Streptococcus pneumoniae Stimulates a STING- and IFN Regulatory Factor 3-Dependent Type I IFN Production in Macrophages, which Regulates RANTES Production in Macrophages, Cocultured Alveolar Epithelial Cells, and Mouse Lungs

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**Streptococcus pneumoniae** Stimulates a STING- and IFN Regulatory Factor 3-Dependent Type I IFN Production in Macrophages, which Regulates RANTES Production in Macrophages, Cocultured Alveolar Epithelial Cells, and Mouse Lungs

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**Streptococcus pneumoniae** is the leading cause of community-acquired pneumonia. In this study, we examine an innate immune recognition pathway that senses pneumococcal infection, triggers type I IFN production, and regulates RANTES production. We found that human and murine alveolar macrophages as well as murine bone marrow macrophages, but not alveolar epithelial cells, produced type I IFNs upon infection with *S. pneumoniae*. This response was dependent on the pore-forming toxin pneumolysin (PLY) and the capsule. The innate immune system senses pneumococci through pattern recognition receptors (PRRs), such as the membrane-bound TLRs, the PYHIN protein absent in melanoma 2 (AIM2) regulate IL-1 production. Moreover, type I IFNs controlled RANTES production during pneumococcal pneumonia in vivo. In conclusion, we identified an immune sensing pathway detecting *S. pneumoniae* that triggers a type I IFN response and positively regulates RANTES production. The Journal of Immunology, 2012, 188: 811–817.

*Streptococcus pneumoniae* is a Gram-positive, extracellular bacterium and the most important pathogen causing community-acquired pneumonia. Central virulence factors are the pore-forming toxin pneumolysin (PLY) and the capsule. The innate immune system senses pneumococci through pattern recognition receptors (PRRs), such as the membrane-bound TLRs, the cytosolic NOD-like receptors (NLRs), and the PYHIN proteins. The TLRs and some NLRs stimulate the transcription of proinflammatory mediators (1, 2). In contrast, other NLRs and the PYHIN protein absent in melanoma 2 (AIM2) regulate IL-1 family cytokines on a posttranslational level (3, 4). The TLRs TLR2, TLR4, and TLR9 and the NLRs NOD2 and NLRP3 have been shown to control the production of several proinflammatory cytokines during *S. pneumoniae* infection (2, 5–12).

Type I IFNs (IFN α/β) had been described in the 1950s as important modulators of the anti-viral defense (13). During infection, viruses are sensed by, for example, cytosolic RIG-I–like receptors (RLRs) and/or endosomal TLRs leading to type I IFN production dependent on IFN regulatory factor transcription factors. After secretion, IFN α/β binds to the IFN α/β receptor (IFNAR), stimulates the JAK/STAT pathway, and triggers the expression of IFN-stimulated genes (ISGs), some of which fulfill anti-viral functions (14).

It was not until the past couple of years that the role of type I IFNs in bacterial infections became apparent (15). A number of studies demonstrated that bacteria-infected cells produce IFN-β (16–24). In several infection models, this response has been induced to depend on cytosolic sensing of bacterial DNA. Although DAI/ZBP1, RNA PolIII–RIG-I, and IFN, γ-inducible protein 16 (IFI16) have been identified as cytosolic DNA sensors (25–28), their roles in most bacterial infections remain to be characterized. Moreover, cytosolic sensing of RNA by RLRs and cyclic dinucleotides by a yet-to-be-identified receptor has also been implicated in some bacteria-induced type I IFN responses (29–31). Most of these sensing pathways appear to signal via the adapter molecule STING, the kinase TBK1, and the transcription factor IFN regulatory factor 3 (IRF3) (23, 32, 33).
In infections with bacteria, type I IFNs can either contribute to or detract from appropriate immune responses, possibly depending on the type of pathogen examined. IFNAR-deficient mice have been shown to be more resistant to infections with *Listeria monocytogenes* and *Mycobacterium tuberculosis*, whereas the mice were more susceptible to some extracellular bacteria (22, 33–36).

In this study, we show that pneumococci are sensed by a cytosolic innate immune pathway that appears to detect bacterial DNA within macrophages. The subsequently produced type I IFNs regulate RANTES production by macrophages and alveolar epithelial cells in an autocrine and/or paracrine manner.

