S. pneumoniae is a common colonizer of the upper respiratory tract, with the potential to cause mild diseases, like otitis media and sinusitis, or life-threatening diseases, such as pneumonia, sepsis, or meningitis. It is estimated by the WHO that more than 800,000 children under the age of 5 years die annually as a consequence of pneumococcal infection (1). The main targets of pneumococcal diseases are young children (1) and people over the age of 65 years (2), immunocompromised individuals, and people infected with HIV (3). Severe and deadly pneumococcal pneumonia also occurs in close temporal proximity after influenza A virus (IAV) infection. We show that IAV infection upregulates TLR3 in DCs, which sensitizes the cells to endosomal pneumococcal RNA. This new insight contributes to unlock the interplay between pneumococci, IAV, and humans.
DCs are a central part of the immune response, because they link innate and adaptive immunity. They are located in the mucosal linings of the lungs and constantly sample antigens. Upon encounter with a pathogen, PRRs are activated and induce the DCs to present large amounts of antigen on their surface and to produce proinflammatory cytokines. DCs are the main producers of interleukin-12 (IL-12), an important proinflammatory cytokine which drives the differentiation of T helper 1 (TH1) cells and induces the production of interleukin-12 (IL-12), an important proinflammatory cytokine. DCs are the main producers of interleukin-12 (IL-12), an important proinflammatory cytokine which drives the differentiation of T helper 1 (TH1) cells and induces the production of interleukin-12 (IL-12), an important proinflammatory cytokine. DCs are the main producers of interleukin-12 (IL-12), an important proinflammatory cytokine which drives the differentiation of T helper 1 (TH1) cells and induces the production of interleukin-12 (IL-12), an important proinflammatory cytokine.

RESULTS

IL-12p70 production by DCs requires internalization of pneumococci into the endosomal compartment and the adaptor molecule TRIF. Cytokine induction was investigated after infection of human DCs with the T4R strain. The nonencapsulated strain T4R was used as an alternative to opsonization of the encapsulated wild-type T4 strain, since it was shown previously that both induce comparable amounts of uptake and cytokine induction in DCs (35). Hence, by using T4R we could avoid introducing a higher degree of complexity into our model. At a low multiplicity of infection (MOI) of 1, we did not observe cytokotoxic effects on DCs (see Fig. S1A in the supplemental material). Additionally, IL-12p70 secretion was abolished in DCs treated with the uptake inhibitors cytochalasin D and wortmannin, indicating that pneumococci activate an intracellular receptor to induce cytokine responses (see Fig. S1B in the supplemental material).

TRIF is an important adaptor molecule for signaling from the endosomal compartment, since it mediates the signals from TLR3 and TLR4 into the cell. We studied the impact of TRIF on IL-12p70 production by silencing TRIF in DCs via small interfering RNA (siRNA). Upon stimulation with T4R, we found that IL-12p70 production was significantly reduced by the TRIF knockdown (Fig. 1A). The TRIF dependency was also confirmed by studying the expression of IL-12p40 using real-time PCR (RT-PCR) (Fig. 1B). The knockdown was confirmed functionally by stimulating DCs with PolyIC (a TLR3 agonist) or R848 (a TLR7/8 agonist), and RT-PCR analysis showed a 60% knockdown of the TRIF transcript (Fig. 1C to E). Our results demonstrated an important role of the endosomal adaptor molecule TRIF for IL-12 production in DCs challenged with T4R.

IL-12p70 production in DCs in response to pneumococci depends on TLR3 but not TLR4. Since TRIF mediates the signals from both TLR3 and TLR4 into the cell and pneumolysin has been suggested to activate TLR4, we first set out to investigate whether TLR4 is activated by T4R in our infection model. We silenced TLR4 with siRNA in DCs and found that IL-12p70 production in response to T4R was independent of TLR4 (Fig. 2A). Similarly, the expression of IL-12p40 did not require TLR4 (Fig. 2B). The knockdown was confirmed functionally by stimulating with lipopolysaccharide (LPS, a TLR4 agonist) or R848, and RT-PCR analysis indicated that 70% of the RNA transcript was silenced (Fig. 2C to E). These data show that IL-12p70 production in DCs does not require the receptor TLR4.

