Role Played by Toll-Like Receptors 2 and 4 in Lipoteichoic Acid–Induced Lung Inflammation and Coagulation

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Background. The cell wall of Streptococcus pneumoniae consists of lipoteichoic acid (LTA), which is released when pneumococci are killed by either the host immune system or antibiotic treatment. Release of excessive amounts of LTA has been implicated in the toxic sequelae of severe gram-positive infection by virtue of its proinflammatory properties. Several in vitro studies have shown that LTA is recognized by Toll-like receptor (TLR) 2 and CD14. Our objective here was to investigate the inflammatory properties of S. pneumoniae LTA in vivo and the role played by TLR2, TLR4, and CD14 therein.

Methods. Wild-type (WT), TLR2 knockout (KO), TLR4 KO, TLR2×4 double-KO, and CD14 KO mice were intranasally inoculated with highly purified pneumococcal LTA.

Results. LTA induced a dose-dependent inflammatory response and activation of the coagulation and fibrinolytic pathways in a TLR2-dependent fashion. Surprisingly, TLR4 KO mice also displayed a somewhat diminished pulmonary inflammatory and coagulant response compared with WT mice, possibly as a result of absent TLR4 signaling through LTA-induced release of endogenous mediators.

Conclusion. Pneumococcal LTA induces a profound inflammatory response and activation of the coagulation pathway in the lungs in vivo through a TLR2-dependent route, which likely is amplified by endogenous TLR4 ligands.

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The in vivo effect of *S. pneumoniae* LTA has never been investigated. Particularly relevant are the effects of *S. pneumoniae* LTA within the intact pulmonary compartment, considering that the pneumococcus is the most common pathogen in community-acquired pneumonia [1, 2]. Therefore, in the present study we sought to determine the effect of highly purified LTA from *S. pneumoniae* Fp23 in the mouse lung in vivo and the role played by CD14, TLR2, and TLR4 therein. We studied the pulmonary effects of pneumococcal LTA not only on lung inflammation but also on bronchoalveolar coagulation, considering that an altered balance between coagulation and fibrinolysis has been implicated in the pathogenesis of pneumonia and lung injury [16, 17] and that staphylococcal LTA has been found to induce procoagulant activity in human mononuclear cells in vitro [18].

**METHODS**

**Animals.** Specific pathogen–free 8–10-week-old C57BL/6 mice (wild type [WT]) were purchased from Charles River. TLR2 knockout (KO) mice and TLR4 KO mice were generated as described elsewhere [19, 20] and were backcrossed to a C57BL/6 genetic background 6 times. TLR2×4 double-KO mice were generated by crossing TLR2 KO and TLR4 KO mice. CD14 KO mice, backcrossed to a C57BL/6 genetic background, were obtained from Jackson Laboratory. All mice were bred in the animal facility of the Academic Medical Center in Amsterdam. In all experiments, age- and sex-matched mice were used. All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam.

**Materials.** LTA from *S. pneumoniae* Fp23 (unencapsulated mutant form of TIGR4) was prepared using butanol extraction and hydrophobic interaction chromatography, as described elsewhere [13]. Contamination of LPS in our LTA preparation was <50 pg of LPS per milligram of LTA, as determined by the chromogenic limulus amebocyte lysate assay. Approximately 1 μg of LTA was purified from 3×10^3–3.5×10^5 cfu of *S. pneumoniae*.

**Experimental design.** Mice were lightly anesthetized by inhalation of isoflurane (Upjohn), after which 50 μL of sterile PBS or LTA dissolved in PBS was administered intranasally. Bronchoalveolar lavage (BAL) was performed as described elsewhere [21]. In early experiments we established that virtually all inflammatory responses strongly decrease beyond the 24-h time point, with the possible exception of leukocyte recruitment (authors’ unpublished data). We therefore chose the 6- and 24-h time points to obtain representative information of cytokine and chemokine release (6 h), activation of coagulation and fibrinolysis (6 and 24 h), and leukocyte recruitment (6 and 24 h).

