Immunotolerance during bacterial pneumonia and sepsis
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Lung inflammation induced by lipoteichoic acid or lipopolysaccharide in humans

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Abstract

Recognition of ‘pathogen-associated-molecular-patterns’ by Toll-like receptors (TLRs) is considered to be important for an appropriate immune response against pathogens that enter the lower airways. We studied the effects of two different TLR-agonists relevant for respiratory infections in the human lung: lipoteichoic acid (LTA, TLR2-agonist, component of gram-positive bacteria) and lipopolysaccharide (LPS, TLR4-agonist, component of gram-negative bacteria). Therefore, fifteen healthy subjects were given LPS or LTA: by bronchoscope sterile saline was instilled into a lungsegment followed by instillation of LTA or LPS into the contralateral lung. After 6 hours a bronchoalveolar lavage was performed and inflammatory parameters were determined. Isolated RNA from purified alveolar macrophages was analyzed by multiplex-ligation-dependent-probe-amplification. Additionally, spontaneous cytokine release by alveolar macrophages was measured. Marked differences were detected between LTA- and LPS-induced lung inflammation. Whereas both elicited neutrophil recruitment, only LPS instillation was associated with activation of neutrophils (CD11b surface-expression, degranulation product levels) and consistent rises of chemo-/cytokine levels. Moreover, LPS but not LTA activated alveolar macrophages, as reflected by enhanced expression of ten different mRNAs encoding pro-inflammatory mediators and increased spontaneous cytokine release upon incubation ‘ex vivo’. Remarkably, only LTA induced C5a release. This is the first study to report the in vivo effects of LTA in men and to compare inflammation induced by LTA and LPS in the human lung. Our data suggest that stimulation of TLR2 or TLR4 results in differential pulmonary inflammation, which may be of relevance for understanding pathogenic mechanisms at play during gram-positive and gram-negative respiratory tract infection.
Introduction

Bacterial pneumonia, often caused by gram-positive pathogens, is the most frequent source of sepsis and has a mortality rate of 22% for patients admitted to the Intensive Care Unit. Because of the high incidence of pneumonia and the accompanying high morbidity and mortality, it is important to gain more insight into the pathogenesis of this prominent infectious disease. Phagocytic cells involved in host defense recognize pathogens through highly conserved motifs (pathogen-associated molecular patterns; PAMPs) leading to activation of intracellular signaling cascades and ultimately resulting in a pro-inflammatory response and activation of the innate immune system. Examples of PAMPs include lipopolysaccharide (LPS), part of the outer membrane of gram-negative bacteria, and lipoteichoic acid (LTA), a major constituent of gram-positive bacteria. PAMPs are recognized by ‘pattern-recognition receptors’ (PRRs) displayed by host cells involved in the innate immune response.

Toll-like receptors (TLRs) are important for an appropriate immune response against pathogens that enter the lower airways. While much has been learned about the pulmonary host response to gram-negative infections and the importance of LPS therein, less is known about the innate immune response against gram-positive pathogens. LPS induces lung inflammation via TLR4 and inhalation or bronchial instillation of LPS induced mild inflammation in the bronchoalveolar space of healthy humans. LTA shares many biological properties with LPS and is able to induce the production of a variety of pro-inflammatory cytokines and chemokines by cells of the innate immune system. From in vitro studies it is known that the cellular recognition and signaling receptor for LTA is TLR2. In addition, in vivo experiments conducted in our laboratory have indicated that LTA induces neutrophil influx and cytokine release via TLR2, i.e. TLR2 gene deficient mice did not mount an inflammatory response in the lung to LTA administered via the airways (Knapp S, unpublished data).

Although many investigations have been published on the effects of LPS in humans, the human response to LTA in vivo has never been studied. Knowledge of the effects of LTA in humans is important considering the prominent place of gram-positive pathogens in both community-acquired and nosocomial infections. In the present study we sought to compare the inflammatory responses elicited by LTA and LPS in the human lung, using the well-established model of segmental instillation. Moreover, to determine the possible differential responsiveness of alveolar macrophages, which are considered major effector cells in pulmonary host defense, we also investigated the effects of LTA and LPS on inflammatory gene expression profiles in isolated alveolar macrophages. This study has been described in an abstract.
Chapter 2

Materials and Methods

Subjects
Twenty-three non-smoking males (age 22.0±0.5 yrs) were recruited by advertising. Screening, consisting of a questionnaire, physical examination, routine blood and urine investigation, electrocardiogram and spirometry, did not reveal any abnormality. The study was approved by the institutional ethics and research committees; written informed consent was obtained from all subjects before enrollment in the study.

