Immunotolerance during bacterial pneumonia and sepsis
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Chapter

Priming of alveolar macrophages upon instillation of lipopolysaccharide in the human lung

JJ Hoogerwerf, AF de Vos, C van ‘t Veer, P Bresser, A de Boer, MWT Tanck, C Draing, JS van der Zee, T van der Poll

Abstract

The airways are continuously exposed to respiratory pathogens, which may result in bacterial pneumonia, one of the most common infectious diseases and the leading cause of sepsis. Considering that recurrent exposure to microbial products can lead to tolerance of immune cells and that this might contribute to the susceptibility to nosocomial infection, we investigated the effect of in vivo lipopolysaccharide (LPS) instillation on the responsiveness of alveolar macrophages. In eight healthy humans sterile saline was instilled into a lung segment by bronchoscope, followed by instillation of LPS into the contralateral lung; 6 hours later a bilateral bronchoalveolar lavage was performed and purified alveolar macrophages were ex vivo stimulated with LPS or lipoteichoic acid (LTA), triggering Toll-like receptor (TLR) 4 and 2 respectively. In vivo LPS-exposed alveolar macrophages were primed as reflected by increased ex vivo LPS- and LTA-induced interleukin-1β and -6 gene expression and production compared to in vivo saline-exposed alveolar macrophages. LPS instillation did not influence the surface expression of TLR4 or TLR2. Furthermore, LPS instillation did not impact on the expression of a number of extracellular and intracellular regulators of TLR signaling. However, p38 mitogen-activated protein kinase (MAPK) remained phosphorylated in alveolar macrophages upon LPS instillation. The current data demonstrate that LPS instillation in the human lung primes alveolar macrophages for further stimulation with either LPS or LTA possibly by sustained p38MAPK activation.
Introduction

Bacterial pneumonia is one of the most common infectious diseases and the leading cause of sepsis. Recognition of pathogens in the alveolar compartment by Toll-like receptors (TLRs) results in activation of immune responses and elimination of pathogens. Immune responses in the lung are tightly regulated, since the lungs are continuously exposed to environmental pollutants and respiratory pathogens and must restrain excessive inflammatory responses to prevent tissue damage. Disproportionate dampening of the immune response will lead to an increased susceptibility to nosocomial infections in critically ill patients. This immune dysregulation is - amongst other mechanisms - thought to result from hyporesponsiveness of immune cells upon recurrent multiple exposures to microbial products, often referred to as tolerance to endotoxin (lipopolysaccharide; LPS). In recent years, various negative regulators of TLR signaling have been shown to contribute to endotoxin tolerance, including ST2, A20, IL-1R-associated kinase (IRAK)-M, MyD88 short (MyD88s), MAP kinase phosphatase (MKP)-1 and suppressor of cytokine signaling (SOCS)-3. Regulation of Nuclear Factor kappa B (NF-κB) and p38 mitogen-activated protein kinase (MAPK) further influences the ability to produce cytokines. Importantly, A20 is a direct negative feedback inhibitor of TLR induced NF-κB signalling by the rapid transcription of A20 upon NF-κB activation and subsequent blockade of TRAF6 by A20. The phosphatase MKP-1 is a highly dynamic and crucial regulator of p38MAPK activity by the direct dephosphorylation of phospho-p38MAPK by MKP-1. In addition, triggering receptors expressed by myeloid cells (TREM) can impact on TLR signalling by macrophages: whereas TREM-1 is able to amplify TLR signalling, TREM-2 can inhibit cellular activation by TLR ligation.

Many investigations have been published on endotoxin tolerance of monocytes in the systemic compartment during human sepsis, trauma or major surgery. In contrast, however, alveolar macrophages, obtained from the lung during a systemic inflammatory response induced by intravenous LPS or sepsis, do not display tolerance and may even be primed for ex vivo rechallenge, suggesting that endotoxin tolerance is a compartmentalized phenomenon. Although alveolar macrophages pre-exposed to LPS or Escherichia coli in vitro produce diminished levels of cytokines on subsequent challenge with LPS, hence are “tolerized”, contrasting results have been reported in models investigating the influence of an in vivo local challenge in the lung on the responsiveness of alveolar macrophages. In the present study, we sought to investigate the influence of in vivo LPS challenge on alveolar macrophages in humans, using the well-established model of bronchial instillation.
Materials and Methods

Subjects

Eight non-smoking males (age 21.3 ± 0.6 yrs, mean ± SEM) 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 subjects were part of a simultaneous study investigating the inflammatory response upon bronchial instillation of LPS or LTA in the human lung21, which was approved by the institutional ethics and research committees. Written informed consent was obtained from all subjects before enrollment in the study.