**Assays.** Tumor necrosis factor (TNF–α), interleukin (IL)–1β, IL–6, and macrophage inflammatory protein (MIP)–2 were measured by ELISA (R&D Systems). The total protein level was measured using a BCA protein kit (Pierce). Thrombin-antithrombin complexes (TATc; Dade Behring), D-dimer (Asserachrom D-dimer; Roche), and plasminogen activator (PA) inhibitor type 1 (PAI-1) [22–24] were measured by ELISA. PA activity was measured as described elsewhere [25]. Myeloperoxidase (MPO) was measured by ELISA (Hycult Biotechnology BV).

**Histopathological analysis.** After lungs were fixed in 10% formalin and embedded in paraffin, 4-μm sections were stained with hematoxylin-eosin or Ly6G monoclonal antibody (Phar- ming) for granulocyte staining, as described elsewhere [26].

**Statistical analysis.** Data were analyzed using GraphPad Prism for Windows (version 4.00; GraphPad Software) and the Mann-Whitney U test or, where applicable, 1-way analysis of variance. Data are expressed as mean ± SE values. Differences were considered statistically significant at *P* < .05.

**RESULTS**

**Dose-dependent inflammatory response to LTA.** To determine the pulmonary inflammatory response to pneumococcal LTA in vivo, we first inoculated WT mice with 0, 10, or 100 μg of LTA via the intranasal route (table 1). BAL fluid (BALF) was harvested 6 h later, because we had previously established that this time point provides representative information on induction of inflammation and coagulation in models of lung inflammation [27–29]. Intranasal inoculation with LTA at 100 μg induced an increase in total cell count, although this increase was not statistically significant. Surprisingly, although neutrophil counts increased dose dependently, macrophage counts decreased dose dependently. The inflammatory response in the lungs, as reflected by neutrophil influx and release of cytokines (TNF–α, IL–1β, and IL–6) and a chemokine (MIP–2) in BALF, increased dose dependently on LTA administration; the responses to 100 μg of LTA were significantly stronger than the responses to 10 μg of LTA. Moreover, 100 but not 10 μg of LTA elicited local activation of coagulation and fibrinolysis, as indicated by increases in BALF concentrations of TATc, PAI-1, and D-and a decrease in the BALF concentration of PA. Further experiments were done with 50 μg of LTA. Considering that the LTA preparation used contained <50 pg of LPS per milligram of LTA (see Methods), 50 μg of LTA contained <2.5 pg of LPS. In separate experiments we established that intranasal administration of 2.5 pg of LPS did not induce neutrophil influx or cytokine or chemokine release in WT mice (data not shown), confirming previous findings from our laboratory [27].

**Role played by TLR2, TLR4, and CD14 in LTA-induced lung inflammation.** To investigate the role of TLR2, TLR4, and CD14 in the pulmonary inflammatory response to *S. pneumoniae* LTA, we inoculated WT, TLR2 KO, TLR4 KO, TLR2×4 double-KO, and CD14 KO mice intranasally with 50 μg of LTA and killed them 6 h later (figures 1 and 2). TLR2 KO and TLR4 KO mice displayed an equally strongly reduced inflammatory response after intrapulmonary delivery of LTA. Neither mouse strain demonstrated neutrophil influx...
(P < .001 vs. WT mice) in BALF, whereas surprisingly more macrophages were retrieved from BALF from these KO strains (P < .001 vs. WT mice, for both). Levels of TNF-α, IL-1β, IL-6, and MIP-2 in BALF from TLR2 and TLR2×4 double-KO mice were strongly diminished (P < .01 to P < .001 vs. WT mice). CD14 KO mice displayed a reduced neutrophil influx (P < .01) and lower IL-1β levels in BALF (P < .05) than did WT mice, whereas BALF TNF-α and MIP-2 concentrations tended to be lower (P = .12 and P = .07, respectively). Neutrophil influx on LTA administration was not significantly altered in TLR4 KO mice. Remarkably, however, TLR4 KO mice did display reduced BALF levels of IL-1β, IL-6, and MIP-2 compared with those in WT mice (P < .05, for all), although the differences with WT mice clearly were not as profound as for TLR2 KO mice. Total protein levels in BALF, indicative of pulmonary vascular leakage, tended to be lower in both TLR2 KO and TLR2×4 double-KO mice (P = .05 and P = .06 vs. WT mice, respectively) but were unaltered in CD14 KO and TLR4 KO mice (mean ± SE for WT, 564 ± 44 μg/mL; for TLR2 KO, 454 ± 30 μg/mL; for TLR4 KO, 631 ± 77 μg/mL; for TLR2×4 double-KO, 465 ± 28 μg/mL; for CD14 KO, 583 ± 45 μg/mL; n = 7–8 per group).