### Materials and Methods

#### Reagents

Purified PLY was kindly provided by Timothy J. Mitchell (University of Glasgow). Cytochalasin D, chloroquine, and ammonium chloride were purchased from Sigma Aldrich, and bafilomycin A1 was obtained from Calbiochem. Polyanosinic-polycytidylic acid (poly(C)) was purchased from Amersham Biosciences.

#### Bacterial strains and PLY

*S. pneumoniae* serotype 2 strains D39, D39Δply, Δcps, and Δcps/Δply (37), serotype 3 strain NCTC7978, and serotype 4 strains TIGR4, TIGR4Δcps, and TIGR4Δcps/Δply were used. The capsule locus (38) of strain TIGR4 was removed by insertion-deletion mutagenesis as described previously (39, 40). A PLY-negative mutant of the TIGR4Δcps without capsule was generated by insertion-duplication mutagenesis using a pJD9 derivative containing an internal fragment of the ply gene as described previously (41).

Host cells were infected with pneumococci at different multiplicities of infection (MOIs) as indicated for 6 h (mRNA analysis) or 16 h (protein analysis and coculture) unless otherwise indicated. Bacterial extracts were prepared as described previously (18). Briefly, *S. pneumoniae* cultures were grown until OD600 = 0.7. The pellet was treated with lysozyme (1 mg/ml) and ultrasound (2 min, 50% pulse chase). Afterward, the debris was pelleted, and the supernatant was adjusted to 2 mM MgCl2, 50 mM KCl, and 20 mM Tris-HCl. Subsequently, the extracts were digested with DNase (100 U/ml), and tibiae of wild-type (wt), Cardif mice and *S. pneumoniae* type I IFN responses in mice.

#### Cells and infections

Bone marrow-derived macrophages (BMMs) were prepared from femurs and tibiae of wild-type (wt), *Cardif*−/− (MAVS−/−) (43), IRF3−/− (44), NOD2−/− (45), TLR2,3,4,7,9−/− (46), or IFNAR−/− (23) mice that were all infected with PLY. Samples were tested for normal distribution using the Kolmogorov–Smirnov test. Data are expressed as mean ± SEM. Samples were tested for normal distribution using the Kolmogorov–Smirnov test. Statistical analysis between two groups was performed using Student’s *t* test. Analysis of more than two groups was performed using ANOVA followed by Bonferroni’s multiple comparison test. Samples that did not meet the criteria of the ANOVA were analyzed with the Mann–Whitney *U* test or the Kruskal–Wallis test followed by Dunn’s multiple comparison test, respectively, and *p* values <0.05 were considered significant: *p* < 0.05, ***p* < 0.01, ****p* < 0.001.

### Results

#### Type I IFN responses in *S. pneumoniae*-infected cells were dependent on PLY

To investigate the type I IFN response to *S. pneumoniae* infection, we infected murine BMMs with either D39 wt pneumococci, D39 deficient in PLY, D39 lacking the capsule (CPS), or D39 double-deficient in PLY and CPS. Infection with wt and CPS-negative pneumococci led to a dose-dependent induction of IFN-β mRNA and protein (Fig. 1A, 1B). In contrast, bacteria deficient in PLY failed to stimulate a type I IFN response.

#### Mouse pneumonia model

Animal procedures were approved by local and institutional authorities (LAGeSo Berlin, Charité Berlin). IFNAR−/− and wt mice on the C57BL/6 background were anesthetized by i.p. ketamine (1.6 mg) and xylazine (0.5 mg) and intranasally inoculated with 1 × 106 CFU *S. pneumoniae* serotype 3 (NCTC7978) in 20 μl PBS as described previously (11, 49).