Study design

Ten milliliters of sterile saline was instilled into a lung subsegment (either the right middle lobe or lingula) followed by instillation of LPS (in 10 ml saline) from *Escherichia coli* (United States Pharmacopeial Convention, Lot G, Bureau of Biologics, United States Food and Drug Administration, Rockville, MD) into the contralateral lung, using a flexible video bronchoscope. The subjects were randomized to left or right lungs for saline or LPS instillation. The subjects received LPS at a dose of 4 ng/kg body weight, which was identical to that used in previous studies22,23. 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-challenged subsegment of the contralateral lung. Cell differentials were performed on cytospins stained with a modified Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland).

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 (Miltenyi Biotec, Bergisch Gladbach, Germany) at 4°C. Of note, our laboratory previously reported that bronchial LPS instillation does not influence CD71 expression by alveolar macrophages24. Cells were
washed again in autoMACS buffer and purified by autoMACS (Miltenyi Biotec). Total and viable cell counts were determined before and after the isolation procedure using a Burker-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 either incubated in RPMI 1640 medium (Life Technologies, Rockville, MD) supplemented with 2 mM L-glutamine, penicillin, streptomycin and 10% FCS for further stimulation or dissolved in RNAeasy lysis Buffer (buffer RLT, QIAGEN, Hilden, Germany) and stored at -80°C until used for RNA isolation. Hence, in each subject alveolar macrophages were harvested from a lung segment instilled with LPS (further indicated by LPS-AM) and from a contralateral lung segment instilled with sterile saline (further indicated by saline-AM).

Ex vivo stimulation of alveolar macrophages

1 x 10^5 alveolar macrophages (either LPS-AM or saline-AM) were stimulated with 0.1, 1 or 10 μg/ml LPS (from Escherichia coli O55:B5; 200 ng/ml; Sigma-Aldrich) or 0.1, 1 or 10 μg/ml lipoteichoic acid (LTA) and medium control for 2 or 20 hours at 37°C and 5% CO₂ in 24-wells plates (Greiner, Alphen a/d Rijn, Netherlands). The LTA preparation (from Staphylococcus aureus (DSM 20233) was >99% pure and was prepared as described before21,25. After 2 hours stimulation, alveolar macrophages were dissolved in RNAeasy lysis Buffer (buffer RLT, QIAGEN, Hilden, Germany) and RNA was isolated using RNeasy Mini kit (QIAGEN, Hilden, Germany).

Assays

Interleukin (IL)-1β, IL-6, IL-10, IL-12p70 and tumor necrosis factor (TNF)-α were measured in supernatants from 20 hour ex vivo stimulations using a cytometric bead array (BD biosciences, San Diego, CA).

Multiplex ligation-dependent probe amplification

Expression of TNF-α, IL-1β and IL-6 mRNA’s was analyzed by multiplex ligation-dependent probe amplification (MLPA) as described previously24,26-28. All samples were tested with the same batch of reagents. 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 interest24,26-28. LPS did not influence β2 microglobulin mRNA expression (data not shown).
p38MAPK and NF-κB phosphorylation

Total cell lysates of isolated alveolar macrophages were prepared using a Bio-Plex cell lysis kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions and were stored at minus 80°C for further analysis. Phosphorylation of p38MAPK and NF-κB was analyzed using Bio-Plex multiplex beads detecting NF-κBp65-phosphoSer326 and p38MAPK-phosphoThr180/Tyr182 in combination with the Bio-Plex phosphodetection reagent kit (Bio-Rad Laboratories, Hercules, CA). Bead fluorescence was evaluated in a Luminex 100TM analyzer (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions. Data are presented as the ratio of phosphoprotein to total cellular protein.

