

Toll-like receptor 2 promotes bacterial clearance during the initial stage of pulmonary infection with *Acinetobacter baumannii*

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Abstract. Toll-like receptor 2 (TLR2) has been identified as a sensor for bacterial lipoprotein. To determine the role of TLR2 in host defense against *Acinetobacter baumannii* infection, wild-type (WT) and TLR2-deficient mice were infected intranasally with *A. baumannii*. Body weight, cytokine and chemokine levels in bronchoalveolar (BAL) fluid and lung histopathology were examined. Body weight changes in TLR2-deficient mice were comparable to those of WT mice throughout the experimental period. However, TLR2-deficient mice exhibited an increased bacterial load in the lungs and increased levels of interleukin-6 and chemokine (C-X-C motif) ligand 2 in BAL fluids compared with WT mice 1 day after infection. Histopathological features of lung tissue in WT and TLR2-deficient mice were comparable between WT and TLR2-deficient mice. Results of the present study demonstrate that TLR2 may have a minimal role in the host defense against *A. baumannii* at the early stages of infection.

Introduction

Acinetobacter baumannii has emerged as a major causative agent of nosocomial infection in hospitals worldwide (1,2). According to the data from a national surveillance study performed in the United States, *A. baumannii* was shown to be the etiological agent in 6.9% of nosocomial pneumonias in 2004, which was an ~72% increase compared with data collected in 1986 (3). Pulmonary compartments are the most favored site of *A. baumannii* infection, and

ventilatory-associated and community-acquired pneumonia are extremely common. The mortality of ventilatory-associated pneumonias due to *A. baumannii* may be as high as 75% (4). Furthermore, emergence of multi-drug resistant *A. baumannii* has become a significant health concern leading to limited therapeutic options in recent decades (1,2). The increased incidence of antibiotic resistance in these bacteria underlines the importance of understanding the host immune response against *A. baumannii* to prevent and control infection.

Innate immune systems provide the first line of defense against pathogenic bacteria, by recognition of pathogen-associated molecular patterns. Toll-like receptors (TLRs) are the best-studied family of pattern recognition receptors (PRRs) and are comprised of N-terminal leucine-rich repeats, a transmembrane domain and a C-terminal Toll/interleukin (IL)-1 receptor domain. TLRs 1, 2, 4, 5 and 6 are present on the cell surface, whereas TLRs 3, 7 and 9 are localized in the endosome (5). Following the recognition of specific molecular patterns found in microbial pathogens, TLRs trigger downstream signaling pathways [myeloid differentiation primary-response protein 88 (MyD88)-dependent and/or TIR domain-containing adapter-inducing interferon- β -dependent pathways], and produce proinflammatory cytokines and antimicrobial factors to eradicate the invading pathogens. Engagement of TLRs initiates signaling through intracellular pathways that lead to activation of nuclear factor- κ B (NF- κ B), mitogen-activated protein kinases (MAPKs) and interferon regulatory factors (6,7).

TLR4 is critical for host defense against Gram-negative bacterial infection as it recognizes lipopolysaccharide (LPS) (7). TLR4 mediates bacterial clearance and production of proinflammatory cytokines and chemokines in *A. baumannii* infection (8). In addition to TLR4, TLR2 is involved in host innate immunity against microbial infections by recognizing peptidoglycans of the bacterial cell wall and activating NF- κ B and MAPKs through the MyD88-dependent pathway (9-11). Although *A. baumannii* is known to induce TLR2-stimulatory activity (12,13), the role of TLR2 in *A. baumannii*-induced immune responses remains unclear. Previously, an *in vivo* study demonstrated that TLR2 deficiency leads to improved bacterial clearance and increased production of chemokines and myeloperoxidase in the lungs of mice infected with *A. baumannii* (8). By contrast, TLR2 is essential for optimal immune responses

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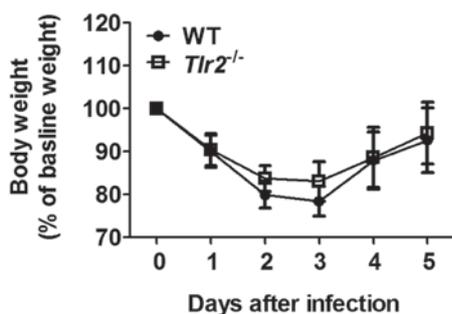


