Role of Toll-like receptor 2 in recognition of *Legionella pneumophila* in a murine pneumonia model

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Legionella pneumophila is an intracellular organism and the major aetiological agent of Legionnaires' disease. Although recent progress has identified Toll-like receptors (TLRs) as receptors for recognition of pathogen-associated molecular patterns in a variety of micro-organisms, understanding the contribution of TLRs to the host response in *L. pneumophila* infection is still limited. This study examined the roles of TLR2 and TLR4 in murine *L. pneumophila* pneumonia and an *in vitro* infection model using bone-marrow-derived macrophages. TLR2-deficient mice, but not TLR4-deficient mice, demonstrated higher lethal sensitivity to pulmonary challenge with *L. pneumophila* than wild-type mice ($P < 0.05$). Although no differences in pulmonary bacterial burden were observed among the mouse strains examined, lower values of macrophage inflammatory protein-2 (MIP-2), keratinocyte-derived cytokine and interleukin (IL)-6 and higher IL-12 levels were noted in lung homogenates of TLR2-deficient mice compared with the wild-type control and TLR4-deficient mice. Recruitment of inflammatory cells, particularly neutrophils, was severely disturbed in the lungs of TLR2-deficient mice. Reduced MIP-2 production was demonstrated in bone-marrow-derived macrophages from TLR2-deficient mice in response to live *L. pneumophila* and purified LPS of this strain, but not *Escherichia coli* LPS. These data highlight the involvement and importance of TLR2 in the pathogenesis of *L. pneumophila* pneumonia in mice. The results showed that TLR2-mediated recognition of *Legionella* LPS and subsequent chemokine-dependent cellular recruitment may be a crucial host innate response in *L. pneumophila* pneumonia.

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INTRODUCTION

Legionella pneumophila is a Gram-negative intracellular pathogen that often causes a serious and life-threatening pneumonia in humans (Marston *et al.*, 1994; Reingold, 1988). An epidemiological survey suggested that 17 000–50 000 patients are hospitalized with *Legionella* disease annually in the USA (Marston *et al.*, 1994; Reingold, 1988) and high mortality rates reaching 10–50% have been

observed in these patients, especially in immunocompromised individuals (el-Ebiary *et al.*, 1997; Pedro-Botet *et al.*, 1998; Tkatch *et al.*, 1998).

Legionella usually infects humans via inhalation of contaminated aerosols from waterborne environmental sources. In lung tissue, the bacteria multiply in several types of host cell, including macrophages, monocytes and alveolar epithelial cells (Horwitz & Silverstein, 1980; Mody *et al.*, 1993; Nash *et al.*, 1984). Previous studies have demonstrated the important role of Th1 cytokines, such as interferon (IFN)- γ , interleukin (IL)-12 and IL-18, in *L. pneumophila* infection models (Brieland *et al.*, 1998, 2000; Gebran *et al.*, 1994; Salins *et al.*, 2001; Skerrett & Martin, 1994) and in human

Abbreviations: BAL, bronchoalveolar lavage; BALF, BAL fluid; BM-M ϕ s, bone-marrow-derived macrophages; EU, endotoxin units; IFN, interferon; IL, interleukin; KC, keratinocyte-derived cytokine; MIP-2, macrophage inflammatory protein-2; TLR, Toll-like receptor.

samples from pneumonia patients (Tateda *et al.*, 1998). Recently, we reported a crucial role of neutrophils as a source of IL-12 in driving Th1-type host responses in *L. pneumophila* pneumonia (Tateda *et al.*, 2001a). Influx of inflammatory cells, macrophages and neutrophils to the site of infection is believed to be critical for host defence responses and immune processes (Carratala *et al.*, 1994). Although robust production of chemokines, including macrophage inflammatory protein-2 (MIP-2), keratinocyte-derived cytokine (KC) and LPS-induced CXC chemokine, has been demonstrated in a murine model of *Legionella* pneumonia (Tateda *et al.*, 2001b), the pathogenesis of the disease, particularly the mechanisms by which this organism is sensed by host cells and by which the innate immune response is triggered, remains to be elucidated.