Flow cytometric analysis of alveolar macrophages

Expression of CD14, TLR4, TLR2, TREM-1, TREM-2 and ST2 on alveolar macrophages was analyzed using unseparated BALF cells by flow cytometric analysis using fluorochrome-conjugated mouse anti-human CD14, CD15, HLA-DR (BD Pharmingen, San Diego, CA), TLR4 and TLR2 antibody (eBioscience, San Diego, CA), TREM-1, TREM-2 (R&D systems, Minneapolis, MN) and ST2 antibody (MBL, Woburn, MA) in combination with isotype controls. Alveolar macrophages were selected using side scatter (SSC), HLA-DR and CD14 (HLA-DR+/CD14+ cells were defined as macrophages). Using this gate definition macrophages can be discriminated from neutrophils (HLA-DR-/CD15+) and lymphocytes (SSC low/HLA-DR+).

Evaluation of mRNA levels by quantitative Real-Time PCR

Isolated RNA was treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed using oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) according to recommendations of the suppliers. Real-Time (RT)-PCR was performed on cDNA samples that were 4-fold diluted in H₂O using FastStart DNA Master SYBR Green I (Roche Diagnostics) with 2.5 mM MgCl₂ in a LightCycler (Roche Diagnostics) apparatus. Primers used are indicated in Table 4.1 and were added to a final concentration of 500 nM. PCR conditions were: 5 min 95°C hot start, followed by 40 cycles of amplification (95°C for 15 s, 60°C for 5 s, 72°C for 20 s). For quantification, standard curves were constructed by PCR on serial dilutions of a concentrated cDNA and data were analyzed using the LightCycler software as described by the manufacturer. Gene expression is presented as a ratio of the expression of the house keeping gene β2 microglobulin²⁸,²⁹.

Statistical analysis

Values are expressed as means ± 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. 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.

Table 4.1 Primer sequences used for Real-Time PCR using the LightCycler.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Fragment Size</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A20</strong></td>
<td>Forward, TCCAGAACACCATTCCGTG</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td>Reverse, TGAGGTGCTTTTGTTGGTTTC</td>
<td></td>
</tr>
<tr>
<td><strong>IRAK-1</strong></td>
<td>Forward, GTACATCAGACGCGGAAGGC</td>
<td>186</td>
</tr>
<tr>
<td></td>
<td>Reverse, AGTGGCTTGCTGGGTCTTCT</td>
<td></td>
</tr>
<tr>
<td><strong>IRAK-M</strong></td>
<td>Forward, GTACATCAGACGCGGAACCT</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>Reverse, GACATGAATCCAGGCTCTCT</td>
<td></td>
</tr>
<tr>
<td><strong>MKP-1</strong></td>
<td>Forward, CAACCCAGAACGACACATCA</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>Reverse, CTCGGCTCTGTGGCTCACAA</td>
<td></td>
</tr>
<tr>
<td><strong>MyD88</strong> (short specific)</td>
<td>Forward, GGGACACAGCATTGGGCATA</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>Reverse, ACATTCCCTGCTTGAGGTT</td>
<td></td>
</tr>
<tr>
<td><strong>SOCS-3</strong></td>
<td>Forward, CGACTGAGACGACAGAACC</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td>Reverse, GAGGAGGTGCTCAGATGG</td>
<td></td>
</tr>
<tr>
<td><strong>β2-microglobulin</strong></td>
<td>Forward, CTGGCGCTACTCTCTCTTCTTCT</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>Reverse, TGCTCCACCTTTTTTCTCTCT</td>
<td></td>
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</table>

Results

*Ex vivo* LPS-induced cytokine release and gene expression by alveolar macrophages after *in vivo* bronchial instillation of LPS or sterile saline