#### Quantitative RT-PCR analysis

Total cellular RNA was isolated, transcribed to cDNA, and amplified by quantitative PCR using Gene Expression Master Mix (Applied Biosystems) and the following primers and probes. hGAPDH: forward 5′-TGAGACTGAGATGAGACCCTCTGGCTGTTTCCCCATTA-3′, reverse 5′-GAAATCAAAGCAATGTTGCTGTT-3′, MIP-2: forward 5′-GGGGAATCATCCAGAGACATCGTTGCTGTT-3′, reverse 5′-GAGGTAGCTGAGGAGAATTCGTTGCTGTT-3′, MIP-1β: forward 5′-AGAGGAAGACATCCTGCTTCGTTTCTTCCATACGCTCTCCCTTTA-3′, reverse 5′-GCTGTTCGTTGCTGTTGCTGTT-3′, hGTPase: forward 5′-AGAGGAAGACATCCTGCTTCGTTTCTTCCATACGCTCTCCCTTTA-3′, reverse 5′-GCTGTTCGTTGCTGTTGCTGTT-3′, hIRF7: forward 5′-AGAGGAAGACATCCTGCTTCGTTTCTTCCATACGCTCTCCCTTTA-3′, reverse 5′-GCTGTTCGTTGCTGTTGCTGTT-3′, hISG15: forward 5′-AGAGGAAGACATCCTGCTTCGTTTCTTCCATACGCTCTCCCTTTA-3′, reverse 5′-GCTGTTCGTTGCTGTTGCTGTT-3′, hIκBα: forward 5′-AGAGGAAGACATCCTGCTTCGTTTCTTCCATACGCTCTCCCTTTA-3′, reverse 5′-GCTGTTCGTTGCTGTTGCTGTT-3′.

#### Supplemented media

For coculture experiments, AECs were seeded on the lower side of Transwell inserts and subsequently cocultured with AECs in a 24-well plate. Coculture was maintained for the next 24 h and then infected with *S. pneumoniae* for 16 h. Postinfection, AECs and AECs containing supernatants were collected. Studies with human AMs and PBMCs were approved by the local ethics committee. Cells were isolated and cultured as previously described (48).
Type I IFN responses to pneumococcal infection appear to be dependent on intracellular recognition of bacterial DNA

Having established that the uptake of bacteria is necessary for triggering the pathway examined, we tested whether the intracellular delivery of bacterial extracts was capable of stimulating IFN-β production. We found that extracts of wt and PLY-negative bacteria were able to elicitable comparable type I IFN responses upon transfection into the cells (Fig. 3A), corroborating our hypothesis that triggering of IFN-β production is mediated by an intracellular sensing mechanism and not solely dependent on PLY. Digestion with DNase, but not RNase A, RNase H, or proteinase K, completely abrogated the IFN-stimulating activity of the bacterial extracts (Fig. 3B). Moreover, transfection of pneumococcal DNA into the BMMs led to a strong IFN-β upregulation (Fig. 3C), suggesting that cytosolic recognition of bacterial DNA is involved in type I IFN responses. To (indirectly) test whether DNA is present in the cytosol during infection, we examined whether AIM2 is activated by S. pneumoniae. AIM2 is a well-characterized actin-polymerization inhibitor cytochalasin D before infection. We found that cytochalasin D treatment strongly reduced the S. pneumoniae-stimulated IFN-β expression (Fig. 2A). Moreover, pneumococci-induced type I IFN responses were also diminished in cells treated with the phagosomal acidification inhibitors bafilomycin A1, chloroquine, or ammonium chloride (Fig. 2B). Similar results were also obtained when using the TIGR4Δcps pneumococcal strain (Fig. 2C, 2D). Thus, the uptake of pneumococci and the acidification of the phagosome are required for triggering IFN-β expression.

Phagocytosis of pneumococci and acidification of the phagosome are required for IFN-β production in S. pneumoniae-infected cells

To assess whether type I IFN responses were indeed dependent on bacterial uptake, BMMs were left untreated or treated with the

![Image](http://www.jimmunol.org/)

