Host-pathogen interactions during (myco)bacterial respiratory tract infections
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Differential roles of CD14 and Toll-like Receptors 4 and 2 in murine *Acinetobacter* pneumonia

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Abstract

Rationale: *Acinetobacter baumannii* is an opportunistic bacterial pathogen that is increasingly associated with Gram-negative nosocomial pneumonia, but the molecular mechanisms that play a role in innate defenses during *A. baumannii* infection have not been elucidated.

Objective: To gain first insight into the role of CD14, Toll-like receptor 4 and 2 in host response to *A. baumannii* pneumonia.

Methods: Respective gene-deficient mice were intranasally infected with *A. baumannii* and bacterial outgrowth, lung inflammation and pulmonary cytokine/chemokine responses were determined. To study the importance of lipopolysaccharide in the inflammatory response, mice were also challenged with *A. baumannii* lipopolysaccharide.

Measurements and Main Results: Bacterial counts were increased in CD14 and Toll-like receptor 4 gene-deficient mice and only these animals developed bacteremia. The pulmonary cytokine/chemokine response was impaired in Toll-like receptor 4 knock-out mice and the onset of lung inflammation was delayed. In contrast, Toll-like receptor 2 deficient animals displayed an earlier cell-influx into lungs combined with increased macrophage inflammatory protein-2 and monocyte chemoattractant protein-1 concentrations, which was associated with accelerated elimination of bacteria from the pulmonary compartment. Neither CD14 nor Toll-like receptor 4 gene-deficient mice responded to intranasal administration of lipopolysaccharide, whereas Toll-like receptor 2 knock-out mice were indistinguishable from wild-type animals.

Conclusions: Our results suggest that CD14 and Toll-like receptor 4 play a key role in innate sensing of *A. baumannii* via the LPS moiety, resulting in effective elimination of the bacteria from the lung, whereas Toll-like receptor 2 signaling seems to counteract the robustness of innate responses during acute *A.baumannii* pneumonia.
Introduction

Members of the genus *Acinetobacter* have recently gained increased recognition as bacterial pathogens that have the potential to cause severe infections in critically ill patients in intensive care units (1, 2). One of the species within the genus, *A. baumannii*, has gained particular notoriety as one of the leading causes of opportunistic nosocomial infections worldwide (3-7). The predominant site of *A. baumannii* infection is the pulmonary compartment and 15-25% of ventilator-associated pneumonias (VAP) are attributable to this pathogen (8, 9). The crude mortality of VAP due to *A. baumannii* has been shown to be as high as 75% (8, 10). There is additional evidence that *A. baumannii* induces community-acquired pneumonia, predominately among young alcoholics in tropical climates (11). Pneumonia induced by *A. baumannii* is frequently associated with a sudden and severe onset that in most cases requires mechanical ventilation and systemic complications including septic shock have been repeatedly described (11). The high rate of antibiotic resistance and widespread colonization of skin, mucosal membranes and medical equipment makes *A. baumannii* a pathogen of high importance and concern (12-18). Given the increased clinical importance of *A. baumannii* and the lack of knowledge regarding host defense mechanisms against this opportunistic pathogen, we developed a murine model of *A. baumannii* pneumonia to address this.

The first line of defense against invading bacteria is provided by the innate immune system, which recognizes pathogen-associated molecular patterns, conserved microbial patterns shared by large groups of pathogens, but not found in higher eukaryotes (19-21). In recent years it has become evident that both the recognition and the subsequent response to pathogens are mainly transferred by members of the Toll-like receptor (TLR) family (for review see: (22-25)). Of the 11 described TLRs, TLR4 and TLR2 are the key receptors signaling the presence of bacteria. TLR4 signaling is triggered by the interaction with lipopolysaccharide (LPS), the major cell-wall component of Gram-negative bacteria (26). CD14, a glycosylphosphatidylinositol-anchored molecule, is an important player in the LPS-signaling process, by enhancing LPS binding to MD-2 (27). This process, in turn, enables LPS binding to TLR4. In the absence of either CD14 or TLR4 the LPS-induced inflammatory responses are greatly reduced (28, 29). TLR2, in contrast to TLR4, has received attention primarily as an important pattern recognition receptor for Gram-positive bacteria, although it might also contribute to the host innate immune defense against Gram-negative pathogens (30-34). TLR2 recognizes peptidoglycan and lipoproteins, which are major constituents of the cell-wall of Gram-positive bacteria but, to a lesser degree, are also present in Gram-negative microorganisms. Here, we provide first insight into the role of these important signaling receptors in *A. baumannii* pneumonia.
Materials and Methods