Materials
LTA, from *Staphylococcus aureus* (DSM 20233), was produced as described\(^26,27\). The LTA preparation was >99% pure, no contamination with lipopeptides was detected using photometric measurements (UV absorption) and NMR examination. LTA contained <50 pg endotoxin/mg LTA as determined by the Limulus amoebocyte lysate assay\(^26,27\). This preparation has been tested extensively for its potency to induce cell activation and inflammation in vitro and in animals in vivo\(^12,17,28\). LPS was derived from *Escherichia coli* (United States Pharmacopeial Convention, Lot G, Bureau of Biologics, United States Food and Drug Administration, Rockville, MD). The in vivo response of humans to this LPS preparation is dependent on TLR4\(^29\).

Study design
Sterile saline (10 ml) was instilled into a lung subsegment (the right middle lobe or lingula) followed by instillation of either LTA or LPS (in 10 ml saline) into the contralateral lung, using a flexible video bronchoscope. First a dose-escalating study was done with LTA, since this compound had never been administered to humans before. Four subjects received LTA 4 ng/kg bodyweight, followed by four subjects receiving 20 ng/kg. After this initial study and based on the degree of inflammatory responses, seven subjects received LTA 100 ng/kg. Eight subjects received LPS 4 ng/kg, which was identical to that used in previous studies\(^9,30\). Vital variables were measured on an hourly basis; volunteers had no complaints and were completely ambulant.

Bronchoalveolar lavage
A bilateral bronchoalveolar lavage (BAL) was performed 6 hours post-challenge in a standardized fashion according to the guidelines of the American Thoracic Society, using a flexible video bronchoscope. Seven successive 20 ml aliquots of pre-warmed 0.9% saline were instilled in the saline-challenged subsegment of the lung and each aspirated immediately with low suction. This procedure was repeated in the LPS- or LTA-challenged subsegment of the contralateral lung. Cell differentials were
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performed on cytopsins stained with a modified Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland).

Assays

Myeloperoxidase (MPO), bactericidal/permeability-increasing protein (BPI), elastase, epithelial cell-derived neutrophil attractant 78 (ENA-78), growth-related gene-alpha (GRO-α), soluble TNF receptor type I (R&D Systems, Minneapolis, MN) and C5a (BD Biosciences, San Diego, CA) were determined by ELISA. IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α were measured using a cytometric bead array. Interferon-γ-inducible protein (IP)-10 and monocyte chemoattractant protein (MCP)-1 were detected in BALF using multiplex bead flow assays (Luminex, Bio-rad Laboratories, Inc., Hercules, CA).

Flow cytometric analysis of neutrophils

Expression of CD11b on neutrophils in BALF was determined by flow cytometric analysis using fluorochrome-conjugated mouse anti-human CD15, HLA-DR and CD14 (BD Pharmingen, San Diego, CA) and CD11b antibody (eBioscience, San Diego, CA) in combination with isotype controls. Neutrophils were selected using side scatter (SSC), CD14 and CD15 (CD14+/CD15+ cells were defined as neutrophils). Using this gate definition, neutrophils can be discriminated from alveolar macrophages (CD14+/CD15-) and lymphocytes (SSC low/HLA-DR+).

Isolation of alveolar macrophages

BAL fluid (BALF) was immediately centrifuged for 10 min at 1200 rpm at 4°C. BALF cells were passed over a 40 µm nylon filter (BD Falcon, Bedford, MA) and resuspended in ice-cold sterile automated magnetic cell sorting and separation (autoMACS) buffer (PBS, 0.5% bovine serum albumin, 2 mM EDTA; pH=7.4). Subsequently, cells were incubated for 15 min with CD71 microbeads (Milenyi Biotec, Bergisch Gladbach, Germany) at 4°C. Cells were washed again in autoMACS buffer and purified by autoMACS (Milenyi Biotec). Total and viable cell counts were determined before and after the isolation procedure using a Burk-Turk haemocytometer and trypan blue (Emergo, Landsmeer, the Netherlands). Additionally, cytopsins were prepared before and after autoMACS and stained with Giemsa. Total and differential cell counts revealed a recovery of 35-40% macrophages and a purity of isolated macrophages of >95% in all groups. After isolation, alveolar macrophages were dissolved in RNeasy lysis Buffer (buffer RLT, QIAGEN, Hilden, Germany) and stored at -80°C until used for RNA isolation.
Multiplex ligation-dependent probe amplification