To determine the effect of pre-exposure to LPS *in vivo* on the capacity of alveolar macrophages to produce cytokines upon *ex vivo* restimulation with LPS, alveolar macrophages were isolated from BALF obtained from lung segments instilled with either saline (saline-AM) or LPS (LPS-AM) *in vivo* 6 hours earlier and incubated with either control medium or LPS for 2 (for cytokine gene expression) and 20 hours (for release of cytokines). Upon incubation with medium only, LPS-AM displayed an increased spontaneous release of IL-1β and IL-6 (but not TNF-α) when compared with saline-AM (Figure 4.1A, C, E). Both saline-AM and LPS-AM produced increased amounts of TNF-α, IL-6 and IL-1β upon *ex vivo* stimulation with LPS (10 µg/ml) as compared to incubation with medium only, although for LPS-AM the difference between IL-1β release upon incubation of medium or LPS did not reach statistical significance (P=0.07)(Figure 4.1A, C, E). Notably, *ex vivo* LPS-induced levels of IL-1β and IL-6 by LPS-AM were much higher than those measured after LPS stimulation of saline-AM (Figure 4.1C and E). The capacity to release TNF-α upon incubation with LPS *ex vivo* did not differ between LPS-AM and saline-AM (Figure 4.1A). *Ex vivo* LPS
stimulation of saline-AM did not lead to enhanced mRNA expression of either TNF-α, IL-1β or IL-6 (Figure 4.1B, D, F). In contrast, relative to saline-AM, LPS-AM displayed significantly enhanced mRNA expression of IL-6 and IL-1β, both after incubation with medium and after re-stimulation with LPS (10 µg/ml) ex vivo; in addition, re-stimulation of LPS-AM with LPS induced increased IL-1β and IL-6 mRNA expression when compared to incubation with medium only (Figure 4.1D and F). Ex vivo stimulation with 0.1 and 1 µg/ml LPS resulted in similar results (data not shown). IL-10 and IL-12p70 levels were below detection in all samples (data not shown). Thus, the spontaneous release of IL-1β and IL-6 by LPS-AM was stronger than that by saline-AM, and LPS-AM demonstrated an enhanced capacity to further increase IL-1β and IL-6 production upon ex vivo restimulation when compared with saline-AM. Indeed, analysis of LPS-induced IL-1β and IL-6 release relative to spontaneous release of these cytokines (i.e. LPS-induced cytokine levels minus levels after incubation with medium only) revealed that both IL-1β and IL-6 release elicited by LPS stimulation ex vivo was higher by LPS-AM than by saline-AM (IL-1β: 35 ± 11 vs. 3 ± 1 pg/ml, P<0.05; IL-6: 378 ± 94 vs. 112 ± 31 pg/ml, P=0.05). Together these results suggest that in vivo exposure to LPS primes macrophages in the alveolar compartment, as reflected by increased ex vivo LPS-induced IL-1β and IL-6 gene expression and production.

Ex vivo LTA-induced cytokine release and gene expression in alveolar macrophages after in vivo bronchial instillation of LPS or sterile saline

To investigate whether pre-exposure to LPS in vivo also primes alveolar macrophages for further stimulation by other TLR agonists, saline-AM and LPS-AM were stimulated ex vivo with LTA (TLR2 agonist) or incubated with medium only. Saline-AM produced increased amounts of TNF-α upon stimulation with LTA 10 µg/ml ex vivo (Figure 4.2A), whereas LTA did not induce IL-1β or IL-6 release from saline-AM (Figure 4.2C, 4.2E). LTA stimulation modestly enhanced the secretion of TNF-α and IL-1β by LPS-AM when compared with incubation with medium only (not significant; Figure 4.2A, 4.2C). In contrast, LTA strongly enhanced IL-6 release by LPS-AM relative to that detected after incubation of LPS-AM with medium only (Figure 4.2E). Ex vivo LTA stimulation of saline-AM did not result in enhanced mRNA expression of TNF-α, IL-1β or IL-6 (Figure 4.2B, 4.2D, 4.2F). In contrast, in LPS-AM LTA increased IL-1β mRNA expression relative to incubation with medium only (Figure 4.2D). Ex vivo stimulation of alveolar macrophages with 0.1 and 1 µg/ml LTA did neither induce cytokine release nor enhanced mRNA expression (data not shown). When corrected for higher spontaneous release of IL-1β and IL-6 by LPS-AM (by subtracting cytokine concentrations measured after incubation with medium only from those detected after incubation with LTA), IL-6 release elicited by LTA stimulation ex vivo was higher by LPS-AM when compared with saline-AM (IL-6: 231 ± 101 vs. 21 ± 11 pg/ml, P<0.05), whereas IL-1β release upon ex vivo LTA stimulation tended to be higher (11 ± 4 vs. 0.5 ± 0.2 pg/ml, P=0.09).
Figure 4.1  Ex vivo LPS-induced cytokine release and gene expression by alveolar macrophages after in vivo instillation of LPS or sterile saline in the bronchoalveolar space.