Figure 1. Body weight changes with *A. baumannii* infection in WT and TLR2-deficient mice. WT and TLR2-deficient mice were infected intranasally with *A. baumannii* (3×10^7 CFU) and body weight changes were monitored daily. WT, wild-type; TLR2, toll-like receptor 2; CFU, colony-forming units.

against *A. baumannii* in macrophages and lung epithelial cells (12,14). Therefore, the present study sought to confirm the role of TLR2 in bacterial colonization, cytokine and chemokine production and lung histopathology, by *A. baumannii* infection in mice.

Materials and methods

Mice. TLR2-deficient mice on C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and wild-type (WT) C57BL/6 mice were from Koatech (Pyeongtaek, Korea). The animal studies were conducted under approved protocols by the Institutional Animal Care and Use Committee of Konyang University (Daejeon, Korea).

Bacterial infection. *A. baumannii* strain KCCM 35453 (ATCC 15150) was purchased from Korean Culture Center of Microorganisms (Seoul, Korea). For bacterial preparation, single colonies were inoculated into 5 ml Luria Bertani (LB) broth and grown overnight at 37°C under agitation. A 1:5 dilution of the culture grown overnight was allowed to grow in fresh medium at 37°C under agitation for a further 2 h. Bacteria were washed and resuspended with sterile phosphate-buffered saline (PBS) to 10^9 colony-forming units (CFU)/ml. Mice were anesthetized by intraperitoneal injection of 10 mg/kg rompun (Bayer Korea Ltd., Seoul, Korea) and 50 mg/kg zoletil (Virbac Korea Co., Ltd., Seoul, Korea), and 30 μ l prepared bacteria was inoculated intranasally. Infected mice were monitored daily for body weight changes and clinical signs. At 1, 3 and 5 days after inoculation, mice were sacrificed and bronchoalveolar lavage (BAL) fluid was collected.

Bacterial count in lung tissue. Following BAL fluid collection, the right lobes of lungs were collected in sterile PBS for bacterial counting. The extracted lobes were weighed, homogenized, serially diluted and 100 μ l homogenate was spread onto LB agar plates. Following overnight culture in a 37°C incubator, the colony was counted and the number of bacteria (measured as CFU/g lung tissue) was calculated.

Measurement of cytokines and chemokines. The levels of IL-6, tumor necrosis factor- α (TNF- α), chemokine (C-X-C motif) ligand 2 (CXCL2) and chemokine (C-C motif) ligand 2

(CCL2) from BAL fluid of *A. baumannii*-infected mice were determined using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA).

Histopathological examination. The left lobes of lungs were harvested and fixed in 10% neutral formalin for histopathological observation. The tissues were routinely processed with alcohol and xylene series and embedded in paraffin. Subsequently, 3- μ m sections were prepared, stained with hematoxylin and eosin and examined by microscopy.

Statistical analysis. The differences in mean values among different groups were tested and the values were expressed as the mean \pm standard deviation. All of the statistical calculations were carried out using Microsoft Excel (Microsoft, Redmond, WA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Clinical signs and body weight changes by *A. baumannii* infection in mice. *A. baumannii* infection caused mild physical signs of ill health in WT and TLR2-deficient mice, manifested as weight loss, ruffled fur, decreased movement and huddling. These were without any significant difference 1 day after infection (data not shown). These clinical signs improved from 3 days after infection. The weight loss was prominent in the early stages of infection and continued until 3 days after infection. Following this, the majority of mice gradually regained body weight and had recovered to within a normal range by 5 days after infection (Fig. 1).

Bacterial clearance in the lungs of mice infected with *A. baumannii*. To determine the role of TLR2 in bacterial clearance *in vivo*, the lung tissue was extracted from *A. baumannii*-infected WT and TLR2-deficient mice and the number of bacteria was counted. The pulmonary bacterial loads were significantly higher in TLR2-deficient mice compared with WT mice 1 day after infection (Fig. 2A). However, at 3 and 5 days after infection, the loads were comparable in WT and TLR2-deficient mice (Fig. 2B and C). These results indicate that TLR2 may contribute to bacterial clearance in *A. baumannii*-infected mice at the early stages of infection but not at the later stages.