Mice
Pathogen-free 7-9 wk-old C57/BL6 mice were obtained from Harlan Sprague-Dawley (Horst, the Netherlands), CD14 gene deficient (CD14−/−) mice from Jackson Laboratories (Bar Harbor, ME) (35). TLR4−/− and TLR2−/− mice were generated as described (29, 32). All mice were bred in the animal facility of the Academic Medical Center in Amsterdam and backcrossed 6 times to C57/BL6 background. Age and sex matched C57/BL6 wild type mice were used as controls. The institutional Animal Care and Use Committee approved all experiments.

Induction of pneumonia
*Acinetobacter baumannii* (strain RUH 2037, allocated to the European clone I, (36)) was isolated during an *Acinetobacter* outbreak in 1986 from sputum of a patient suffering from pneumonia. Bacteria were grown to midlogarithmic phase at 37°C using Luria Bertani broth (Difco, Detroit, MI), washed and resuspended in sterile isotonic saline (10^6 to 10^8 CFU/50µl). Mice were anesthetized by inhalation of isoflurane (Upjohn, Ede, The Netherlands) and 50 µl were inoculated intranasally (i.n.). At indicated timepoints mice were sacrificed and bacterial counts were determined as described (34, 37). In some experiments a broncho-alveolar lavage was performed, total cell numbers were counted using a coulter counter (Beckmann Coulter, Fullerton, CA) and differential cell counts were done on cytospin preparations stained with Giemsa.

Cytokine/chemokine and myeloperoxidase measurements
Lungs were homogenized as described (34, 37, 38). Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10 and monocyte chemoattractant protein-1 (MCP-1) were measured using the cytometric bead array (BD Bioscience, St. Jose, CA). IL-1β, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2 were measured using ELISAs (R&D Systems, Minneapolis, MN) as was myeloperoxidase (MPO) (HyCult Biotechnology, Uden, the Netherlands).

Histologic examination
Lungs were harvested at indicated timepoints, fixed in 10% formaline and embedded in paraffin. Four µm sections were stained with hematoxylin and eosin (H&E), and analyzed by a pathologist blinded for groups. To score lung inflammation and damage, the entire lung surface was analyzed regarding the presence of: interstitial inflammation, edema, endothelialitis, bronchitis and pleuritis as described (34, 38): Granulocyte staining was done as described previously (34, 37).
LPS pneumonitis
LPS (100 ng) purified from A. baumannii strain 24 (RUH 872, allocated to the European clone I (39, 40)) was administered (in 50 µl saline) i.n. to mice that were anesthetized by inhalation of isoflurane (Upjohn). Six hours later, a broncho-alveolar lavage was performed, total cell numbers were counted using a hemocytometer (Türk chamber) and differential cell counts were done on cytospin preparations stained with Giemsa (41).

Statistical analysis
Differences between groups were calculated by Mann-Whitney U test or one-way analysis of variance where appropriate using GraphPad software (San Diego, CA). Values are expressed as mean ± SEM. A P-value < 0.05 was considered statistically significant.