Alveolar macrophages, obtained from bronchoalveolar lavage fluid 6 hours after instillation of saline in a lung segment and LPS (4 ng/kg body weight, N=8) in the contralateral lung, were incubated ex vivo with 10 µg/ml LPS or medium only. TNF-α (A), IL-6 (C) and IL-1β (E) were determined in supernatants after 20 hours stimulation. mRNA expression of TNF-α (B), IL-6 (D) and IL-1β (F) was determined in RNA isolated from alveolar macrophages after 2 hours stimulation. Open dots indicate in vivo saline challenged alveolar macrophages, filled dots indicate in vivo LPS challenged alveolar macrophages. † indicates P<0.05 vs. ex vivo medium control (RPMI); * indicates P<0.05 vs. ex vivo saline-challenged control.
Figure 4.2  Ex vivo LTA-induced cytokine release and gene expression by alveolar macrophages after in vivo instillation of LPS or sterile saline in the bronchoalveolar space.

Alveolar macrophages, obtained from bronchoalveolar lavage fluid 6 hours after instillation of saline in a lung segment and LPS (4 ng/kg bodyweight, N=8) in the contralateral lung, were incubated ex vivo with 10 µg/ml LTA or medium only. TNF-α (A), IL-6 (C) and IL-1β (E) were determined in supernatants after 20 hours stimulation. mRNA expression of TNF-α (B), IL-6 (D) and IL-1β (F) was determined in RNA isolated from alveolar macrophages after 2 hours stimulation. Open dots indicate in vivo saline challenged alveolar macrophages, filled dots indicate in vivo LPS challenged alveolar macrophages. † indicates P<0.05 (‡‡ P<0.001) vs. ex vivo medium control (RPMI); * indicates P<0.05 vs. in vivo saline-challenged control.
Phosphorylation of transcriptional factors in alveolar macrophages

Enhanced cytokine production may be the result of augmented gene transcription and/or prolonged mRNA stability, which is regulated by NF-κB and p38MAPK respectively. Therefore, we investigated the effect of bronchial LPS instillation on the phosphorylation of NF-κB and p38MAPK in alveolar macrophages. Remarkably, p38MAPK was still robustly activated in alveolar macrophages 6 hours after in vivo LPS challenge (Figure 4.3A). However, NF-κB phosphorylation was not increased upon LPS challenge compared to saline (Figure 4.3B).

Figure 4.3 p38MAPK and NFκB phosphorylation in alveolar macrophages upon instillation of LPS or sterile saline in the bronchoalveolar space.
Six hours after instillation of saline in a lung segment and LPS (4 ng/kg bodyweight, N=8) in the contralateral lung, bronchoalveolar lavage fluid was obtained and isolated alveolar macrophages were stored as cell lysates. Phospho-p38MAPK (A) and pNFκB (B) levels were determined by a Luminex based Multiplex assay (FI/ml). Data are presented as the ratio phosphoproteins to total protein levels x 10^(-2). ** indicates P<0.005 vs. saline.

Bronchial instillation of LPS does not influence surface expression of CD14, TLR4 or TLR2 on alveolar macrophages

One mechanism by which pre-exposure of alveolar macrophages to LPS in vivo could enhance their responsiveness to LPS and LTA, is increased expression of TLR4 (LPS receptor) or TLR2 (LTA receptor) on LPS-AM relative to saline-AM. To address this possibility we analyzed the surface expression of TLR4 and TLR2, together with the ligand binding part of the LPS receptor complex CD14, on alveolar macrophages harvested from lung segments instilled with saline or LPS in vivo by flow cytometry. LPS instillation did not alter CD14, TLR4 or TLR2 surface expression on alveolar macrophages (Figure 4.4).
Chapter 4

Figure 4.4 Expression of CD14, TLR4 and TLR2 on alveolar macrophages upon instillation of LPS or sterile saline in the bronchoalveolar space.

Six hours after instillation of saline in a lung segment and LPS (4 ng/kg bodyweight, N=8) in the contralateral lung, bronchoalveolar lavage fluid was obtained and CD14 (A), TLR4 (B) and TLR2 (C) surface expression was determined on alveolar macrophages by flow cytometry. Differences between LPS and saline challenged alveolar macrophages were not significant.