To confirm and extend these data, we conducted additional studies in WT, TLR2, and TLR4 KO mice, obtaining BALF 6 and 24 h after intranasal administration of 50 μg of LTA. Again we

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<th>Table 1. Induction of a dose-dependent inflammatory response by lipoteichoic acid (LTA) in the lungs of wild-type (WT) mice.</th>
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**NOTE.** WT mice were inoculated intranasally with 0, 10, or 100 μg of LTA and killed 6 h later. Data are mean ± SE values (n = 5 per group) and were analyzed by 1-way analysis of variance. IL, interleukin; MIP, macrophage inflammatory protein; PA, plasminogen activator; PAI, PA inhibitor; TATc, thrombin-antithrombin complexes; TNF, tumor necrosis factor.

\(^{a}P < .01\) vs. control.

\(^{b}P < .05\) vs. 10 μg of LTA.

\(^{c}P < .01\) vs. 10 μg of LTA.

Figure 1. Total cell counts, neutrophil counts, and macrophage counts in bronchoalveolar lavage fluid from wild-type (WT) (black bars), Toll-like receptor (TLR) 2 knockout (KO) (white bars), TLR4 KO (gray bars), TLR2×4 double-KO (horizontally striped bars), and CD14 KO (hatched bars) mice 6 h after inoculation with 50 μg of lipoteichoic acid. Data are mean ± SE values (n = 7–8 per group). \(*P < .01\) and \(1P < .001\) vs. WT mice (Mann-Whitney U test).
established that the recruitment of neutrophils into BALF was strongly TLR2 dependent: TLR2 KO mice did not display neutrophil influx at either 6 or 24 h (P < .001 vs. WT mice), whereas the number of neutrophils recovered from BALF from TLR4 KO mice did not differ from that for WT mice (figure 3). Again, macrophage counts were higher in BALF from TLR2 and TLR2×4 double-KO mice (P < .001 vs. WT mice, for both). In addition, to obtain insight into the capacity of LTA to elicit neutrophil degranulation and the role played by TLR2 and TLR4, we measured MPO concentrations in cell-free BALF supernatants. Although MPO was not detectable in BALF from healthy mice (data not shown), LTA induced a time-dependent rise in BALF MPO concentrations, reaching maximal values at 24 h (figure 3). Local MPO release was delayed and strongly attenuated in TLR2 KO mice (P < .001 vs. WT mice). Interestingly, although BALF MPO levels in TLR4 KO and WT mice were indistinguishable at 6 h, at 24 h TLR4 KO mice demonstrated higher BALF MPO levels than did WT mice (P < .05). In these experiments, cytokine and chemokine release elicited by LTA again proved to be largely TLR2 dependent (figure 4). In accordance with the findings presented in figure 2, relative to WT mice, TLR4 KO mice showed reduced cytokine and chemokine release in BALF, significantly so for MIP-2. Figure 5 shows representative slides of lung tissue from WT, TLR2 KO, and TLR4 KO mice obtained 6 and 24 h after inoculation of LTA. As described elsewhere, LTA-induced lung pathology and neutrophil recruitment were reduced in TLR2 KO mice.