Results

A. baumannii pneumonia model
To enable the investigation of host defense mechanisms in A. baumannii pneumonia in vivo we first developed a suitable mouse model. A well-documented strain of A. baumannii isolated from a clinical case of Acinetobacter pneumonia was selected for this purpose and mice (n=5 per group) were infected i.n. with inocula ranging from $10^6$ to $10^8$ CFU. Mice were sacrificed after 1, 4, 24, 48 or 72h to follow the inflammatory response over time. As depicted in Fig. 1, lung bacterial counts temporarily increased until 4h post-infection, and gradually declined thereafter (Fig. 1). However, bacteria were still detectable in lung tissue 72h after inoculation. Likewise, pulmonary TNF-α production reached peak levels at t=4h and quickly decreased thereafter, while the number of infiltrating polymorphonuclear cells (PMNs) (determined as MPO-concentration) remained at a constant level between 4 and 48h post-infection (Fig. 1). Histological examination of lung tissue illustrated the early onset of pneumonia as reflected by dense pulmonary infiltrates (Fig. 2). Of the mice challenged with $10^8$ CFU of A. baumannii, two died after 24h. Based on these pilot experiments, subsequent studies were performed with a bacterial inoculum of $10^7$ CFU and mice were sacrificed at 4h (peak of cytokine response and bacterial counts) and 24h (peak of PMN influx and onset of bacterial clearance) following challenge.
CD14 and TLR4 contribute to clearance of A. baumannii

Having established a murine model of A. baumannii pneumonia we next studied host innate defense pathways possibly involved in the observed inflammatory responses. CD14 and TLRs are important pattern recognition receptors that contribute to the initiation of an adequate inflammatory response during infections and, hence, to an effective host immune defense. Because CD14 and TLR4 are known to recognize LPS from Gram-negative bacteria, we first investigated their respective roles during A. baumannii pneumonia in vivo. Wild-type and gene-deficient mice were inoculated with $10^7$ CFU of A. baumannii and sacrificed after 4 and 24h. At 4h post-inoculation, significantly higher lung bacterial counts were found in CD14$^{-/-}$ and TLR4$^{-/-}$ mice as compared to wild-type animals (Fig. 3). At 24h, wild-type mice had lower bacterial counts than either group of gene-deficient mice, though this difference was only statistically significant for TLR4$^{-/-}$ animals (Fig. 3). To investigate systemic bacterial dissemination, blood samples were cultured for the presence of A. baumannii. Whereas blood cultures were sterile at 4h in all mice, approximately 50% of mice lacking either CD14 or TLR4 had positive blood cultures at t=24h (6/10 and 4/9 mice, respectively) as compared to wild-type animals (0/8 mice). We next examined whether TLR2 might also play a role in host innate defense against A. baumannii. TLR2$^{-/-}$ and wild-type mice were infected and lung bacterial counts enumerated. No difference was found at t=4h after infection but, somewhat surprisingly, a significantly lower number of bacteria was observed in lungs of TLR2$^{-/-}$ mice 24h after induction of pneumonia (Fig. 4). The observed difference in bacterial clearance between wild-type mice and mice lacking TLR2 was still observed at 44h post-inoculation (Fig. 4). Taken together, the above results suggested that CD14 and TLR4 contribute to lung bacterial clearance and prevent systemic dissemination of A. baumannii, whereas TLR2 signaling counteracts elimination of the bacteria from the lungs.
Humoral & Cellular factors
To gain further insight into the impaired bacterial clearance in mice lacking CD14 or TLR4, we next investigated the pulmonary cytokine and chemokine responses to A. baumannii. Early after infection (4h) TLR4−/− mice displayed a reduced ability to produce IL-6, TNF-α, KC and MIP-2 as compared to wild-type animals (p<0.05 in all cases), whereas IL-1β, IL-10 and MCP-1 levels were comparable to wild-type mice (Table I). Cytokine concentrations did not differ between TLR4−/− and wild-type mice at 24h, whereas KC levels remained lower in the TLR4-deficient animals than in the wild types (Table I). Pulmonary cytokine and chemokine concentrations in CD14−/− and wild-type mice were largely similar (data not shown). The onset of pulmonary inflammation, as assessed by histological examination of lungs at t=4h, was delayed in the absence of TLR4 (inflammation score at t=4h: 9.3±0.6 for wild-type and 7.1±0.6 for TLR4−/− mice; p<0.05) while no difference was observed between CD14−/− mice and their wild-type counterparts (inflammation score at t=4h: 10.5±0.9 in wild-type and 11.5±0.9 in CD14−/− mice; n.s.). At this early time point in particular TLR4−/− mice demonstrated a reduced influx of PMNs into BALF,
whereas CD14^{-/-} mice displayed moderately, albeit insignificantly, reduced PMN numbers in BALF (Table II, p < 0.05 TLR4^{-/-} versus wild-type mice). In line with increased bacterial counts, mice lacking CD14 had significantly higher pulmonary inflammation scores than their wild-type counterparts 24h after infection (10.0±0.6 for wild type and 12.4±0.4 for CD14^{-/-} mice; p<0.05). Likewise, the proportion of mice with confluent pneumonia was higher in TLR4^{-/-} animals (14% and 50% of wild type and TLR4^{-/-} mice, respectively, displayed areas of confluent pneumonia) although no difference between the lung inflammation score of wild type or TLR4^{-/-} animals was found at t=24h (12.1±0.7 in wild type vs. 12.0±0.7 in TLR4^{-/-} mice). In TLR2^{-/-} mice, the most striking finding was a significant increase in pulmonary MIP-2 and MCP-1 concentrations 4h post-infection, while TNF-α, IL-1β, IL-6 or IL-10 levels did not differ when compared to wild-type mice (Fig. 5). KC concentrations were decreased in lungs of TLR2^{-/-} mice at this early time-point (data not shown). Moreover, TLR2^{-/-} mice displayed an earlier and more pronounced inflammatory cell influx into the lungs (Fig. 6). The early recruitment of PMNs into the pulmonary compartment was confirmed by Ly-6 immunohistochemical stainings and the detection of higher pulmonary MPO concentrations in TLR2^{-/-} (Fig. 5 and Fig. 6 (insets)). Of note, TLR2^{-/-} mice did not have an increased influx of PMNs into BALF (Table II).