Since IL-12p70 production was independent of TLR4 but required TRIF, we next tested whether pneumococci are recognized by TLR3, an endosomal receptor shown to recognize dsRNA but that has not previously been identified as a receptor for pneumococci. Upon silencing of TLR3 with siRNA, a significant reduction in IL-12p70 production was observed in DCs challenged with T4R (Fig. 2B). In accordance with this finding, the expression of IL-12p40 was reduced by the TLR3 knockdown (Fig. 2C). The knockdown was confirmed functionally by stimulating DCs with PolyIC or R848, and RT-PCR analysis showed that 80% of the transcript was silenced (Fig. 3C to E). To confirm the TLR3 dependency observed in our siRNA studies, we treated DCs with a TLR3/dsRNA complex inhibitor and found a strong reduction in IL-12p70 in response to T4R (Fig. 3F). Additionally, we opsonized the encapsulated strain T4 and tested whether the capsule influenced the recognition of pneumococci by TLR3. We found that the IL-12p70 secretion by DCs infected with T4 could be inhibited by the TLR3/dsRNA complex inhibitor in a similar manner as T4R-infected DCs (Fig. 3G). The inhibitor also reduced IL-12p70 production in DCs stimulated with PolyIC but not in R848-stimulated cells, confirming the specificity of the inhibitor (Fig. 3H and I). Our data showed that pneumococci can be recognized by DCs via TLR3 and its adaptor TRIF and that the TLR3/TRIF axis is important for IL-12p70 production.

Upregulation of TLR3 by IAV leads to increased IL-12p70 production in IAV-pneumococcus coinfections. We previously described a type 1 IFN-dependent cytokine boost in IAV-pneumococcus coinfections. IAV triggers type 1 IFN production in infected DCs, which in turn primes the surrounding DCs to secrete increased amounts of IL-12p70 (33). We set out to inves-
igate whether this cytokine boost is caused by an IAV-triggered upregulation of TLR3 expression. We found an increase in TLR3 expression, which peaked at 8 h post-IAV infection, both in IAV singly infected DCs as well as in DCs first infected with IAV and then 4 h later with T4R (Fig. 4A and B). T4R single infection had no effect on TLR3 expression (Fig. 4A and B). IAV single infection had no effect on IL-12p40 expression, in contrast to coinfection with T4R, where the IL-12p40 level started to increase 4 h after T4R was added to the DCs (Fig. 4C). IL-12p40 expression in T4R singly infected DCs also increased after 4 h, but at a lower level (Fig. 4C). To test whether type I IFNs caused the increased TLR3 expression in coinfected DCs, we also measured TLR3 expression in DCs treated with IFN-\( \alpha \), as the primary stimulus enhanced the IL-12p70 production, and this boost was significantly inhibited by the TLR3/dsRNA complex inhibitor (see Fig. S2B in the supplemental material).