Role played by TLR2 and TLR4 in LTA-induced pulmonary coagulation. Finally, to obtain insight into the role played by TLR2 and TLR4 in LTA-induced activation of coagulation and fibrinolysis in the lungs, we measured the levels of TATc, PAI-1, PA activity, and D-dimer in BALF harvested 6 and 24 h after local LTA challenge (figure 6). In WT mice, LTA reproduced the hemostatic alterations found in the lungs of patients with pneumonia, that is, elevated levels of TATc, PAI-1, and D-dimer with concurrently decreased levels of PA activity [30, 31]; these changes were dose dependent (table 1 and figure 6). In TLR2 KO mice, these LTA effects were almost completely absent: BALF levels of TATc, PAI-1, and D-dimer were much lower than in WT mice (P < .01 to P < .001), whereas PA activity remained at ~100% (P < .001 vs. WT mice). TLR4 KO mice displayed a somewhat diminished hemostatic response in their bronchoalveolar space; in particular, BALF TATc concentrations were lower than in WT mice (P < .01 to P < .001), whereas PAI-1 and PA activity were modestly but significantly altered at 24 h (P < .05).

**DISCUSSION**

LTA is an important component of the pneumococcal cell wall and a potent inducer of cell activation in vitro via a TLR2- and partially CD14-dependent route [12, 13]. LTA, which is released when pneumococci are killed by autolysis, host defense mechanisms, antibiotics, or a combination of these, has been implicated in the toxic sequelae of pneumococcal infections [5], but to date no studies of

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**Figure 2.** Role played by Toll-like receptor (TLR) 2, TLR4, and CD14 in lipoteichoic acid (LTA)–induced cytokine release. Cytokine and chemokine concentrations in bronchoalveolar lavage fluid from wild-type (WT) (black bars), TLR2 knockout (KO) (white bars), TLR4 KO (gray bars), TLR2×4 double-KO (horizontally striped bars), and CD14 KO (hatched bars) mice 6 h after inoculation with 50 μg of LTA. Data are mean ± SE values (n = 7–8 per group). *P < .05, †P < .01, and ‡P < .001 vs. WT mice (Mann-Whitney U test). IL, interleukin; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor.

**Figure 3.** Total cell counts, macrophage counts, neutrophil counts, and myeloperoxidase (MPO) concentrations in bronchoalveolar lavage fluid from wild-type (WT) (black bars), Toll-like receptor (TLR) 2 knockout (KO) (white bars), and TLR4 KO (gray bars) mice 6 and 24 h after inoculation with 50 μg of lipoteichoic acid. Data are mean ± SE values (n = 7–8 per group). *P < .05, †P < .01, and ‡P < .001 vs. WT mice (Mann-Whitney U test).
Indeed, stimulation of human peripheral blood mononuclear cells with pneumococcal LTA originally was related to differences in their structures [11, 12]. However, stimulation with pneumococcal LTA [11, 12] was less potent than S. aureus LTA. This difference was attributed to differences in their structures [11, 12].

Several studies have compared the biological potency of pneumococcal LTA in vivo, showing that pneumococcal LTA was less potent than S. aureus LTA; this difference originally was related to differences in their structures [11, 12]. Indeed, stimulation of human peripheral blood mononuclear cells with S. aureus LTA induced more TNF-α production than did stimulation with pneumococcal LTA [11, 12]. However, these earlier investigations used pneumococcal LTA derived from S. pneumoniae strains R6 or R36A. These strains lack D-alanine [13, 32], which appears to be essential for the immunostimulatory potency of LTA: D-alanine containing LTAs from S. aureus and S. pneumoniae Fp23 proved equally potent in inducing cytokine release in human whole blood [13]. Our findings demonstrate comparable biological inflammatory properties of S. aureus and S. pneumoniae Fp23 LTA in mice in vivo:

both S. pneumoniae Fp23 LTA and S. aureus LTA induce a dose-dependent neutrophil influx and cytokine release in BALF [28, 33, 34]. In addition, in some studies S. aureus LTA also induced at least a tendency to decrease the number of alveolar macrophages [34]. Overall, however S. aureus LTA was shown to be more potent than S. pneumoniae LTA in vivo [28, 33].