**FIGURE 1.** S. pneumoniae infection leads to the induction of type I IFNs dependent on the virulence factor PLY. A, C57BL/6 BMMs were infected with S. pneumoniae D39 wt, Δply, Δcps, or ΔcpsΔply (MOI 0.025 and 2.5) for 6 h. B, BMMs from wt mice were infected with S. pneumoniae D39 wt for 18 h. C, Human AMs were infected with S. pneumoniae D39 wt and Δply (MOI 2.5) for 6 h. D, BMMs from wt mice were infected with S. pneumoniae TIGR4 wt (MOI 0.025 and 2.5) for 6 h. E, BMMs from wt mice were infected with S. pneumoniae TIGR4Δcps or ΔcpsΔply (MOI 0.025 and 2.5) for 6 h. F, BMMs from wt mice were infected with S. pneumoniae D39 wt, D39Δcps, TIGR4 wt, or TIGR4Δcps (MOI 2.5) for 1 h. Intracellular bacteria were measured as described in the Materials and Methods. G, PBMCs were stimulated with recombinant PLY (1 μg per well) or polyI:C (pIC; 0.25 μg per well) for 16 h. IFN-β mRNA levels were determined by quantitative RT-PCR. IFN-β in supernatants was quantified by ELISA. Data shown are representatives of three (A, C–E, G) independent experiments carried out in duplicate (A–C, G) or triplicate (D–F).

![Image](http://www.jimmunol.org/)

**FIGURE 2.** Type I IFN response to pneumococcal infection is dependent on bacterial uptake and phagosomal acidification. A, BMMs were pretreated with 2 μM cytochalasin D for 30 min before infection with S. pneumoniae D39 for 6 h (MOI 0.025 and 2.5). B, BMMs were pretreated with 200 μM bafilomycin A1, 50 μM chloroquine, or 5 mM ammonium chloride for 30 min and then infected with S. pneumoniae D39 for 6 h (MOI 2.5). C, BMMs were pretreated with 2 μM cytochalasin D for 30 min before infection with S. pneumoniae TIGR4Δcps for 6 h (MOI 2.5). D, BMMs were pretreated with 200 μM bafilomycin A1, 50 μM chloroquine, or 5 mM ammonium chloride for 30 min and then infected with S. pneumoniae TIGR4Δcps for 6 h (MOI 2.5). IFN-β mRNA levels were determined by quantitative RT-PCR. Data shown are representative of three (A, B) or two (C, D) independent experiments carried out in duplicate (A, B) or triplicate (C, D).
cytosolic DNA sensor that activates inflammasome-dependent IL-1β production rather than IFN-β responses in bacteria- and DNA virus-infected cells (50–53). Our results show that two siRNAs targeting AIM2 strongly reduced the pneumococci-stimulated IL-1β production in C57BL/6 BMMs, indicating that DNA can in principle be recognized by cytosolic PRRs during pneumococcal infection (Fig. 3D, 3E). Overall, cytosolic recognition of DNA appears to be involved in triggering type I IFN responses in S. pneumoniae-infected cells.

**STING and IRF3 mediate IFN-β production in S. pneumoniae-infected cells.**

Next, we examined the host cell molecules involved in sensing S. pneumoniae leading to type I IFN induction. We found that cells deficient in the TLRs 2, 3, 4, 7, and 9 were fully capable of eliciting an IFN-β response (Fig. 4A), but were defective in producing inflammatory mediators after stimulation with respective TLR agonists (Supplemental Fig. 1A–D). Similarly, cells lacking NOD2 showed an unaltered type I IFN induction after pneumococcal infection (Fig. 4B) but failed to upregulate IL-1β mRNA after MDβ stimulation (Supplemental Fig. 1E). BMMs deficient for the RLR adapter MAVS were fully capable of eliciting an IFN-β response (Fig. 4C), but did not respond to poly(C) or 5′-triphosphorylated RNA (Supplemental Fig. 1F). Considering that DNA sensing might be involved (see above), we tested whether the adapter molecule STING, which is implicated in most cytosolic DNA-sensing pathways, mediates the *S. pneumoniae*-stimulated IFN-β production. Transfection of STING-specific siRNA led to a strong inhibition of STING expression (Fig. 4D) and to a reduced type I IFN response in BMMs infected with D39 wt (Fig. 4E) or TIGR4Δcps (Fig. 4F). In contrast, our RNA interference experiments did not argue for a major role of the DNA sensors DAI and IFI16 in pneumococci-induced IFN-β production (Fig. 4G–J), although a mild reduction of bacteria-stimulated IFN-β production by different siRNAs targeting DAI was repeatedly observed. The type I IFN induction in *S. pneumoniae*-infected cells was, however, dependent on the transcription factor IRF3 (Fig. 4K, 4L). Taken together, the *S. pneumoniae*-stimulated IFN-β production depends on STING and IRF3. The data further suggest that a yet-to-be-identified DNA sensor different than DAI/ZBP1, RNA PolIII–RIG-I, and IFI16 upstream of STING is involved.