**Pneumococcal RNA is a sufficient stimulus to induce TLR3 activation and IL-12p70 production in human DCs.** To understand whether pneumococcal RNA alone is sufficient to induce IL-12p70 production, total pneumococcal RNA from T4R or RNA digested with a cocktail of the endonucleases RNase A and RNase T1 was transfected into DCs. In combination, RNases A and T1 cleave RNA behind the C, G, and U residues, which results in nearly complete degradation of RNA. Total pneumococcal RNA was indeed a sufficient stimulus to induce IL-12p70 production in DCs, and RNA digested with the cocktail of RNases A and T1 lost its ability to activate DCs, indicating that there were no other components in our RNA preparation that activate the cells (Fig. 5A). To determine whether total RNA activates the same pathway as whole bacteria, DCs were transfected with pneumococcal RNA and also treated with the TLR3/dsRNA complex inhibitor. Stimulation of IL-12p70 secretion by transfected pneumococcal RNA was found to be TLR3 dependent (Fig. 5B). Additionally, HEK293 cells expressing a luciferase reporter system as well as TLR3 or TLR4 were transfected with pneumococcal RNA. A significant increase in luciferase activation was observed in TLR3-expressing, but not in TLR4-expressing, HEK293 cells (Fig. 5C). Furthermore, we used IAV as a primary stimulus and total RNA from T4R as the secondary stimulus, but we could only observe a nonsignificant trend to increased IL-12p70 production relative to bacterial RNA alone (see Fig. S2C in the supplemental material).

Our results showed that pneumococcal RNA can induce production of IL-12p70 by DCs and that pneumococcal RNA can act as a stimulus for TLR3.

**Pneumococcus-induced IL-12p70 production by DCs requires RNA as a signal.** To investigate the role of RNA during the infection of DCs with whole pneumococci, we treated T4R with UV radiation or heat. We found heat treatment, in contrast to UV
treatment, abolished bacterial induction of IL-12p70 (Fig. 6A). Treatment with UV preserved bacterial RNA, whereas the RNA was degraded during heat killing (see Fig. S3A in the supplemental material). Heat, in contrast to UV treatment, is also expected to melt double-stranded regions in RNA. UV-killed T4R triggered IL-12p70 production in amounts comparable to those produced via live T4R, and a 10-fold increase in the MOI of UV-killed bacteria increased cytokine secretion without affecting the viability of DCs (Fig. 6B). Similar to live bacteria, the IL-12p70 production in response to UV-killed bacteria was dependent on bacterial uptake (see Fig. S3B in supplemental material).

To study whether depletion of RNA from whole bacteria could decrease IL-12p70 production, we next treated UV-killed T4R with the cocktail of RNase A and RNase T1 prior to DC challenge. We used UV-killed bacteria for the RNA digestion, since the enzymatic treatment induced death in live bacteria (data not shown), which also could result in reduced cytokine production by DCs. The RNase treatment of UV-killed T4R led to a significantly reduced cytokine secretion by DCs in a dose-dependent manner, and the effect was specific for degradation of RNA, since pretreatment of LPS with the endonucleases did not affect cytokine secretion (Fig. 6C). In contrast, treatment of UV-killed T4R with DNase I did not affect IL-12p70 production (Fig. 6D). Hence, pneumococci equipped with intact RNA activate IL-12p70 production in DCs. Collectively, our results demonstrate that pneumococcal RNA is a stimulus for the TLR3/TRIF pathway, which is required to induce the production of IL-12p70 by DCs during infection with pneumococci, a pathway that can be primed by IAV coinfection.

**DISCUSSION**

Within recent years, bacterial RNA has emerged as an important trigger of host immune responses to sense live bacteria (36), in both TLR3-independent (36–42) and TLR3-dependent manners (43–45). Clearly, the traditional view of TLR signaling and TLR3 as a sensor of viral dsRNA has to be reconsidered. Here, we describe for the first time a TLR4-independent and TLR3/TRIF-dependent IL-12 production in response to pneumococci, and we propose that the activation of TLR3 plays an important role in coinfections of DCs with IAV and pneumococci. The investigated aspects of DC responses to pneumococci and IAV, as well as the proposed mechanisms involved, are summarized in Fig. 7.

The role of pneumolysin as a TLR4 agonist is still being debated in the literature. Depending on the model system and cell type, it has been reported that pneumolysin activates TLR4 and induces the production of cytokines (12–16), whereas other studies show TLR4-independent cytokine production (46–48). Our results demonstrated that IL-12p70 production in human monocyte-derived DCs is independent of cellular expression of TLR4. Furthermore, we showed that bacterial uptake by human DCs is required for IL-12p70 production, as well as the expression of the endosomal receptor TLR3 and its adaptor molecule TRIF. The TLR3 dependence for IL-12p70 production is independent of capsular expression of the bacteria.