RNA was isolated (RNeasy Mini kit, Qiagen, Hilden, Germany) and analyzed by multiplex ligation-dependent probe amplification (MLPA) as described previously\textsuperscript{31,32} using an inflammation-specific kit developed in collaboration with MRC-Holland (Amsterdam, the Netherlands) for the simultaneous detection of 40 target genes. Levels of mRNA for each gene were expressed as a normalized ratio of the peak area divided by the peak area of the β2 microglobulin mRNA, to give the relative abundance of mRNAs of the genes of interest\textsuperscript{31,32}.

Spontaneous cytokine release by alveolar macrophages

Purified alveolar macrophages (1x10\textsuperscript{5}) in RPMI 1640 medium (Life Technologies, Rockville, MD) supplemented with 2 mM L-glutamine, penicillin/streptomycin and 10\% FCS were incubated for 20 hours at 37°C and 5\%CO\textsubscript{2} in 24-wells plates (Greiner, Alphen a/d Rijn, Netherlands).

Ex vivo cytokine release by human alveolar macrophages upon increasing doses of LTA and LPS

Purified alveolar macrophages (1x10\textsuperscript{5}) from BALF obtained from saline-stimulated lung segments were stimulated with either 0.1, 1.0 or 10 μg/ml LPS (Ultrapure LPS \textit{E. coli} 0111:B4; Invivogen, San Diego, CA) or 0.1, 1.0 or 10 μg/ml LTA (\textit{S. aureus}; DSM 20233) and incubated for 20 hours at 37°C and 5%CO\textsubscript{2} in 24-wells plates.

Statistical analysis

Values are expressed as mean±SEM. Data were checked for normal distribution and equal variances using the residuals. Depending on the results of these tests, data were analyzed either parametrically or nonparametrically. Statistical comparisons were made by paired t-test or Wilcoxon signed rank test to establish significance between separate datasets. Dose-dependant increases were analyzed by Kruskal-Wallis test (Figure 2.6). These analyses were performed using SPSS (version 12.0.1; SPSS Inc., Chicago, IL). A P-value of less than 0.05 was considered to represent a statistically significant difference.

Results

Clinical signs

Instillation of saline in one lung segment and LTA or LPS in the contralateral lung was well tolerated and was not associated with clear symptoms. A modest rise in body temperature was recorded 6 hours after bronchial instillation, which reached
significance in volunteers who received LPS (0.7 ± 0.2 °C; p<0.05), not in volunteers who received LTA (0.6 ± 0.4 °C). Both LTA and LPS caused a significant rise in neutrophil counts in blood (LTA from 2,6 ± 0,2 to 4,1 ± 0,8 x 10^9/l; LPS from 2,9 ± 0,2 to 4,7 ± 0,5 x10^9/l; both P<0.05). Bronchial instillation of LPS was associated with a modest increase in plasma IL-6 concentrations to 4,5 ± 1,4 pg/ml (P<0.05), whereas plasma IL-6 did not change after administration of LTA (data not shown).

**Leukocyte recruitment**

Instillation of LTA resulted in a profound dose-dependent increase in the total number of cells recruited into BALF 6 hours post-challenge compared to instillation of saline in the contralateral lung (Figure 2.1A). The increase in total cell number was due to a dose-dependent rise in the number of neutrophils (Figure 2.1B; P<0.001 versus saline). Remarkably, instillation of LTA decreased the number of macrophages (Figure 2.1C; P<0.05 versus saline). LPS challenged volunteers developed similar increases in cellularity and neutrophils in the LPS-instilled segments compared with their control segments (Figure 2.1A and 2.1B, P<0.0001 versus saline); however, LPS did not influence the number of macrophages (Figure 2.1C). The number of lymphocytes was not altered after either LTA or LPS instillation (data not shown).

![Figure 2.1](image.png)

**Figure 2.1.** LTA instillation induces a profound dose-dependent recruitment of neutrophils into the bronchoalveolar space.