The first line of defence 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 (Medzhitov, 2001; Medzhitov & Janeway, 2000; Zhang *et al.*, 2000). Over the last few 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 (Aderem & Ulevitch, 2000; Akira & Takeda, 2004; Beutler, 2004; Janeway & Medzhitov, 2002). At least 11 TLRs have been described so far, among which TLR2 and TLR4 are the best-investigated family members. TLR4 was originally recognized as a receptor for LPS of Gram-negative bacteria, whereas TLR2 has been designated the major receptor for Gram-positive bacteria by virtue of its capacity to recognize major cell-wall constituents, such as peptidoglycan, lipoteichoic acid and lipoproteins (Medzhitov, 2001; Takeda *et al.*, 2003). However, recent progress in this field has demonstrated that LPS from certain bacteria, such as *Bacteroides*, *Pseudomonas*, *Rhizobium* and *Legionella*, is recognized mainly by TLR2, rather than by TLR4 (Erridge *et al.*, 2004; Girard *et al.*, 2003). Consistent with these results, Lettinga *et al.* (2002) demonstrated that, in pulmonary *L. pneumophila* infection, C3H/HeJ mice, which display a non-functional gene encoding TLR4, were indistinguishable from control C3H/HeN mice in multiple parameters such as bacterial growth, cytokine production and histopathological changes in the infected lungs. More recently, Akamine *et al.* (2005) demonstrated that intracellular growth of *L. pneumophila* was enhanced within bone-marrow-derived macrophages (BM-M ϕ s) from TLR2-deficient mice, but not from TLR4-deficient mice, although the lethal sensitivity of TLR2-deficient mice and an association between cellular responses to *L. pneumophila* LPS and pathogenesis of the disease remain to be clarified.

The present study was designed to investigate further the role(s) of TLR2 in the pathogenesis of *L. pneumophila* pneumonia. The data obtained from TLR-deficient mice clearly demonstrated that TLR2, but not TLR4, is a critical molecule for the elaboration of an antibacterial host

response against *L. pneumophila*. The relative contribution and importance of TLRs for detecting live *L. pneumophila* and its LPS component were compared in BM-M ϕ s from TLR2- and TLR4-deficient and wild-type mice.

METHODS

Mice. Specific-pathogen-free C57BL/6 mice (6–8 weeks old) were purchased from Charles River (Yokohama, Japan). TLR2- and TLR4-deficient mice (C57BL/6 Jcl) were purchased from Oriental Bio-Service (Tokyo, Japan). All animals were housed in a pathogen-free environment and received sterile food and water in the Toho University School of Medicine (Tokyo, Japan).

Bacteria. Clinical isolates of *L. pneumophila* Suzuki (Tateda *et al.*, 2001a), a serogroup 1 strain originally isolated at Toho University Hospital, were grown for 3–4 days at 37 °C on buffered charcoal yeast extract (BCYE) agar supplemented with L-cysteine and ferric nitrates. A single colony was transferred to 3 ml buffered yeast extract broth and incubated overnight at 37 °C with constant shaking. The bacterial suspension was transferred to fresh buffered yeast extract broth as serial fivefold dilutions and incubated overnight under the same conditions as those described above. After confirmation of bacterial motility, the concentration of bacteria in the broth was determined by measuring the OD₆₀₀. Post-exponential-phase bacteria were used as challenge organisms (Byrne & Swanson, 1998). The numbers of viable bacteria in the challenge suspensions were determined by plating and incubating organisms on BCYE agar for 4 days.

Escherichia coli LPS and preparation of *L. pneumophila* LPS. Commercially available *E. coli* LPS (O55:B5; Difco) was used to stimulate BM-M ϕ s from TLR2- and TLR4-deficient and wild-type control mice. LPS of *L. pneumophila* Suzuki was prepared as described previously (Girard *et al.*, 2003; Yoshizawa *et al.*, 2005). For purification of LPS, the phenol re-extraction step was included to remove lipoprotein contaminants from the preparations. Purified LPS demonstrated an activity of 429 500 endotoxin units (EU) ml⁻¹, which is equivalent to approximately 2×10^9 – 4×10^9 c.f.u. ml⁻¹. As a weighable pellet of purified LPS was not obtained, LPS concentration was expressed in EU ml⁻¹. Protein contamination in the purified LPS was determined to be <5 µg ml⁻¹ in 429 500 EU ml⁻¹ using a protein assay (Bio-Rad).

Induction of *L. pneumophila* pneumonia in mice. Animals were anaesthetized intramuscularly with 7 mg ketamine and 15 mg xylazine kg⁻¹. The trachea was exposed and 30 µl bacterial suspension, or saline as a control, was injected directly into the trachea with a sterile 26-gauge needle. The skin incision was closed with surgical staple.

Lung harvesting for analysis. At designated time points, mice were sacrificed by CO₂ asphyxia. Before lung removal, the pulmonary vasculature was perfused with 1 ml saline via the right ventricle. After removal, whole lungs were homogenized in 1 ml saline using a tissue homogenizer (Omni International) under a vented hood. Portions of homogenates (10 µl) were inoculated onto BCYE agar after serial 1:10 dilutions in saline. The remaining homogenates were incubated on ice for 30 min and then centrifuged at 1400 g for 10 min. The supernatants were collected, passed through a 0.45 µm filter (Kanto Chemical) and stored at -40 °C until use.

Bronchoalveolar lavage (BAL) and collection of BAL fluid (BALF). Mice were sacrificed on day 1 after inoculation with bacteria and BAL was performed. The trachea was exposed and intubated using a 1.7 mm outer diameter polyethylene catheter. BAL was performed by instilling 2 ml PBS containing 5 mM EDTA and

BALF was pooled for each animal. Leukocyte numbers were counted with a haemocytometer. Cytospins were prepared, stained with May-Giemsa and differential cell counts were performed. The remaining BALF was stored at -40°C until further analysis.