Production of cytokines and chemokines by *A. baumannii* in BAL fluids. To examine whether TLR2 has a role in the production of cytokines and chemokines following *A. baumannii* infection, levels of cytokines (IL-6 and TNF- α) and chemokines (CXCL2 and CCL2) from BAL fluids of *A. baumannii*-infected mice were measured 1, 3 and 5 days after infection. The levels of IL-6 and CXCL2 were significantly higher in the BAL fluid of TLR2-deficient mice than in WT mice 1 day after infection (Fig. 3A and B). Although there was no statistically significant difference, the TNF- α level was also slightly higher in the BAL fluid of TLR2-deficient mice compared with WT mice (Fig. 3C).

Histopathology of the lung of *A. baumannii*-infected mice. Histopathological features of lung tissue from WT and

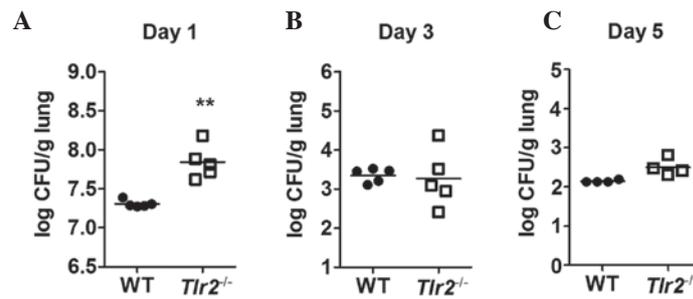


Figure 2. Bacterial clearance in the lung of mice infected with *A. baumannii*. Bacterial numbers of WT and TLR2-deficient mice were measured in lung homogenates (A) 1, (B) 3 and (C) 5 days after infection. * $P < 0.01$, vs. WT group. WT, wild-type; TLR2, toll-like receptor 2.

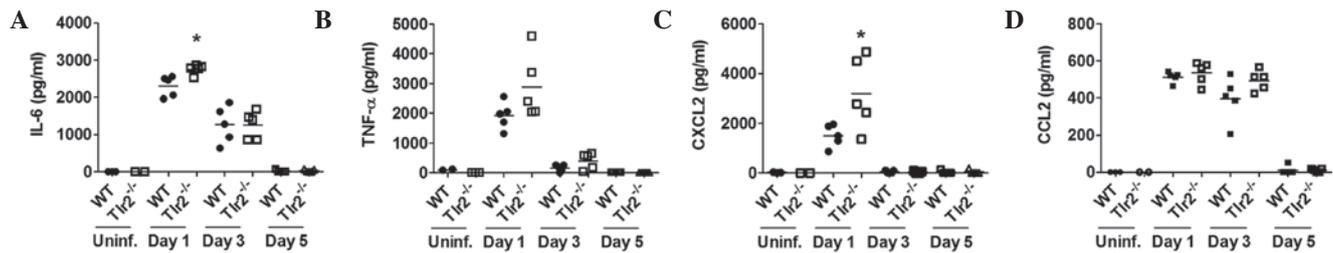


Figure 3. The production of cytokines and chemokines by *A. baumannii* in BAL fluids. WT and TLR2-deficient mice were infected with *A. baumannii* and BAL fluids were collected 1, 3 and 5 days after infection. The levels of (A) IL-6, (B) TNF- α , (C) CXCL2 and (D) CCL2 in BAL fluids were measured by ELISA. * $P < 0.01$, vs. WT group. BAL, bronchoalveolar lavage; TLR-2, toll-like receptor 2; IL-6, interleukin-6; TNF- α , tumor necrosis factor- α ; CXCL2, chemokine (C-X-C motif) ligand 2; CCL2, chemokine (C-C motif) ligand 2; WT, wild-type.