Total leukocyte counts (A), neutrophil counts (B) and macrophage counts (C) were determined in bronchoalveolar lavage (BALF) fluid 6 hours after instillation of saline in a lung segment and either LTA (4, 20 or 100 ng/kg bodyweight, N=4 for 4 and 20 ng/kg, N=7 for 100 ng/kg) or LPS (4 ng/kg bodyweight, N=8) in the contralateral lung. Open bars indicate neutrophil or macrophage counts after saline instillation, dark grey bars indicate LTA instillation and grey bars indicate LPS instillation. * P<0.05, ** P<0.001, *** P<0.0001 vs. saline.
Neutrophil activation

LPS elicited a rise in the BALF concentrations of the neutrophil degranulation products MPO, BPI and elastase (P<0.05 versus saline; Figure 2.2A-C). LPS-induced neutrophil activation was further illustrated by enhanced CD11b expression on the surface of neutrophils in BALF as measured by flow cytometry (Figure 2.2D; P<0.001 versus saline). These neutrophil responses were highly variable after LTA instillation, and as a consequence none of these neutrophil activation markers were altered by LTA in a statistically significant way.

![Figure 2.2 Neutrophil degranulation after LTA and LPS instillation and LPS-induced up-regulated surface expression of CD11b on neutrophils.](image)

Concentrations of neutrophil degranulation products elastase (A), MPO (B) and BPI (C) were measured in BALF obtained 6 hours after instillation of saline and either LTA (100 ng/kg bodyweight, N=7) or LPS (4 ng/kg bodyweight, N=8) in human volunteers. Expression of adhesion molecule CD11b on the surface of neutrophils was measured by flow cytometry. Open bars indicate saline-instilled lung segments, dark grey bar indicates LTA (100 ng/kg bodyweight) instilled lung segments and grey bar indicates LPS (4 ng/kg bodyweight) instillation. * P<0.05, ** P<0.001 vs. saline.

Chemotactic proteins

LPS elicited the release of CXC chemokines IL-8, GRO-α, and ENA-78 (Figure 2.3A-C; P<0.05 versus saline), whereas LTA showed a trend toward increased chemokine levels, reaching significance for GRO-α only (P<0.05 versus saline). On the contrary, levels of IP-10 (Figure 2.3D), an interferon-γ inducible CXC chemokine not active on neutrophils, and the CC chemokine MCP-1 (Figure 2.3E) increased upon LPS instillation (P<0.005 versus saline) but not LTA instillation. Instillation of LTA resulted in an
increased level of complement factor C5a (P=0.05 versus saline); LPS instillation however, did not influence C5a concentrations (Figure 2.3F).

**Figure 2.3** Increased release of neutrophil attractants upon bronchial challenge with LTA or LPS. Concentrations of CXC chemokines IL-8 (A), GRO-α (B), ENA-78 (C), IP-10 (D), CC chemokine MCP-1 (E) and complement factor C5a (F) were assessed in bronchoalveolar lavage fluid 6 hours after challenge with saline and either LTA (100 ng/kg bodyweight, N=7) or LPS (4 ng/kg bodyweight, N=8). Open bars indicate saline instillation, dark grey bar indicates LTA instillation and grey bar indicates LPS instillation. * P<0.05, ** P<0.01 vs. saline.

**Cytokines**

To further study the innate immune response upon LTA, cytokine expression was measured at the protein level in BALF 6 hours post-challenge. TNF-α concentrations were increased after either LPS or LTA challenge (Figure 2.4A; P<0.05 versus saline), although the former clearly was more potent; soluble TNF receptor type I (Figure 2.4B), IL-1β (Figure 2.4C) and IL-6 (Figure 2.4D) levels only increased after LPS-challenge (P<0.005 versus saline). IL-10 and IL-12 remained undetectable (data not shown).
Gene expression profiles in alveolar macrophages

To reveal a possible differential responsiveness of alveolar macrophages to LPS and LTA in vivo, multiplex ligation-dependant probe amplification was performed on RNA isolated from purified alveolar macrophages harvested 6 hours after instillation of LTA or LPS (Table 2.1). Compared to instillation of normal saline, bronchial instillation of LPS resulted in enhanced alveolar macrophage expression of mRNAs encoding the pro-inflammatory mediators IL-6, IL-8, IL-1α, IL-1β, IL-1RA, MCP-1, MIP-1α and MIP-1β (P<0.01 versus saline). Moreover, LPS upregulated the expression of mRNAs encoding proinflammatory signaling factors IkBα and nuclear factor kappa-B subunit 1 (NFκB1). In contrast, no significant increases were observed after instillation of LTA. Macrophage expression of mRNAs encoding the inflammatory mediators TNF-α, IFN-γ, IL-10, IL-12, IL-18, MCP-2 and NFκB2 were either very low or undetectable (data not shown).