TLRs are a family of pattern-recognition receptors that are capable of recognizing conserved molecular patterns expressed by pathogens (reviewed in [6]). TLR2 has been implicated as the major pattern-recognition receptor for gram-positive bacteria by virtue of its capacity to recognize products of gram-positive organisms, such as LTA and peptidoglycan [9, 11, 12]. To investigate whether pneumococcal LTA induces a TLR2-dependent inflammatory response in vivo, we inoculated LTA in WT and TLR2 KO mice. Neutrophil recruitment and cytokine and chemokine production were strongly reduced in TLR2 KO mice compared with WT mice. Together with the fact that the early inflammatory response to intact pneumococci in the lower airways depends at least in part on TLR2 signaling [26], these data strongly support a role for LTA in the initiation of lung inflammation during respiratory tract infection by S. pneumoniae. Of note, this early interaction between TLR2 and LTA and possible other TLR2 ligands expressed by S. pneumoniae is not essential for induction of antibacterial defense mechanisms, as indicated by studies from our and another laboratory showing that TLR2 deficiency does not affect the growth of pneumococci or the outcome in mouse models of S. pneumoniae pneumonia [26, 35, 36]. Taken together, these data show that, even though TLR2 can be compensated for by other receptors during pneumococcal pneumonia, recognition of pneumococcal LTA in vivo is clearly TLR2 dependent.

Interestingly, inoculation of pneumococcal LTA in WT mice resulted in a reduced recovery of alveolar macrophages from BALF. It is conceivable that local instillation of LTA into the lungs causes adhesion of alveolar macrophages to the respiratory epithelium, thereby making them less easy to harvest by BAL. Clearly, this response was TLR2 dependent, because it did not occur in TLR2 KO or TLR2×4 double-KO mice. Further studies are warranted to study the mechanisms underlying this phenomenon.

Remarkably, compared with WT mice, TLR4 KO mice tended to display a diminished neutrophil influx and cytokine production 6 h after inoculation of LTA. Moreover, MIP-2 in particular was decreased in TLR4 KO mice compared with WT mice. Earlier studies showed contradictory results regarding the recognition of LTA by TLR2 [8, 11–13, 19, 37], possibly due to contamination of the LTA preparations with LPS [38]. In our study, a role for possible LPS contamination is highly unlikely for several reasons. First, neutrophil influx or cytokine/chemokine production was not induced by inoculation with the LPS dose that, based on the limulus amebocyte lysate assay, could maximally contaminate the LTA preparation, and this observation confirms findings from a previous report [27]. Second, neutrophil recruitment and cytokine and chemokine production were similar in TLR2 and TLR2×4 double-KO mice, which ar-

Figure 4. Role played by Toll-like receptor (TLR) 2 and TLR4 in the early and late inflammatory response to lipoteichoic acid (LTA). Cytokine and chemokine concentrations in bronchoalveolar lavage fluid from wild-type (WT) (black bars), TLR2 knockout (KO) (white bars), and TLR4 KO (gray bars) mice 6 and 24 h after inoculation with 50 μg of LTA. Data are mean ± SE values (n = 7–8 per group). *P < .05 and †P < .001 vs. WT mice (Mann-Whitney U test). IL, interleukin; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor.
guages against LPS-TLR4 signaling. Third, polymyxin B (an established inhibitor of LPS effects) did not influence cytokine release in human whole blood induced by the LTA preparation that we used [13]. Of note, controversy exists about the inflammatory properties of LTA and whether these are at least in part mediated by possible contaminating lipoproteins [39, 40].

A possible explanation for the reduced inflammation in TLR4 KO mice could be the release of endogenous TLR ligands during LTA-induced inflammation. Several such endogenous mediators have been identified as TLR4 ligands, including fragmented hyaluronan, oxidation products, biglycans, and heat-shock proteins (reviewed in [41]); these could synergize in a TLR4-dependent way with LTA to cause an enhanced inflammatory response. However, no one has yet studied whether any of these factors are in fact induced by LTA stimulation. Other evidence for indirect effects of LTA in lungs in vivo comes from our finding of MPO release in BALF. Indeed, pneumococcal LTA [13], like S. aureus LTA [42], did not induce MPO release from isolated neutrophils in vitro. LTA-induced cytokines and chemokines are probably involved in these secondary effects relating to neutrophil degranulation.