Bronchial instillation of LPS does not influence surface expression of TREM-1, TREM-2 or ST2 on alveolar macrophages

Several macrophage surface receptors have been identified that influence the response to TLR ligation. Among these, TREM-1 has been shown to amplify TLR signaling \(^{30,31}\), whereas TREM-2 \(^{32}\) and ST2 \(^{33}\) can inhibit TLR signaling. Therefore, we were interested in the potential impact of bronchial LPS instillation on the expression of these surface receptors on alveolar macrophages. However, LPS instillation did not influence the expression of TREM-1, TREM-2 or ST2 on alveolar macrophages when compared with saline instillation (Figure 4.5).
Figure 4.5  Expression of TREM-1, TREM-2 and ST2 on alveolar macrophages upon instillation of LPS or sterile saline in the bronchoalveolar space.
Six hours after instillation of saline in a lung segment and LPS (4 ng/kg bodyweight, N=8) in the contralateral lung, bronchoalveolar lavage fluid was obtained and TREM-1 (A), TREM-2 (B) and ST2 (C) surface expression was determined on alveolar macrophages by flow cytometry. Differences between LPS and saline challenged alveolar macrophages were not significant.

Expression patterns of intracellular regulators of TLR-cascade in alveolar macrophages

Several intracellular regulators have been identified that serve to inhibit TLR signaling. We were interested in the effect of bronchial LPS in the expression of these negative TLR regulators in alveolar macrophages and therefore determined mRNA expression of IRAK-M, MyD88s, MKP-1, SOCS-3 and A20 in alveolar macrophages purified from BALF obtained 6 hours after LPS or saline instillation. In addition, we analyzed mRNA expression of IRAK-1, which is an important positive mediator of signaling through both TLR4 and TLR2. In vivo LPS challenge did not alter the mRNA expression of IRAK-M, IRAK-1, MyD88s, MKP-1 or SOCS-3 (Figure 4.6A-E). Remarkably, A20 mRNA expression was upregulated in alveolar macrophages upon in vivo LPS challenge (Figure 4.6F).
Figure 4.6  Gene expression patterns of regulators of TLR signaling in alveolar macrophages upon instillation of LPS or sterile saline in the bronchoalveolar space.

Six hours after instillation of saline in a lung segment and LPS (4 ng/kg bodyweight, N=8) in the contralateral lung, bronchoalveolar lavage fluid was obtained and RNA was isolated from alveolar macrophages. mRNA expression of IRAK-M (A), IRAK-1 (B), MyD88s (C), MKP-1 (D), SOCS-3 (E) and A20 (F) was determined by quantitative Real-Time PCR using the LightCycler. Gene expression is presented as a ratio of the expression of the house keeping gene β2 microglobulin $\times 10^{-3}$. * indicates P<0.05 vs. saline.

Discussion

The airways are continuously exposed to respiratory pathogens, which may cause bacterial pneumonia when pathogens pass innate defenses in the upper airways and enter the alveolar space. Considering that recurrent exposure to microbial products can lead to tolerance of immune cells and that this might render the host more susceptible to nosocomial infections, we were interested to investigate the effect of
an *in vivo* LPS challenge into the human bronchoalveolar space on the responsiveness of alveolar macrophages to subsequent *ex vivo* stimulation with LPS or LTA. Our results demonstrate that LPS instillation into a pulmonary segment did not render alveolar macrophages less responsive to either LPS or LTA, but rather primed these cells, as manifested by enhanced cytokine production.