Figure 2.4 Enhanced cytokine response upon LTA and LPS bronchial challenge. TNF-α (A), soluble TNF receptor type I (B), IL-1β (C) and IL-6 (D) concentrations were measured in BALF obtained 6 hours after instillation of saline and either LTA (100 ng/kg bodyweight, N=7) or LPS (4 ng/kg bodyweight, N=8) in human volunteers. Open bars indicate saline instillation, dark grey bar indicates LTA instillation and grey bar indicates LPS instillation. * P<0.05, ** P<0.01 vs. saline.
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Table 2.1 mRNA expression profile of alveolar macrophages after LTA or LPS instillation.

<table>
<thead>
<tr>
<th>Group</th>
<th>LTA 100 ng/kg body weight</th>
<th>LPS 4 ng/kg bodyweight</th>
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<tbody>
<tr>
<td></td>
<td>saline</td>
<td>LTA</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.07 ± 0.05</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>IL-1α</td>
<td>0.35 ± 0.13</td>
<td>0.58 ± 0.17</td>
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<td>IL-1β</td>
<td>0.29 ± 0.15</td>
<td>0.81 ± 0.31</td>
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<tr>
<td>IL-1ra</td>
<td>0.63 ± 0.22</td>
<td>2.34 ± 0.98</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.36 ± 0.22</td>
<td>1.31 ± 0.55</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>0.41 ± 0.24</td>
<td>0.98 ± 0.35</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1.13 ± 0.64</td>
<td>2.28 ± 0.81</td>
</tr>
<tr>
<td>IkBα</td>
<td>0.26 ± 0.05</td>
<td>0.45 ± 0.13</td>
</tr>
<tr>
<td>NFκB1</td>
<td>0.19 ± 0.05</td>
<td>0.25 ± 0.04</td>
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LTA, lipoteichoic acid; LPS, lipopolysaccharide. RNA was analyzed by multiplex ligation-dependent probe amplification (MLPA) after isolation from alveolar macrophages purified from bronchoalveolar lavage fluid 6 hours after instillation of saline in a lung subsegment and LTA (100 ng/kg bodyweight, N=7) or LPS (4 ng/kg bodyweight, N=8) in the contralateral lung of human volunteers. Data are means ± SEM. * P<0.01 vs. saline; ** P<0.001 vs. saline.

Ex vivo cytokine release by alveolar macrophages

The gene expression profiles of alveolar macrophages purified from subjects challenged with either LPS or LTA, together with the (relative) inability of LTA to induce cytokine release in BALF in vivo, suggested that alveolar macrophages might be less activated by LTA. To obtain further proof for this we incubated purified alveolar macrophages harvested 6 hours after LPS or LTA instillation for 20 hours at 37°C and measured spontaneous cytokine release in the supernatants. Due to the relatively low yield of alveolar macrophages after LTA instillation (most cells were used for RNA isolation), these incubations could only be done for 3 LTA challenged subjects (versus 8 LPS instilled subjects). When compared to alveolar macrophages harvested from the saline control lung, alveolar macrophages from the LPS challenged lung spontaneously released significant quantities of IL-1β, IL-6 and IL-8, but not of TNF-α (Figure 2.5). In contrast, alveolar macrophages obtained from the LTA instilled lung released only small amounts of all 4 cytokines in all 3 subjects (Figure 2.5). Remarkably, in LTA challenged subjects a variable response was seen with regard to alveolar macrophages from the saline control lung, where 2 of 3 subjects showed enhanced release of cytokines. Of note, however, due to the low number of observations no firm conclusions can be drawn with regard to the activation state of alveolar macrophages harvested from the saline control lung in LTA challenged subjects.
Figure 2.5  Spontaneous cytokine release by purified alveolar macrophages. IL-1β (A, B), IL-6 (C, D), IL-8 (E, F) and TNF-α (G, H) concentrations were measured in supernatant of 20h-incubated purified alveolar macrophages harvested from BALF 6 hours after instillation of saline and either LTA (100 ng/kg bodyweight, N=3) or LPS (4 ng/kg bodyweight, N=8) in human volunteers. * P<0.05, ** P<0.01 vs. saline.