In murine-derived DCs infected with T4R, we previously found a much smaller effect on IL-12p70 secretion than in T4R-infected human DCs (35). Additionally, a redundancy of TLRs in vivo has also been found in mice in relation to pneumococcal...
infections for the receptors TLR1, TLR2, TLR4, and TLR6 (17, 49, 50). In these studies, only TLR9 was identified as a nonredundant receptor in a murine pneumococcal pneumonia model (17). Monocyte-derived human DCs do not express TLR9 (reviewed in reference 51), and the effects that we find in vitro might therefore be partially masked in murine in vivo models by the activation of other cell types and other TLRs, especially TLR9, explaining why we see only modest differences in infected TLR3 deficient mice as compared to wild-type mice (data not shown).

Here, we also identified a stimulus for IL-12p70 production by human DCs in vitro. The presence of bacterial RNA was required and sufficient to induce IL-12 responses, and we found that completely digested pneumococcal RNA lost its capacity to induce IL-12p70 secretion. Moreover, we demonstrated that pneumococcal RNA activates TLR3.

We propose that the TLR3-dependent induction of IL-12p70 is of special importance in coinfections with IAV, as severe bacterial diseases, and in particular those caused by pneumococci, often occur 1 to 2 weeks after the onset of IAV infections (5). We previously showed that IAV-infected DCs express type I IFNs, which prime the surrounding DCs to secrete increased amounts of IL-12p70 upon subsequent challenge with pneumococci (33). Viral infection, as well as type I IFNs or Poly(I:C), are known to upregulate TLR3 expression (52–55). The role of TLR3 in IAV infections has been described elsewhere (54), as has the importance of TLR3 for the priming which leads to severe secondary infections with S. pneumoniae (56), but so far the contribution of TLR3 during the secondary infection with pneumococci has not been studied.

Here, we show in vitro that IAV upregulates TLR3 expression in DCs and that the enhanced IL-12p70 production in coinfected cells depends on TLR3. We attribute only a partial role in the upregulation of TLR3 to type I IFNs, since IFN-α alone has no impressive effect on the upregulation of TLR3 in DCs. Therefore, there are likely additional soluble factors involved in the priming of human DCs by IAV.

In summary, we propose a model in which pneumococci are phagocytosed by DCs and degraded in the endolysosomal compartment. The released pneumococcal RNA activates TLR3 and

FIG 3 TLR3 is required for IL-12p70 production by DCs challenged with pneumococci. DCs were treated with siRNA against TLR3 or random control siRNA (A to E) or treated with a TLR3/dsRNA complex inhibitor (F to I). The cells were infected with T4R (A, B, and F), opsonized T4 (G), or stimulated with Poly(I:C) (C and H) or R848 (D and I) to confirm the knockdown. IL-12p70 secretion was measured in the cell culture supernatant by ELISA; each dot represents the results from one donor (A, C, D, and F to I). Fold changes in gene expression of IL-12p40 or TLR3 relative to control cells were measured using RT-PCR (B and E). Graphs show the means ± standard errors of the means of results for 6 (A, C, D, and F to I), 2 (B), or 3 (E) experiments. Statistical analysis was performed using a Wilcoxon matched-pairs signed-rank test (for ELISAs) or Student’s t test (for RT-PCRs). *, P < 0.05. n.s., not significant.
TRIF, which subsequently leads to IL-12 expression and secretion. In the coinfection setting, IAV primes DCs to secrete type I IFNs and other soluble factors, which trigger changes in the surrounding DCs, including enhanced expression of TLR3, which prime the cells to react with increased IL-12p70 production in the secondary infection with pneumococci (Fig. 7).