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**Table II**

<table>
<thead>
<tr>
<th>Group</th>
<th>Lung Inflammation Score</th>
<th>Confluent Pneumonia (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>10.0±0.6</td>
<td>14%</td>
</tr>
<tr>
<td>CD14^{-/-}</td>
<td>12.4±0.4</td>
<td>50%</td>
</tr>
<tr>
<td>TLR4^{-/-}</td>
<td>12.1±0.7</td>
<td>14%</td>
</tr>
</tbody>
</table>

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**Figure 3: Reduced bacterial clearance in TLR4^{-/-} and CD14^{-/-} mice.** Wild-type and (A) TLR4^{-/-} or (B) CD14^{-/-} mice (n=7-10 per group at each time-point) were inoculated with 10^7 CFU of *A. baumannii* and lung bacterial counts were determined after 4 and 24 h. Results are expressed as mean±SEM; *P<0.05 vs. WT.

**Figure 4: Accelerated bacterial clearance in TLR2^{-/-} mice.** Wild-type and TLR2^{-/-} mice (n=8 per group at each time-point) were inoculated with *A. baumannii* (10^7 CFU) and lung bacterial counts were determined after 4, 24 and 44h. Results are expressed as mean±SEM, *P<0.05 vs. wild-type.
TLRs in *A. baumannii* infection

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>Wild-type</th>
<th>TLR4&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Wild-type</th>
<th>TLR4&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>9.8 ± 1.6</td>
<td>4.9 ± 1.0*</td>
<td>1.1 ± 1.8</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>IL-1β</td>
<td>8.5 ± 2.8</td>
<td>11.4 ± 2.4</td>
<td>3.0 ± 0.5</td>
<td>3.2 ± 0.8</td>
</tr>
<tr>
<td>IL-6</td>
<td>5.8 ± 1.2</td>
<td>2.8 ± 0.6*</td>
<td>0.9 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>MCP-1</td>
<td>6.2 ± 0.9</td>
<td>7.0 ± 1.4</td>
<td>4.3 ± 0.4</td>
<td>4.1 ± 0.6</td>
</tr>
<tr>
<td>MIP-2</td>
<td>34.9 ± 10.7</td>
<td>13.6 ± 1.9*</td>
<td>2.9 ± 0.6</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>KC</td>
<td>21.4 ± 1.2</td>
<td>5.7 ± 1.2*</td>
<td>5.4 ± 0.9</td>
<td>1.2 ± 0.2*</td>
</tr>
</tbody>
</table>

Table I: Concentration (ng/ml) of cytokines/chemokines in wild-type and TLR4<sup>−/−</sup> mice. Wild-type and TLR4<sup>−/−</sup> mice (n=7-9 per strain) were i.n. inoculated with 10<sup>7</sup> CFU of *A. baumannii*. At indicated time points, mice were sacrificed and cytokine and chemokine levels were measured in lung homogenates. Data are mean±SEM; *P<0.05 vs. wild-type.