Ex vivo dose response to LTA and LPS of human alveolar macrophages

Following the observation that alveolar macrophages might be less or not responsive to in vivo LTA stimulation, we stimulated normal human alveolar macrophages ex vivo with either 0.1, 1.0 and 10 μg/ml LPS or with 0.1, 1.0 and 10 μg/ml LTA. In concordance with the in vivo findings, alveolar macrophages did not produce or hardly produced any cytokines upon ex vivo stimulation, whereas a significant dose
dependant increase in cytokine production could be found upon ex vivo LPS stimulation (Figure 2.6).

![Figure 2.6](image)

**Figure 2.6** Ex vivo LPS- and LTA-induced cytokine release by purified human alveolar macrophages. TNF-α (A) and IL-6 (B) concentrations were measured in supernatant of 20h-incubated purified alveolar macrophages harvested from BALF 6 hours after instillation of saline (N=8). * P<0.05, ** P<0.01 for dose-dependant increase in one group, analyzed by Kruskal-Wallis.

**Discussion**

Bacterial pneumonia is one of the most common infectious diseases and the leading cause of sepsis. In light of the prominent attribution of gram-positive pathogens pneumonia, we were interested to investigate the effect of LTA, an important proinflammatory component of gram-positive bacteria, in the human lung. This study reports for the first time a detailed analysis of the in vivo effects of LTA on lung inflammation in human volunteers. Our results demonstrate that LTA instillation into a pulmonary segment results in localized inflammation, manifested by neutrophil influx and elevated levels of neutrophil attractants, in the absence of detectable activation of alveolar macrophages. Previously, enhanced inflammatory responses in the lung have been observed in response to local administration of LPS in vivo9,11. We here demonstrate that the inflammatory profile upon LTA instillation clearly differs from local LPS challenge.

The LTA used in this study was purified based on a relatively novel technique making use of a gentle extraction procedure using butanol27. Many studies involving LTA, especially those using commercially available preparations, are confounded by contaminating products33 and by the fact that purification methods using a phenol extraction step result in decomposition of LTA especially in the loss of its alanine substituents27. The bioactive LTA preparation used here activated monocytes to release cytokines and is free of impurities such as LPS27.
In the LTA dose escalation study we sought to find a dose at which LTA elicited a comparable influx of neutrophils into the bronchoalveolar compartment as LPS given at a dose (4 ng/kg) used in previous studies in human subjects. This dose response study established that 100 ng/kg LTA was required to provoke neutrophil influx to a similar extent as LPS. This difference in relative potency between LTA and LPS was expected in light of earlier studies examining the pulmonary effects of these bacterial constituents in mice. Of note, in this research model we also demonstrated that instillation of either LTA or LPS in the human lung resulted in activation of coagulation and inhibition of fibrinolysis.

In line with previous studies, the number of macrophages recovered from BALF was not influenced 6 hours after LPS challenge. Remarkably, instillation of LTA tended to decrease the number of macrophages compared to saline, an effect that already was apparent at the lowest LTA dose. Two studies in mice documented a modest decrease in the number of alveolar macrophages after intrapulmonary delivery of LTA, although this was not found in other murine studies. Although a clear explanation for this phenomenon is lacking, it is possible that LTA induces increased adhesiveness of alveolar macrophages to the respiratory epithelium thereby reducing their recovery during BAL. Our finding that LPS stimulation caused neutrophil activation as shown by the release of degranulation markers elastase, MPO and BPI, confirms previous studies. LTA, however, only showed a tendency towards elevation. It remains to be established whether highly purified LTA can directly activate neutrophils. Whereas some investigations reported activation of purified human neutrophils upon exposure to LTA in vitro, another study was not able to detect such an effect. Of note, neutrophils do express the receptors required for LTA signaling, i.e. CD14, TLR2 and TLR6.

Surprisingly, unlike LPS, LTA elicited modest inflammatory responses in the contralateral saline-challenged lung, which was especially true for neutrophil influx and C5a release. Previously, others have demonstrated that focal lung injury often results in neutrophil emigration at distant sites. Focal instillation of Staphylococcus pneumoniae induced neutrophil emigration in the contralateral region in rabbits. Motosugi et al. showed that focal hydrochloric (HCl) aspiration induced adhesion-complex CD11b/CD18-independent neutrophil emigration on the side of the lung damage and CD11b-dependant neutrophil emigration in the contralateral lung in rats. In the current study, neutrophils obtained from both the saline- and LTA-challenged lung segments in LTA-volunteers showed equal CD11b neutrophil surface expression. However, in LPS-volunteers CD11b neutrophil surface expression was profoundly upregulated in the LPS-challenged lung compared to their saline-challenged lung segment. This finding suggests that LTA- and LPS-induced neutrophil adhesion and migration are differentially mediated in the human lung, which is in line with animal experiments showing that gram-negative bacteria elicit neutrophil emigration.
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requiring CD11b/CD18-adhesion complex, while neutrophil emigration induced by gram-positive bacteria does not require the CD11b/CD18 adhesion pathway in the lung.

