and RNA preparation. Bacterial cell extracts were obtained by vortexing of GBS (grown to the mid-log phase) in the presence of glass beads 425–600 μm in diameter (Sigma). In preliminary experiments, this procedure was optimized to minimize RNA degradation, as detected by electrophoresis through agarose gels. Treatments with RNAse (Genomed), DNAse (Qiagen) and proteinase K (Sigma) were done according to the manufacturers' instructions. Total GBS RNA was prepared from extracts obtained as described above with the RNeasy Mini kit according to the manufacturer's protocol (Qiagen). Purified mRNA was obtained from total RNA by sequential removal of rRNA and small RNA with the Microbe Express kit and the Megaclear kit, respectively, according to manufacturer's protocols (Ambion). Total and rRNA were separated by electrophoresis through agarose gels, followed by slicing of 23S and 16S bands and electroelution. Small RNA (under 200 nucleotides, enriched in tRNA and 5S rRNA) was obtained from total RNA with the microRNA Purification kit according to the manufacturer's protocol (Norgen). The purity of all RNA preparations was assessed by electrophoresis through agarose gels. GBS extracts and RNA preparations were 'complexed' with DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; Sigma) as described[9] before cell stimulation.

Statistics. Differences in cytokine concentrations were analyzed by one-way analysis of variance and Student-Keuls-Newman test. Survival data were

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analyzed with Kaplan-Meier survival plots followed by the log-rank test (JMP Software; SAS Institute) on an Apple Macintosh computer. Differences in organ colony-forming units were evaluated with Mann-Whitney rank-order test. *P* values of less than 0.05 were considered statistically significant.

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