Figure 5: Early onset of pulmonary inflammation in TLR2<sup>−/−</sup> animals. Wild-type (Wt) and TLR2<sup>−/−</sup> mice (n=8 per group) were inoculated i.n. with *A. baumannii* (10<sup>7</sup> CFU). After 4h, MIP-2 (left), MCP-1 (middle) and MPO (right) concentrations were measured in lung homogenates. Results are mean±SEM, *P<0.05 versus wild-type.

**The role of *A. baumannii* LPS in pulmonary infection**

So far, our studies were performed using whole bacteria. Given the involvement of LPS-signaling receptors CD14 and TLR4 in the innate response within the pulmonary compartment, we next inoculated mice i.n. with 100 ng of purified *A. baumannii* LPS of strain 24. The LPS of this strain has been shown previously to possess the same immunochemical properties as that of strain RUH 2037 (40), which was used in the studies described above. Broncho-alveolar PMN influx and cytokine/chemokine concentrations were assessed 6h after LPS administration. As expected, CD14 and TLR4 were crucial for the induction of the inflammatory response; mice lacking either signaling receptor did not mount a substantial PMN influx or TNF-α or IL-6 release (Fig. 7; IL-6 data not shown). These results confirm that *A. baumannii* LPS is a major immunostimulatory component that leads to a pro-inflammatory response during pneumonia with whole *A. baumannii* bacteria. Accordingly, we did not find a role for TLR2 in LPS-induced pneumonia. Neither
PMN-influx nor alveolar TNF-α or IL-6 concentrations differed between TLR2⁻/⁻ and wild-type mice (Fig. 7; IL-6 data not shown).

<table>
<thead>
<tr>
<th></th>
<th>Cell count/ml</th>
<th>AM %</th>
<th>PMN %</th>
<th>Lymphocytes %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>5051 ± 605</td>
<td>5.5 ± 0.7</td>
<td>93.7 ± 0.8</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>TLR2⁺/⁻</td>
<td>2616 ± 130</td>
<td>6.6 ± 1.2</td>
<td>92.4 ± 1.2</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>TLR4⁻/⁻</td>
<td>412 ± 45</td>
<td>62.8 ± 8.2</td>
<td>36.3 ± 8.3</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>CD14⁻/⁻</td>
<td>1664 ± 231 ⁎</td>
<td>12.9 ± 3.2</td>
<td>84.9 ± 3.2</td>
<td>2.1 ± 0.4</td>
</tr>
</tbody>
</table>

Table II: Cell count and differentials in BALF. Wild type, TLR2⁻/⁻, TLR4⁻/⁻, and CD14⁻/⁻ mice (n=6-8 per strain) were inoculated with A. baumannii. After 4h BALF cells were enumerated and differential counts were performed on cytospin preparations. Data are mean±SEM; ⁎ P<0.05 vs. wild type, ⁎⁎ P<0.05 vs. TLR2⁻/⁻ mice

Figure 6: Pulmonary inflammation is increased in TLR2⁻/⁻ mice. Representative lung histology slides of wild-type (A) and TLR2⁻/⁻ (B) mice 4 h after infection with 10⁷ CFU of A. baumannii. The insets demonstrate immunostaining for granulocytes, confirming the dense granulocytic infiltration. Pictures are representative for at least 8 mice per group. H&E staining: magnification x 10; inset (Ly-6 staining): magnification x 20.