**MATERIALS AND METHODS**

**Bacterial and virus strains used.** The encapsulated serotype 4 strain TIGR4 (T4; ATCC BAA-334) (57) of *S. pneumoniae* was used, as well as the unencapsulated isogenic mutant T4R (58). Bacteria were grown on blood agar plates at 37°C and 5% CO2 overnight. Colonies were inoculated into C+Y medium and grown until exponential phase (optical density at 620 nm [OD620], 0.5). Dilutions were made to obtain the desired concentration of bacteria, and viable counts were performed to retrospectively confirm the bacterial numbers. The X31 strain of IAV (59) was originally propagated in chicken eggs, purified, and concentrated on a sucrose gradient. The virus was further propagated for one generation in Madine-Darby canine kidney (MDCK) cells, purified, and concentrated on a sucrose gradient (Virapur). Virus titers were determined by performing Avicel (FMC Biopolymer) plaque assays on MDCK cells described elsewhere (60).

**Culturing of human DCs.** Monocytes were purified from buffy coats of healthy donors (Karolinska University Hospital) by using a RosetteSep monocyte purification kit (Stem Cell Technologies) and Ficoll-Hypaque Plus (Amersham Biosciences) gradient centrifugation. Human DCs were seeded at 0.8 x 10^6 to 1.5 x 10^6 cells/ml in R10 (RPMI 1640, 2 mM l-glutamine, 10% fetal bovine serum [FBS]) supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF; 40 ng/ml) and IL-4 (40 ng/ml) (both from PeproTech) for 6 days. Cells were given fresh media and cytokines at a ratio of 1:1 on day 4 and cultured until day 6. The human DC phenotype was assessed by examination of CD11c and CD1a expression via staining with allophycocyanin (APC)-conjugated mouse anti-human CD11c and fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD1a (BD Pharmingen). DCs used in these experiments were above 90% CD1a^+^/CD11c^+^.

**Inhibitors and reagents.** LPS, PolyI:C, R848, cytochalasin D, and wortmannin were purchased from Sigma, and the TLR3/dsRNA complex inhibitor was purchased from PBL Assay Science. Cytochalasin D (0.5 mM), wortmannin (0.1 mM), and the TLR3/dsRNA complex inhibitor (270 Î¼M) were applied 20 min prior to infection. LPS (100 ng/ml), PolyI:C (10 Î¼g/ml), R848 (10 Î¼g/ml), and IFN-Î± (500 U/ml) were used for stimulation of TLR4, TLR3, TLR7/8, and IFNAR, respectively.

**siRNA knockdown.** DCs (6 x 10^6) were electroporated with 5 Î¼M siRNA against TRIF (s45113, s45114, and s45115), TLR4 (s14194, s14195, and s14196), TLR3 (s235 and s236), or random control siRNA (4390843 and 4390846) (all from Life Technologies) on day 4 of DC differentiation. The cells were electroporated with the Bio-Rad Gene Pulser (square wave, 500 V, 0.5 ms with a single pulse), immediately resuspended in fresh culture medium containing IL-4 and GM-CSF, and incubated for a further 2 days.

**In vitro infection of DCs.** DCs were seeded in 96-well plates (1 x 10^5 per well) and exposed to pneumococci. If not otherwise stated, an MOI of 1 was used, and extracellular bacteria were killed with 200 Î¼g/ml gentamicin after 2 h of infection and maintained in culture until the experiment was ended after 18 h. For opsonization, bacteria were incubated in the presence of pneumococcal antiserum for serotype 4 from Statens Serum Institut for the duration of the uptake period. For coinfection experiments, DCs were exposed to IAV at a MOI of 1 under serum-free conditions for 1 h and in the presence of serum for 3 h. Cells were pelleted, medium was removed, and T4R was added in fresh R10 medium. After a 2-h incubation, extracellular bacteria were killed with 200 Î¼g/ml gentamicin and maintained in the cell culture until the experiment was ended after 18 h.