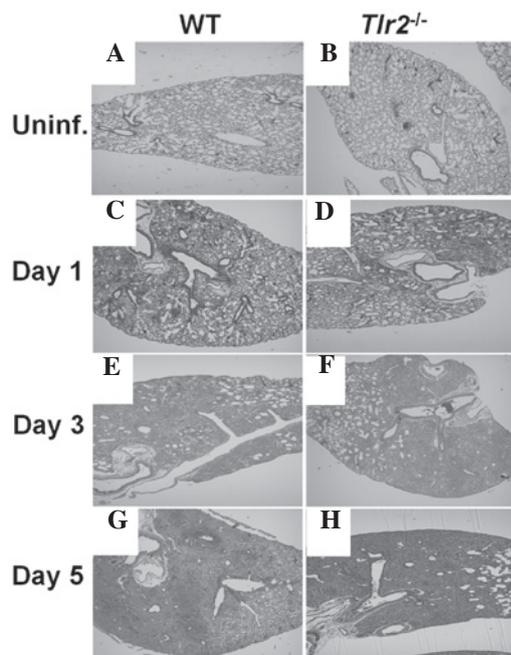


Figure 4. Histopathology in the lung of *A. baumannii*-infected mice. WT and TLR2-deficient mice were infected with 3×10^7 CFU *A. baumannii* and representative histological images of the lungs are depicted (hematoxylin and eosin; magnification, $\times 40$). WT, wild-type; TLR2, toll-like receptor 2; CFU, colony-forming units.

TLR2-deficient mice was assessed 1, 3 and 5 days after inoculation with *A. baumannii*. Whilst no infiltration of inflammatory cells was observed prior to *A. baumannii* infection in

the lungs of WT and TLR2-deficient mice (Fig. 4A and B), infection led to polymorphonuclear (PMN) cell infiltration in the alveolar spaces and perivascular and peribronchiolar areas in WT and TLR2-deficient mice from 1 day after infection (Fig. 4C and D). Infiltration of these cells became severe at 3 (Fig. 4E and F) and 5 days (Fig. 4G and H) after infection, and no marked difference in inflammatory cell infiltration was observed between WT and TLR2-deficient mouse lung tissue (Fig. 4). These results indicate that TLR2 may not be involved in the progression of lung inflammation in mice.

Discussion

Inflammatory responses play a pivotal role in lung defense against bacterial pathogens, including *A. baumannii*. This specific response includes the release of pro-inflammatory cytokines and chemokines (8,15,16). IL-6, TNF- α , and IL-1 β are representative proinflammatory cytokines and are associated with the upregulation of cell adhesion molecules and antigen presentation. IL-8 (keratinocyte-derived protein chemokine/CXCL1 in mice), monocyte chemoattractant protein-1/CCL2, and macrophage inflammatory protein-2/CXCL2 act as chemoattractants and are required for recruitment of immune cells from the bloodstream into the airway (17). In addition to immune cells, airway epithelial cells contribute to host resistance against *A. baumannii* by producing antimicrobial peptides, for example defensins (18,19). All of these factors are closely related and cooperate during the immune response against *A. baumannii*.

In the present study, mice lacking functional TLR2 exhibited a selective impaired bacterial clearance compared with

WT mice 1 day after infection, although lung infiltration of immune cells was comparable between the two groups. These results are in accordance with a previous report demonstrating that TLR2 deficiency impaired the nitric oxide-mediated bacterial killing capacity of PMN cells and alveolar macrophages, without any defects in the immune cell influx in pulmonary infection of *Porphyromonas gingivalis* (20). In addition, the possible role of TLR2-dependent local immune responses cannot be excluded. Our previous study revealed that TLR2 is not required for cytokine production in macrophages in response to *A. baumannii* (21). However, *A. baumannii* may induce IL-8 production in human lung epithelial cells and was inhibited by TLR2 small interfering RNA (12). Although TLR2 deficiency demonstrated increased CXCL2 production 1 day after infection, no significant difference was observed between the two groups with regard to PMN cell infiltration into the lung. It is possible that TLR2-independent mechanisms may have compensated for this function, for example stimulation by bacterial-derived formyl peptides of the formyl-peptide receptor, which plays a major role in PMN recruitment to infected alveoli (22).