Discussion

A. baumannii pneumonia poses an increased threat to hospitalized patients, as reflected in the rising number of nosocomial pneumonia cases caused by this bacterial species and the relatively high incidence of mortality among infected individuals (4, 8). In light of the high antibiotic resistance of A. baumannii, knowledge about host defense mechanisms is highly warranted. We hereby introduce an acute A. baumannii pneumonia model that allows the in vivo investigation of these molecular mechanisms during infection with this bacterium. We observed that the release of pro-inflammatory cytokines mediated by CD14 and
TLR4 signaling is crucial to bacterial clearance within the lungs and to the prevention of systemic bacterial spread in vivo. In contrast, TLR2-related pathways delayed early MIP-2 and MCP-1 release as well as pulmonary inflammation, which were accompanied by an impaired elimination of A. baumannii from the lungs.

A. baumannii exhibits some resemblance to Pseudomonas aeruginosa: both are Gram-negative non-glucose fermenters that are strongly associated with nosocomial pneumonia. While many reports have described host defense mechanisms against P. aeruginosa, very little is known about A. baumannii. We have taken first steps to fill this gap by establishing a murine model of A. baumannii infection and thereby encountered some similarities to the P. aeruginosa model we frequently study in our laboratory (42, 43). Thus, both groups of bacteria rapidly induce a robust inflammatory response within the lungs and bacteria are eventually cleared by the host unless high infection doses are administered. Until recently the role of TLRs during this type of acute pneumonia was not entirely clear and we therefore decided to investigate the respective roles of CD14, TLR4 and TLR2 during A. baumannii pneumonia. While this work was in progress, two other groups reported an important role for myeloid differentiation factor (MyD) 88, the main adaptor protein involved in TLR and IL-1 signaling, during P. aeruginosa pneumonia (44, 45). Both reports describe a severely impaired PMN influx and cytokine/chemokine response within the lungs of MyD88−/− mice that was associated with higher bacterial counts in this organ. Since IL-1 and IL-18 pathways...
(which also use MyD88 as adaptor) have been shown to play no supportive role during *P. aeruginosa* pneumonia (42, 43). Power et al. then focused on TLRs and investigated the contribution of TLR2 and 4 to host defense (45). Power et al. made use of C3H/HeJ mice that harbor a mutation in TLR4 that renders this receptor dysfunctional, whereas in the study reported here TLR4/−/− animals backcrossed to a C57/BL6 background were used. Though in most instances the differences are minor, C3H/HeJ mice do not precisely mirror the situation found in TLR4/−/− mice that were used here. We also performed experiments in C3H/HeJ mice (data not shown) and revealed a less prominent role for TLR4 using these mice when compared to TLR4/−/− animals. However, just like Power et al. in *P. aeruginosa* pneumonia (45), we found an impaired early (4h) cytokine and PMN response in TLR4 gene-deficient mice in *A. baumannii* pneumonia. In addition, we also observed an increased bacterial load in TLR4/−/− mice after 4h, while the difference in C3H/HeJ mice did not reach significance at this early timepoint (data not shown). Since LPS is considered the main ligand for TLR4 and CD14, we expanded our studies and used purified LPS from *A. baumannii* to demonstrate that both TLR4 and CD14 are indeed the two crucial receptors involved in the signaling cascade during *A. baumannii* infection in vivo.

To investigate the receptor that counts responsible for the inflammatory response to *A. baumannii* in the absence of TLR4 or CD14, we moved on and examined the role of TLR2, the receptor for bacterial lipoproteins and peptidoglycan. Much to our surprise we found that the absence of TLR2 accelerated PMN influx into lung tissue (as assessed by MPO levels in whole lung homogenates and histology), although PMN counts in BALF of TLR2/−/− mice did not differ from BALF PMN counts in wild-type mice early after infection. Nonetheless, TLR2 deficiency was associated with an improved bacterial clearance from this organ. When Power et al. investigated *P. aeruginosa* pneumonia in TLR2/−/− mice they found a very moderately decreased PMN influx in lung tissue (as indicated by reduced MPO levels) after 4h but no impairment of the cytokine/chemokine responses (45). However, the authors did not report on lung CFU nor did they investigate later timepoints when improved bacterial clearance might have become apparent. We found an early onset of pulmonary inflammation together with increased MIP-2 and MCP-1 concentrations in TLR2/−/− mice 4h after infection.