**FIG 4** The role of TLR3 in IAV-pneumococcal coinfection. DCs were infected with IAV and/or T4R. Expression of TLR3 (A and B) or IL-12p40 (C) was measured by RT-PCR at the indicated time points (A and C) or after 8 h (B). DCs were treated with IAV, PolyI:C, or T4R, with or without the TLR3/dsRNA complex inhibitor, as indicated. IL-12p70 secretion was measured in the supernatant by ELISA (D). Values represent means ± standard errors of the means for results from 3 (A), 4 (B), 2 (C), or 6 (D) experiments. Statistical analysis was performed using Student’s t test (for RT-PCRs) or the Wilcoxon matched-pairs signed-rank test (for ELISAs). * P < 0.05. n.s., not significant.
Assessment of cell viability. The influence of pneumococcal infection on DC viability was determined using annexin V-FITC (BD Pharmingen) and the fixable viability dye (FVD) eFluor780 (eBioscience). Cells were infected as previously described, and 18 h after infection the numbers of apoptotic and necrotic cells were determined by staining cells with annexin V-FITC and the FVD eFluor780 in Annexin V buffer and fixation with 2% paraformaldehyde. Cells were assessed by flow cytometry in a Gallios flow cytometer.

RNA isolation, cDNA synthesis, and quantitative RT-PCR. Total cellular RNA was extracted from infected cells using the RNeasy kit (Qiagen). The concentration and purity of isolated RNA were determined spectrophotometrically with the NanoDrop ND 1000 apparatus. cDNA was synthesized from the isolated RNA using the High Capacity cDNA reverse transcription kit (Applied Biosystems). RT-PCR was performed using the iTaq Universal SYBR green supermix (BioRad). Predesigned primer mixes containing forward and reverse primers for the specific RT-PCR target were purchased from Qiagen (QuantiTect primer assay). The following primers were used: TRIF, Hs_TICAM1_1_SG; TLR4, Hs_TLR4_2_SG; TLR3, Hs_TLR3_1_SG; IL-12p40, Hs_IL12B_1_SG; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hs_GAPDH_1_SG. Each primer pair was validated for specificity by performing melt curve analysis of the PCR product to ensure the absence of primer dimers and unspecific products. For each sample, the mRNA expression level was normalized to the level of GAPDH, and relative expression was determined via the \( \Delta \Delta C_T \) method. Each PCR run included no-template controls. Statistical analysis was performed using a Wilcoxon matched-pairs signed-rank test (for ELISAs) or using Student’s \( t \) test (for the luciferase assay). *, \( P < 0.05 \); **, \( P < 0.005 \); n.s., not significant.

Quantification of cytokines. For cytokine assessment in vitro, culture supernatants were harvested 18 h after infection and frozen at \(-20^\circ C\) or used directly for measurement of IL-12p70 production. DCs were challenged with live, UV-killed, or heat-killed (HK) T4R, with live or UV-killed T4R at the indicated MOI (B), with UV-killed T4R (MOI, 10), with LPS pretreated with a cocktail of RNase A (200 to 20 U/ml) and RNase T1 (8,000 to 800 U/ml) (C), or with UV-killed T4R (MOI, 10) pretreated with DNase I (1,000 to 250 U/ml) (D). IL-12p70 production in the cell supernatant was measured in an ELISA (A to D), and DC viability was measured by flow cytometry (B). Values represent means ± standard errors of the means for results from 6 (A and B) or 3 (C) experiments. IL-12p70 secretion was measured in the cell supernatant. Statistical analysis was performed using a one-way analysis of variance and a Bonferroni posttest. *, \( P < 0.05 \); **, \( P < 0.005 \); ***, \( P < 0.0005 \); n.s., not significant.