CD14 is a glycosylphosphatidylinositol surface-anchored molecule and a pattern-recognition receptor for several conserved bacterial motifs, including LPS, peptidoglycan, and LTA [7, 43, 44]. Membrane-bound CD14 lacks an intracellular domain and requires interaction with other TLRs for signal transduction [45]. CD14 is known to facilitate the recognition of and immune response to LTA in vitro [12]; however, the contribution of CD14 to LTA signaling in vivo was previously unknown. We recently showed that CD14 plays an important role in the pathogenesis of pneumococcal pneumonia by a mechanism that does not rely on TLR signaling: CD14, either cell bound or soluble, facilitated invasive respiratory tract infection by S. pneumoniae [21]. Our current findings demonstrated that the inflammatory response to pneumococcal LTA was only modestly attenuated in CD14 KO mice. Together, these findings suggest that a possible CD14-LTA interaction does not contribute significantly to TLR-dependent lung inflammation during pneumococcal pneumonia.
Infection leads not only to an inflammatory response but also to activation of the coagulation system, which has been considered to reflect an attempt of the host to limit the spread of bacteria and keep the inflammatory reaction local [46]. Local activation of the coagulation system has been implicated in the pathogenesis of bacterial pneumonia [16, 17]. Our laboratory previously showed that both patients and mice with pneumococcal pneumonia display a compartmentalized activation of coagulation, reflected by elevated BALF levels of TATc, with a concurrent inhibition of fibrinolysis within their lungs, reflected by reduced PA activity and elevated BALF PAI-1 concentrations [30, 31]. We have demonstrated here that intrapulmonary delivery of pneumococcal LTA reproduces these findings, implicating this cell-wall constituent as a contributor to the altered hemostatic balance in the lungs during respiratory tract infection with *S. pneumoniae*. Moreover, our data indicate that these local procoagulant responses to LTA are largely TLR2 dependent. The slightly reduced response in TLR4 KO mice may be explained by additional effects of endogenous mediators induced by LTA- TLR2 signaling, which may also play a role in inducing lung inflammation (see above).

It should be noted that the TLR KO mice used in the present study were backcrossed 6 times to a C57BL/6 genetic background, whereas C57BL/6 WT mice were used as controls. Although backcrossing 6 times results in a >99% C57BL/6 back-

Figure 6. Role played by Toll-like receptor (TLR) 2 and TLR4 in the early and late activation of coagulation and fibrinolysis. Bronchoalveolar lavage fluid levels of thrombin-antithrombin complexes (TATc), plasminogen activator inhibitor type 1 (PAI-1), plasminogen activator activity (PAA), and D-dimer in wild-type (black bars), TLR2 knockout (KO) (white bars), and TLR4 KO (gray bars) mice 6 and 24 h after inoculation with 50 μg of lipoteichoic acid. Data are mean ± SE values (n = 7–8 per group). *P < .05, †P < .01, and ‡P < .001 vs. WT mice (Mann-Whitney U test).

In conclusion, we show here for the first time that pneumococcal LTA induces a profound inflammatory response and activation of the coagulation and fibrinolytic pathways in the lungs in a largely TLR2-dependent manner. In addition, we report that, although pneumococcal LTA activates TLR4-deficient cells as potently as WT cells in vitro [12, 13], TLR4 KO mice display a somewhat reduced responsiveness to LTA in vivo, suggesting the involvement of secondary endogenous TLR4 ligands induced by the interaction between LTA and TLR2. These results identify pneumococcal LTA containing D-alanine as a proinflammatory and procoagulant factor during respiratory tract infection with *S. pneumoniae* in vivo.

Acknowledgments

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References