Alveolar macrophages have been reported to be the main source of MCP-1 during pulmonary infection (46) and LPS, via TLR4/CD14 and direct as well as indirect NF-κB activation, is the major trigger for the secretion of MCP-1 (47, 48). MCP-1 has also been described in respiratory epithelial cells 24h after infection with *P. aeruginosa* (49). Moreover, two reports described the highly beneficial role of MCP-1 during Gram-negative infection: early MCP-1 administration contributed to a faster elimination of bacteria whereas late MCP-1 administration (at t=24h)
reduced lung injury and improved the resolution of *P. aeruginosa* pneumonia via enhanced uptake of potentially harmful apoptotic PMNs (49, 50). Likewise, increased MIP-2 levels have been described to improve the PMN influx and phagocytosis of bacteria during Gram-negative pneumonia (51). Accordingly, we found an improved pulmonary clearance of *A. baumannii* in the presence of elevated MCP-1 and MIP-2 concentrations. The robust and early (4h) onset of pulmonary inflammation in the absence of TLR2\(^{-/-}\) might therefore explain the improved bacterial clearance observed at later time points (24 and 44h). Why and how TLR2 precisely prevents the rise in MCP-1 and MIP-2 levels remains unclear. The possibility exists that TLR2 mediates anti-inflammatory pathways that down-regulate MCP-1 production. IL-10 has been shown to reduce MIP-2 and MCP-1 secretion by activated monocytes/macrophages (48, 52, 53) but we did not find any differences in IL-10 concentrations that could explain our findings. Increased MIP-2 levels might even be a consequence of elevated MCP-1 concentrations, as illustrated by the finding of synergistically enhanced MIP-2 release in the presence of MCP-1 and LPS (54). Alternatively, the lack of TLR2 signaling in gene-deficient mice could have been associated with an upregulation of other receptors with mainly pro-inflammatory properties, such as TLR4. This phenomenon has been described in TLR2\(^{-/-}\) mice infected with *P. aeruginosa* that lack pilus expression (55). Another potential explanation could be that differences in the cellular expression profile of TLRs within the respiratory tract are associated with distinct responses. It seems quite well established that LPS induced pulmonary inflammation relies primarily on TLR4 expressing macrophages (56, 57). LPS directly activates alveolar macrophages to secrete pro-inflammatory cytokines such as TNF-\(\alpha\) and IL-1\(\beta\); these, in turn, stimulate respiratory epithelial cells to produce, for example, chemokines (56). Respiratory epithelial cells have been repeatedly shown to be unresponsive when stimulated with LPS alone, although TLR4 mRNA is present in these cells (56, 58-60). In contrast, less is known about pulmonary TLR2, but this signaling receptor is expressed on alveolar macrophages and has gained much attention recently as part of a lipid raft receptor assembly at the apical side of airway epithelial cells (61). This finding strongly indicates the direct involvement of TLR2 in the sampling of and response to pathogens within the lungs. Given the high rate of colonization with *A. baumannii* in critically ill patients, it is tempting to speculate that the reduced inflammatory response we observed to occur in the presence of TLR2 might even prove beneficial for the host, by providing a delicate balance between situations requiring robust response for the rapid clearance of bacteria in individuals with high pulmonary bacterial counts and the risk of systemic dissemination (such as the model of acute infection described herein) and cases in which the low degree of colonization does not warrant such a vigorous response.
A more recent study by Benjamim et al. investigated the immunosuppression and higher susceptibility to nosocomial pulmonary infections following cecal ligation puncture (CLP). Among other findings, the authors described an increased expression of TLR2 within the lungs after CLP (62). Considering our observation of a weakened immune response to A. baumannii in the presence of TLR2, these data suggest that preceding insults such as CLP lead to molecular alterations that include the upregulation of TLR2 and are associated with an impaired ability to combat nosocomial bacteria such as A. baumannii.

In conclusion, we demonstrate that CD14 and TLR4 are indispensable for A. baumannii LPS-mediated signaling, resulting in the effective elimination of A. baumannii from the lungs in vivo, whereas TLR2 reduces the pulmonary inflammatory response and so delays elimination of bacteria from the lungs. Our model of A. baumannii pneumonia will be useful in further studies aimed at elucidating mechanisms involved in innate and adaptive immune responses to this increasingly important nosocomial pathogen.

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References