FIG 5 Purified pneumococcal RNA is sufficient to induce IL-12p70 in DCs and can activate TLR3. DCs were transfected with total RNA from T4R with or without prior digestion with RNase A and T1, or transfected with the enzymes alone, and a proportion of these cells were treated with LPS 4 h posttransfection (A). DCs were transfected with total RNA from T4R and treated with a TLR3/dsRNA complex inhibitor (B). IL-12p70 production was measured in the supernatant by ELISA (A and B). HEK293 cells expressing TLR3 or TLR4 were transfected with total RNA from T4R, stimulated with PolyI:C or LPS, and luciferase activity was measured (C). Values represent means ± standard errors of the means for results from 6 (A and B) or 3 (C) experiments. IL-12p70 secretion was measured in the cell supernatant. Statistical analysis was performed using a Wilcoxon matched-pairs signed-rank test (for ELISAs) or using Student’s \( t \) test (for the luciferase assay). *, \( P < 0.05 \); **, \( P < 0.005 \); n.s., not significant.

FIG 6 RNA is required as a pneumococcal stimulus to induce IL-12p70 production. DCs were challenged with live, UV-killed, or heat-killed (HK) T4R (A), with live or UV-killed T4R at the indicated MOI (B), with UV-killed T4R (MOI, 10), with LPS pretreated with a cocktail of RNase A (200 to 20 U/ml) and RNase T1 (8,000 to 800 U/ml) (C), or with UV-killed T4R (MOI, 10) pretreated with DNase I (1,000 to 250 U/ml) (D). IL-12p70 production in the cell supernatant was measured in an ELISA (A to D), and DC viability was measured by flow cytometry (B). Values represent means ± standard errors of the means for results from 4 (A), 3 (B), 7 (C), or 4 (D) experiments. Statistical analysis was performed using a one-way analysis of variance and a Bonferroni posttest. *, \( P < 0.05 \); **, \( P < 0.005 \); ***, \( P < 0.0005 \); n.s., not significant.
20 U/ml) and RNase T1 (8,000 to 800 U/ml) (Life Technologies) or with DNase I (1,000 to 250 U/ml; Qiagen) in a volume of 50 μl at 37°C for 1 h prior to infection of DCs. Total bacterial RNA was incubated with 25 U/ml RNase A and 1,000 U/ml RNase T1 of the RNase cocktail in a volume of 25 μl for 30 min.

**RNA transfection.** T4R was grown to mid-log phase and treated with phenol. Total RNA was isolated with TRIzol (Life Technologies) followed by DNA digestion with Turbo DNase (Life Technologies). RNA concentration and quality were determined spectrophotometrically with the NanoDrop ND 1000 apparatus, and the absence of DNA contamination was confirmed by PCR. DCs were transfected with 1 μg bacterial RNA per well complexed with N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP; Roche) at a ratio of 1:2.5 and incubated for 18 h. HEK293 cells were transfected with 1 μg RNA per well complexed with Lipofectamine 2000 (Life Technologies) at a ratio of 1:2 and incubated for 6 h.

**Luciferase assay with transfected HEK293 cells.** HEK293 cells stably expressed human TLR4, MD2, and CD14 as well as luciferase under the control of the NF-κB promoter or human TLR3 as well as luciferase under the control of the ELAM promoter (61, 62). The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and penicillin-streptomycin. A total of 2.5 × 10^4 cells were seeded per well of a 96-well plate. After 1 day, the cells were transfected with 1 μg pneumococcal RNA and incubated for 6 h. Cells were lysed, and luciferase activity was measured using the luciferase assay system (Promega) according to the manufacturer’s instructions.

**Visualization of RNA on gels.** Isolated total RNA was loaded on a RNA nanochip and analyzed and visualized with the Agilent 2100 Bioanalyzer.

**Statistical analysis.** Data were statistically analyzed as indicated in the figure legends, using GraphPad Prism 3.04. Significant differences were noted at P levels of <0.05, <0.005, and <0.0005.

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**REFERENCES**