**Type I IFNs produced by S. pneumoniae-infected macrophages modulate RANTES production in an autocrine manner.**

In the following, we tested whether autocrinely produced type I IFNs modulate the expression and release of RANTES in mac-
mrophages infected with pneumococci. RANTES is a key chemokine involved in the host defense to pneumococcal pneumonia (54). We infected BMMs from wt or IFNAR-deficient mice with *S. pneumoniae* D39. Our results show that the production of RANTES was significantly reduced in IFNAR-deficient BMMs (Fig. 5A, 5B). Similarly to IFNAR−/− macrophages, IRF3−/− cells produced reduced amounts of RANTES (Fig. 5C, 5D). This reduced RANTES production in IRF3−/− cells, however, was rescued and even enhanced by the addition of IFN-β. Moreover, IFN-β alone was capable of stimulating a strong RANTES production in wt and IRF3−/− BMMs (data not shown). Taken together, IRF3-dependently produced type I IFNs stimulate RANTES synthesis in *S. pneumoniae*-infected macrophages in an autocrine manner via IFNAR.

**IFN-β production of *S. pneumoniae*-infected AMs regulates innate immune responses in AECs**

To test the hypothesis that type I IFNs produced by macrophages after pneumococcal infection influence innate immune pathways in AECs, we conducted coculture experiments. In vitro, primary murine AMs, but not AECs, produced IFN-β upon *S. pneumoniae* infection (Fig. 6A, 6B). Subsequently, wt AMs were cocultured with either wt or IFNAR-deficient AECs. Cocultured cells were infected with *S. pneumoniae*, and gene expression of well-known ISGs and of RANTES in lung epithelial cells was assessed. Our results show that *S. pneumoniae*-stimulated expression of ISG15 and IFN regulatory factor 7 (IRF7) in epithelial cells was dependent on the presence of IFNAR (Fig. 6C, 6D), indicating that type I IFNs produced by the macrophages regulate ISG expression in these epithelial cells. Upregulation of RANTES mRNA in epithelial cells and RANTES protein concentrations in the supernatants of the cocultured cells were significantly reduced by a lack of IFNAR in the AECs (Fig. 6E, 6F). Overall, type I IFNs produced by pneumococci-infected AMs regulate the expression of signaling molecules and the release of RANTES by cocultured AECs.

**RANTES production during pneumococcal pneumonia depends on type I IFNs**

Finally, we examined the role of type I IFNs in regulating RANTES production during pneumococcal pneumonia. Wild-type mice were intranasally infected with *S. pneumoniae*. We observed a time-dependent increase in mIFN-β mRNA expression in mouse lungs (Fig. 7A). To study the regulation of RANTES production in vivo, we infected C57BL/6 wt and IFNAR−/− mice with *S. pneumoniae* for 48 h. RANTES concentration in the bronchoalveolar lavage was strongly reduced in IFNAR−/− mice (Fig. 7B) and basically absent in control mice treated with PBS (data not shown). Thus, type I IFNs regulate RANTES production during pneumococcal pneumonia.
Discussion