A number of studies have reported that TLR2-deficient mice exhibit increased resistance to pulmonary infection with *A. baumannii* (8) or *pilA* mutant *Pseudomonas aeruginosa* (23), and to systemic challenge with *Yersinia enterocolitica* (24) or *Candida albicans* (25). It is hypothesized that these results may be due to TLR2 signaling having several inhibitory effects on the inflammatory response, by inducing IL-10 or downregulating TLR4 expression. However, the distinct mechanisms have not been clearly identified. In the present study, no bacterial clearance defects were observed in the lungs of TLR2-deficient mice compared with those of WT mice 3 and 5 days after infection. In addition, TLR2-deficient mice exhibited similar inflammatory cell infiltration in the lungs as WT mice with *A. baumannii*, which was not consistent with the previous report (8). This discrepancy may be due to the distinct bacterial origin and different experimental conditions.

Although TLR4 is regarded as the main PRR of Gram-negative bacteria, TLR2 has also been reported to participate in the recognition of some Gram-negative bacteria, including *Helicobacter pylori*, *Porphyromonas gingivalis* and *Bacteroides fragilis* (11,26,27). Therefore, TLR2 serves as a functional receptor for Gram-positive and -negative bacteria and induces production of proinflammatory cytokines (28). In conclusion, results of the present study demonstrated that TLR2 may be involved in the recognition of *A. baumannii*, particularly at the early stages of infection. A previous study revealed that LPS-deficient *A. baumannii* exhibited impaired ability to produce TNF- α in TLR2-deficient macrophages compared with WT cells (14). Therefore, it is necessary to re-analyze the *in vivo* effects of TLR2, using an LPS-deficient strain of *A. baumannii* or by comparing phenotypes between TLR4- and TLR2/4-deficient mice.