The results of the current study should be considered in the context of previously reported investigations. Endotoxin tolerance has been well-described in various animal and human models and in a number of cell types, including monocytes and macrophages. The vast majority of *in vivo* studies reported on tolerance of circulating immune cells upon induction of systemic inflammation. Evidence indicates that alveolar macrophages may respond differently to repeated exposure to bacterial antigens when compared with macrophages from other body sites. Indeed, whereas intravenous administration of LPS to humans results in a diminished capacity of circulating leukocytes to produce proinflammatory cytokines upon restimulation with LPS *ex vivo*, it has been found to lead to priming of alveolar macrophages, as reflected by increased *ex vivo* LPS-induced IL-1 release. Similarly, Fitting et al. demonstrated that alveolar macrophages could escape the tolerization process after intravenous administration of LPS in mice. These studies provided evidence that a systemic LPS challenge does not “tolerize” alveolar macrophages, but may even prime them for enhanced cytokine production in the lungs. Investigations that studied the responsiveness of alveolar macrophages after direct pre-exposure to LPS or other proinflammatory stimuli (*i.e.* *in vitro* or within the bronchoalveolar space *in vivo*) have yielded variable results. LPS stimulation of rabbit or rat alveolar macrophages following *in vitro* pre-exposure to LPS or *E. coli* rendered these cells tolerant for further stimulation. Furthermore, alveolar macrophages obtained from patients with community-acquired pneumonia were shown to be hyporesponsive to further stimulation with LPS. In contrast, alveolar macrophages obtained from mice with acute lung injury following intratracheal hydrochloric acid administration were shown to be primed upon secondary LPS challenge. In line with the latter investigation, our results show that human alveolar macrophages exposed to LPS *in vivo* were primed, rather than “tolerized”, as shown by increased *ex vivo* LPS-induced IL-1β and IL-6 production. In accordance with a primed state, LPS-AM, but not saline-AM, displayed an increase in IL-1β and IL-6 mRNA upon incubation with LPS *ex vivo*.

TNF-α release was not influenced by pre-exposure to LPS. In line, TNF-α release by alveolar macrophages was not significantly increased upon *ex vivo* LPS stimulation following intravenous LPS challenge and no increase in TNF-α was found in BALF of guinea pigs after recurrent intranasal administration of LPS. The differential results on TNF-α production and that of IL-1β and IL-6 by LPS-AM might be influenced by augmented gene transcription and/or prolonged mRNA stability. p38MAPK has been shown to be involved in stabilizing mRNAs encoding IL-6, IL-1β and TNF-α.
Unstimulated macrophages display repressed stability and ensuing rapid degradation of mRNAs of these cytokines, whereas stimulation of these cells leads to activation of p38MAPK, which stabilizes mRNAs of multiple stress-induced factors. Our results show a strong sustained phosphorylation of p38MAPK in alveolar macrophages. Although our data do not provide direct proof, it is tempting to speculate that the increase in spontaneous IL-6, IL-1β and to a lesser extent TNF-α mRNA expression upon LPS instillation may be partially due to p38MAPK dependent enhanced mRNA stability. Different than IL-6 and IL-1β, the somewhat enhanced mRNA expression of TNF-α is not followed by increased cytokine production. The latter may be due to the fact that transcription of TNF-α is more dependant on activation of transcription factor NF-κB, which was unaltered in alveolar macrophages.

Additional evidence that pre-exposure to LPS in vivo results in an increased responsiveness of alveolar macrophages was provided by the ex vivo stimulations of LPS-AM with LTA. Indeed, whereas saline-AM did not respond to LTA stimulation, LPS-AM showed a strongly increased IL-6 release, suggesting that both TLR4 and TLR2 stimulation (such as induced by LPS and LTA respectively) was associated with enhanced responsiveness of these cells. We investigated several mechanisms that might have contributed to this finding. We did not detect enhanced expression of CD14, TLR4 or TLR2 at the surface of LPS-AM, making this an unlikely explanation for the data generated upon ex vivo stimulation. Recently, our laboratory reported on the expression of multiple TLR regulators in blood leukocytes upon intravenous injection of LPS. LPS tolerance observed in this systemic model was associated in time with an increased expression of IRAK-M mRNA with a concurrently decreased expression of IRAK-1 mRNA. Induction of IRAK-M in pulmonary macrophages has also been implicated in an impaired capacity to mount an effective inflammatory response to respiratory tract infection by Pseudomonas in mice suffering from sublethal peritonitis. In our study, bronchial LPS instillation did not result in significant changes in the expression of IRAK-M or IRAK-1; moreover, the levels of SOCS3 and MyD88s mRNAs were not altered in LPS-AM when compared with saline-AM. MKP-1, a phosphatase involved in regulation of p38MAPK activity, was not altered. In this respect it is interesting to note that in vitro induced p38MAPK phosphorylation by LPS in murine alveolar macrophages is fiercely counteracted by MKP-1. The sustained p38MAPK activation in our alveolar macrophages obtained after LPS segmental lung challenge was not associated with MKP-1 transcription. Just recently it has been recognized that acetylation of MKP-1 is an important issue in LPS mediated responses of macrophages. Potentially alveolar macrophages may have a deficit in vivo in their capacity to acetylate MKP-1 which would allow increased p38MAPK phosphorylation, as we observed in our study. Obviously, our studies prompt in depth investigations on the in vivo regulation of p38MAPK activity and its inhibitor MKP-1 in alveolar macrophages.
Of the different negative TLR regulators studied only A20 was significantly upregulated in LPS-AM. Expression of A20 is directly controlled by NF-κB dependent transcription. Thus, the increased expression of A20 indicates earlier NF-κB activity in these LPS-AM. A20 is essential in the termination of TLR responses\textsuperscript{10} which is executed by a rapid 50-100 fold increase of A20 transcription. Thus the ~2.3 fold increase in A20 transcription in LPS-AM in our study should probably be interpreted as the lingering expression of an earlier A20 peak that apparently succeeded in attenuation of NF-κB activity to baseline levels.