Since instillation of LTA caused only moderate if any elevations in the BALF levels of neutrophil active chemokines, it is conceivable that the strongly increased C5a concentrations contributed importantly to the LTA-induced neutrophil recruitment. Interestingly, C5a levels were not influenced by LPS instillation. Since C5aR is mainly expressed by bronchial and alveolar epithelial cells in the human lung, it is tempting to speculate that activation of epithelial cells plays an important role in LTA-induced inflammation. In this respect it is interesting to note that LTA can activate respiratory epithelial cells via the platelet activating factor receptor. This notion is further corroborated by the absence of significant proinflammatory gene expression in alveolar macrophages obtained from LTA challenged lung segments, as well as by the absence of spontaneous cytokine release by these same cells upon ex vivo culture. IP-10 production upon LPS instillation is in line with the fact that IP-10 is produced TRIF/IRF3-dependently and is consequently not increased after TLR2 activation by LTA.

To obtain insight into the specific role of alveolar macrophages in LTA-induced lung inflammation, we determined expression of mRNAs encoding a series of inflammatory mediators in isolated alveolar macrophages. Remarkably, mRNA expression of pro-inflammatory mediators IL-6, IL-1α, IL-1β, IL-8, MCP-1, MIP-1α, MIP-1β and signaling factors IκBα and NFκB1 were not upregulated in alveolar macrophages from LTA-volunteers, whereas the mRNAs encoding these mediators were significantly upregulated in LPS-volunteers. The latter is in line with a previous study wherein healthy subjects inhaled aerolized LPS. Clearly, alveolar macrophages play a different role in LTA versus LPS signaling. In accordance with our previous human study, LPS instillation resulted in detectable TNF-α protein levels in BALF in the absence of detectable TNF-α mRNA levels in alveolar macrophages. Conceivably, TNF-α mRNA was expressed by alveolar macrophages at time points earlier than 6 hours and/or by other cell types.

The current study has several limitations. First, the model of bronchial instillation involves the administration of relatively low doses of LTA and LPS into a single lung segment, which differs significantly from the clinical setting of acute lung injury or pneumonia. Furthermore, the (relative) unresponsiveness of alveolar macrophages to LTA might be a matter of the dose of LTA used in this study. However, this is contradicted by the significantly enhanced neutrophil influx upon LTA instillation (Figure 2.1B) and the inability of human alveolar macrophages to produce cytokines upon incubation with high doses of LTA ex vivo (Figure 2.6). Secondly, a kinetic analysis over multiple time-points of especially LTA effects would be of considerable...
interest, but we chose not to expand the number of time-points in light of the invasive procedure to obtain BALF samples. This is also the reason why a relatively limited number of volunteers was studied. Although in particular the LTA challenge studies would have benefited from a larger number of volunteers, an estimated 30-150 subjects would have been required to have at least 80% power to obtain statistical differences compared to saline effects with regard to distinct inflammatory responses such as neutrophil degranulation and cytokine/chemokine release (based on the interindividually variability in these inflammatory responses). Moreover, one should realize that the inflammatory response 6 hours post-challenge is likely to be a combination of the inflammatory response generated by the initial stimulus and then amplified by secondary endogenous inflammatory mediators generated during the inflammatory response. At present it is unclear whether gram-positive and gram-negative bacteria induce distinct pulmonary inflammatory responses in humans in vivo. Of note, however, intact bacteria express multiple PAMPs that can interact with different TLRs, making a direct comparison with responses to purified TLR2 and TLR4 agonists difficult.

In conclusion, the present study is the first to provide insight into the in vivo effects of LTA in the human lung. Further studies are needed to evaluate the specific contribution of macrophages and respiratory epithelium in LTA-induced inflammation. This novel human model may be used to evaluate pathogenic mechanisms at play during gram-positive respiratory tract infection.
References