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References

- Perez F, Hujer AM, Hujer KM, Decker BK, Rather PN and Bonomo RA: Global challenge of multidrug-resistant *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 51: 3471-3484, 2007.
- Van Looveren M and Goossens H; ARPAC Steering Group: Antimicrobial resistance of *Acinetobacter* spp. in Europe. *Clin Microbiol Infect* 10: 684-704, 2004.
- Gaynes R and Edwards JR; National Nosocomial Infectious Surveillance System: Overview of nosocomial infections caused by gram-negative bacilli. *Clin Infect Dis* 41: 848-854, 2005.
- Fagon JY, Chastre J, Domart Y, Trouillet JL and Gibert C: Mortality due to ventilator-associated pneumonia or colonization with *Pseudomonas* or *Acinetobacter* species: assessment by quantitative culture of samples obtained by a protected specimen brush. *Clin Infect Dis* 23: 538-542, 1996.
- Akira S: Innate immunity to pathogens: diversity in receptors for microbial recognition. *Immunol Rev* 227: 5-8, 2009.
- Kawai T and Akira S: TLR signaling. *Cell Death Differ* 13: 816-825, 2006.
- Takeda K and Akira S: Toll-like receptors in innate immunity. *Int Immunol* 17: 1-14, 2005.
- Knapp S, Wieland CW, Florquin S, *et al*: Differential roles of CD14 and toll-like receptors 4 and 2 in murine *Acinetobacter* pneumonia. *Am J Respir Crit Care Med* 173: 122-129, 2006.
- Flo TH, Halaas O, Lien E, *et al*: Human toll-like receptor 2 mediates monocyte activation by *Listeria monocytogenes*, but not by group B streptococci or lipopolysaccharide. *J Immunol* 164: 2064-2069, 2000.
- Echchannaoui H, Frei K, Schnell C, Leib SL, Zimmerli W and Landmann R: Toll-like receptor 2-deficient mice are highly susceptible to *Streptococcus pneumoniae* meningitis because of reduced bacterial clearing and enhanced inflammation. *J Infect Dis* 186: 798-806, 2002.
- Smith MF Jr, Mitchell A, Li G, *et al*: Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for *Helicobacter pylori*-induced NF- κ B activation and chemokine expression by epithelial cells. *J Biol Chem* 278: 32552-32560, 2003.
- March C, Regueiro V, Llobet E, *et al*: Dissection of host cell signal transduction during *Acinetobacter baumannii*-triggered inflammatory response. *PLoS One* 5: e10033, 2010.
- Erridge C, Moncayo-Nieto OL, Morgan R, Young M and Poxton IR: *Acinetobacter baumannii* lipopolysaccharides are potent stimulators of human monocyte activation via Toll-like receptor 4 signalling. *J Med Microbiol* 56: 165-171, 2007.
- Moffatt JH, Harper M, Mansell A, *et al*: Lipopolysaccharide-deficient *Acinetobacter baumannii* shows altered signaling through host Toll-like receptors and increased susceptibility to the host antimicrobial peptide LL-37. *Infect Immun* 81: 684-689, 2013.
- Qiu H, KuoLee R, Harris G and Chen W: High susceptibility to respiratory *Acinetobacter baumannii* infection in A/J mice is associated with a delay in early pulmonary recruitment of neutrophils. *Microbes Infect* 11: 946-955, 2009.
- Van Faassen H, KuoLee R, Harris G, Zhao X, Conlan JW and Chen W: Neutrophils play an important role in host resistance to respiratory infection with *Acinetobacter baumannii* in mice. *Infect Immun* 75: 5597-5608, 2007.
- Craig A, Mai J, Cai S and Jeyaseelan S: Neutrophil recruitment to the lungs during bacterial pneumonia. *Infect Immun* 77: 568-575, 2009.
- Routsias JG, Karagounis P, Parvulesku G, Legakis NJ and Tsakris A: In vitro bactericidal activity of human beta-defensin 2 against nosocomial strains. *Peptides* 31: 1654-1660, 2010.
- Maisetta G, Batoni G, Esin S, *et al*: In vitro bactericidal activity of human β -defensin 3 against multidrug-resistant nosocomial strains. *Antimicrob Agents Chemother* 50: 806-809, 2006.
- Hajishengallis G, Wang M, Bagby GJ and Nelson S: Importance of TLR2 in early innate immune response to acute pulmonary infection with *Porphyromonas gingivalis* in mice. *J Immunol* 181: 4141-4149, 2008.
- Kim CH, Jeong YJ, Lee J, *et al*: Essential role of toll-like receptor 4 in *Acinetobacter baumannii*-induced immune responses in immune cells. *Microb Pathog* 54: 20-25, 2013.
- Fillion I, Ouellet N, Simard M, Bergeron Y, Sato S and Bergeron MG: Role of chemokines and formyl peptides in pneumococcal pneumonia-induced monocyte/macrophage recruitment. *J Immunol* 166: 7353-7361, 2001.

23. Lorenz E, Chemotti DC, Vandal K and Tessier PA: Toll-like receptor 2 represses nonpilus adhesin-induced signaling in acute infections with the *Pseudomonas aeruginosa* pilA mutant. *Infect Immun* 72: 4561-4569, 2004.
24. Sing A, Rost D, Tvardovskaia N, *et al*: Yersinia V-antigen exploits Toll-like Receptor 2 and CD14 for interleukin 10-mediated immunosuppression. *J Exp Med* 196: 1017-1024, 2002.
25. Netea MG, Suttmuller R, Hermann C, *et al*: Toll-like receptor 2 suppresses immunity against *Candida albicans* through induction of IL-10 and regulatory T cells. *J Immunol* 172: 3712-3718, 2004.
26. Darveau RP, Pham TT, Lemley K, *et al*: *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. *Infect Immun* 72: 5041-5051, 2004.
27. Erridge C, Pridmore A, Eley A, Stewart J and Poxton IR: Lipopolysaccharides of *Bacteroides fragilis*, *Chlamydia trachomatis* and *Pseudomonas aeruginosa* signal via toll-like receptor 2. *J Med Microbiol* 53: 735-740, 2004.
28. Dziarski R and Gupta D: Role of MD-2 in TLR2- and TLR4-mediated recognition of Gram-negative and Gram-positive bacteria and activation of chemokine genes. *J Endotoxin Res* 6: 401-405, 2000.