Finally, bronchial LPS instillation did not influence the surface expression of ST2, TREM-1 or TREM-2 on alveolar macrophages. Of these, ST2 and TREM-2 have been found to inhibit TLR signaling\textsuperscript{32,33}, whereas TREM-1 can amplify TLR responses\textsuperscript{30,31}. Previously, our laboratory reported increased TREM-1 expression on blood leukocytes of humans intravenously injected with LPS\textsuperscript{42} and patients with sepsis caused by the gram-negative bacterium \textit{Burkholderia pseudomallei}\textsuperscript{47}. However, pulmonary macrophages of mice infected with \textit{Burkholderia pseudomallei} showed reduced rather than increased TREM-1 expression\textsuperscript{47}. Together, these data suggest that TREM-1 expression by alveolar macrophages is not easily altered by infectious or inflammatory stimuli and is unlikely to explain our results obtained with LPS-AM. Notably, to the best of our knowledge our study is the first to report on TREM-2 expression by human alveolar macrophages.

Our results indicate that alveolar macrophages recovered from LPS-challenged lung segments exhibited greater spontaneous mRNA expression and production of IL-6 and IL-1β than did macrophages from the saline-challenged lung segments. This finding represents an \textit{in vivo} activated state of alveolar macrophages within the LPS-challenged lung segment, which we have described previously in this model\textsuperscript{21} and is further supported by previous studies reporting increased spontaneous cytokine release by alveolar macrophages upon intravenous LPS administration\textsuperscript{5} and in patients suffering from community-acquired pneumonia\textsuperscript{19}.

The current study has several limitations. First, the model of bronchial instillation involves the administration of relatively low doses of LPS into a single lung segment, which differs significantly from the clinical setting of acute lung injury or pneumonia. Secondly, studies were done with alveolar macrophages obtained at one time point (6 hours) after bronchial instillation with LPS. Although a kinetic analysis over multiple time-points would be of considerable interest, we chose not to expand the number of time-points in light of the invasive procedure to obtain BALF samples. Third, our study does not provide direct evidence for a mechanism underlying the priming effect of LPS on alveolar macrophages. In this respect it should be noted that interventional studies, such as inhibition of p38 MAPK, are difficult to perform in healthy humans for ethical reasons, while \textit{in vitro} investigations using alveolar macrophages may not
adequately reproduce the effects of in vivo exposure to LPS\textsuperscript{17,18}. With respect to the latter, it is possible that the priming effect of LPS in vivo at least in part is caused indirectly by an effect on epithelial cells and/or neutrophils, resulting in the release of mediators that subsequently impact on alveolar macrophages responsiveness. Finally, our study does not exclude a possible confounding immune enhancing effect of the CD71 microbeads used to purify alveolar macrophages.

In conclusion, our data demonstrate that LPS instillation in the human lung primes alveolar macrophages for further stimulation with either LPS or LTA. This is in line with previous reports stating that alveolar macrophages cannot be “tolerized” in vivo and supporting the suggestion that tolerance is a compartmentalized phenomenon\textsuperscript{5,48}. In theory, the absence of tolerance upon a primary stimulus in the lung could be beneficial for host defence against pneumonia; on the other hand, the increased responsiveness of alveolar macrophages may render the host more vulnerable to acute lung injury. Further studies are needed to evaluate the mechanisms underlying the enhanced responsiveness of alveolar macrophages upon bronchial instillation of LPS.
Priming of alveolar macrophages upon instillation of lipopolysaccharide in the human lung

References