In this study, we show that human and murine macrophages produced type I IFNs upon *S. pneumoniae* infection. This response was dependent on bacterial uptake, phagosomal acidification, expression of PLY, a putative cytosolic recognition of DNA, and the host cell molecules STING and IRF3. Type I IFNs produced by macrophages regulated RANTES production within the macrophages in an autocrine manner. Type I IFNs produced by AMs shaped the innate immune response of AECs. Moreover, IFN α/β controlled RANTES production during pneumococcal pneumonia in mice. Our data showed that PLY production by pneumococci was required for IFN-β induction in macrophages, whereas treatment of the cells with purified PLY was not sufficient for activating this pathway. This suggests that PLY itself is not the microbial molecule directly triggering the pathway examined. Rather, it might be involved in the delivery of the type I IFN-stimulating molecule to the cellular compartment where the stimulatory molecule–PRR interaction occurs, as suggested recently for pore-formation toxins of group B streptococci (18). We found that IFN-β production in pneumococci-infected cells was dependent on the bacterial uptake. Moreover, intracellular delivery of *S. pneumoniae* DNA activated a similar type I IFN response, and DNase treatment of pneumococcal extracts abrogated their IFN-β-inducing activity. These experiments indicate that pneumococcal DNA could be the key microbial molecule being sensed by this pathway leading to type I IFN production. Although a clear-cut proof of the relevance of pneumococcal DNA for triggering IFN-β production in infections with viable bacteria is difficult to implement, we provide indirect evidence for DNA being indeed present in the cytosol during *S. pneumoniae* infection and for DNA being sensed by (another) cytosolic DNA sensor (AIM2). This DNA, however, could theoretically also be derived from the host cell, although this seems to be less likely. However, our experiments do not exclude the possibility that sensing of other pneumococcal components, such as cyclic-di-nucleotides, contributes to the type I IFN response in *S. pneumoniae* infection (29, 31). Our data further show that pneumococci-stimulated type I IFN responses were dependent on phagosomal acidification. We speculate that this acidification is involved in the degradation of bacteria within the phagosome leading to the release of DNA, which—via the pore-forming toxin PLY—might be delivered into the host cell cytosol.

IFN-β induction in pneumococcal infection is dependent on STING, a well-characterized adapter molecule downstream of cytosolic DNA sensors, which further supports our conclusion that DNA sensing is critical for triggering this pathway. To date, DAI, RNA PolIII–RIG-I, and IFI16 have been implicated in type I IFN induction upon cytosolic DNA recognition (25–28). Our data with macrophages deficient for the RLR adapter MAVS and macrophages treated with different DAI and IFI16 siRNAs, however, do not argue for a major, nonredundant role of any of these pathways. Pneumococcal DNA might thus be sensed by another yet-to-be-identified cytosolic PRR that signals via STING. Alternatively, the aforementioned pathways could compensate for each other and mediate type I IFN induction during pneumococcal infection cooperatively. Our findings that the *S. pneumoniae*-stimulated type I IFN induction is dependent on PLY and on STING-mediated recognition of bacterial DNA are in agreement with a study that was published when our manuscript was under revision (55).

Similar to D39 pneumococci, TIGR4 bacteria stimulated IFN α/β production depending on bacterial uptake, PLY, and on the host cell molecules STING and IRF3. This type I IFN response induced by TIGR4 wt was, however, much weaker compared with the response to D39 bacteria, which correlated with the bacterial invasiveness into macrophages. We nonetheless think that this type I IFN pathway might be of importance also in infections with TIGR4 pneumococci considering that uptake of these bacteria into macrophages is most likely enhanced by humoral factors during in vivo infections.

Our data indicate that the type I IFNs produced by pneumococci-infected macrophages affect macrophages and neighboring AECs in an autocrine and paracrine manner, leading to an enhanced production of RANTES, which is involved in the host defense to pneumococcal pneumonia (54). We speculate that IFN α/β released by *S. pneumoniae*-infected macrophages binds to IFNAR and activates STAT transcription factors, which in turn enhance transcription of the RANTES promoter (56). In addition, signaling molecules such as IRF7, which are upregulated by paracrine type I IFNs, may enhance the production of inflammatory mediators in cells interacting with pneumococci or neighboring cells. In contrast to this positive regulation of RANTES by type I IFNs, two recent studies showed that type I IFNs negatively regulate KC and CCL2 production in an influenza *S. pneumoniae* coinfection model (57, 58). Collectively, type I IFNs appear to regulate differentially various proinflammatory mediators during infections in the lung, possibly depending on the magnitude of production and on the infection conditions.

Taken together, *S. pneumoniae*-infected macrophages produce type I IFNs dependent on bacterial uptake, expression of bacterial PLY, and a STING- and IRF3-mediated host cell pathway that appears to detect bacterial DNA. Importantly, IFN α/β produced by bacteria-infected macrophages enhances the production of RANTES by the macrophages themselves and by neighboring lung epithelial cells. Type I IFNs are also major regulators of RANTES production during pneumococcal pneumonia in mice.

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Disclosures

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