Preparation of BM-M ϕ s and stimulation with *L. pneumophila* and LPS. BM-M ϕ s were prepared from bone marrow exudates of mouse femurs, as described previously (Celada *et al.*, 1984; Swanson & Isberg, 1995). The cells were seeded overnight in 96-well tissue culture plates (10^4 cells per well) and used for BM-M ϕ experiments. After the medium had been changed, BM-M ϕ s were infected with *L. pneumophila* at the indicated m.o.i. for 2 h. At the end of the infection period (time 0), non-phagocytosed and non-adherent bacteria were removed by two washes with medium. These cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 . At the indicated time points, culture supernatants were collected and the infected macrophages were lysed, as described previously (Yoshizawa *et al.*, 2005). Harvested bacterial suspensions were plated on BCYE agar plates after serial tenfold dilutions and incubated at 37°C for 4 days. In some experiments, BM-M ϕ s were incubated with LPS of *L. pneumophila* or *E. coli* at the indicated concentrations for 24 h. Supernatants of BM-M ϕ cultures were collected and centrifuged at 10 000 r.p.m. for 5 min. Samples were stored at -40°C until used for ELISA.

ELISAs for MIP-2, KC, IL-6 and IL-12. The levels of each cytokine in the culture supernatant of cells and lung homogenates were determined using a commercially available ELISA kit (DuoSet, ELISA development system; R&D Systems), according to the manufacturer's instructions. The ELISA consistently detected MIP-2, KC, IL-6 and IL-12 concentrations of between 20 and 4000 pg ml^{-1} . The ELISA did not cross-react with other chemokines or cytokines.

Statistical analysis. Statistical significance was determined using an unpaired, two-tailed, alternative Student's *t*-test. Survival curves were constructed using the Kaplan-Meier method and analysed using a log rank test. A value of $P < 0.05$ was considered to be significant.

RESULTS

Lethal sensitivity of TLR-deficient mice in *L. pneumophila* pneumonia

C57BL/6 control and TLR2- and TLR4-deficient mice were intratracheally challenged with *L. pneumophila* Suzuki strain (3×10^6 c.f.u. per mouse) and survival was assessed twice daily for 10 days after infection (Fig. 1). Wild-type mice started to die from day 4 after infection, with a survival rate of 70% (14/20) at the end of the observation period. TLR4-deficient mice demonstrated similar kinetics in lethality to wild-type mice, with an overall survival rate of 60% (12/20). In contrast, significantly higher mortality was observed in TLR2-deficient mice, as survival in these mice was only 35% (7/20) ($P < 0.05$). These data suggested a protective role for TLR2, but not TLR4, in this murine model of *L. pneumophila* pneumonia.

Bacterial burden in the lungs of mice with *L. pneumophila* pneumonia

After intratracheal inoculation of bacteria, mice were sacrificed on days 1, 2 and 3 and the number of bacteria in the lungs was determined as described in Methods

Fig. 1. Lethal sensitivity of TLR-deficient mice in *L. pneumophila* pneumonia. C57BL/6 control (■) and TLR2- (▲) and TLR4- (●) deficient mice were challenged intratracheally with *L. pneumophila* Suzuki strain (3×10^6 c.f.u. per mouse) and mortality was observed twice daily for 10 days after infection. *, $P < 0.05$ compared with the wild-type.

Fig. 2. Bacterial burden in the lungs of mice with *L. pneumophila* pneumonia. After intratracheal inoculation of bacteria, mice were sacrificed on days 1, 2 and 3 and the number of bacteria in the lungs was compared in wild-type (filled bars), TLR2-deficient (open bars) and TLR4-deficient (hatched bars) mice, as described in Methods ($n = 5$).

(Fig. 2). Despite a significant difference in lethal sensitivity, there were no differences in bacterial number in the lungs of wild-type and TLR2- and TLR4-deficient mice ($n = 5$). These data suggested that bacterial overgrowth in the lungs did not account for the higher lethal sensitivity observed in TLR2-deficient mice.

MIP-2, KC, IL-6 and IL-12 in the lungs of mice with *L. pneumophila*

Next, we examined the production of MIP-2, KC, IL-6 and IL-12 in the lungs of wild-type and TLR2- and TLR4-deficient mice at the indicated time points (Fig. 3). IL-12 is

Fig. 3. Levels of MIP-2, KC, IL-6 and IL-12 in the lungs of mice with *L. pneumophila* pneumonia. Production of MIP-2 (a), KC (b), IL-6 (c) and IL-12 (d) was examined in the lungs of wild-type (filled bars), TLR2-deficient (open bars) and TLR4-deficient (hatched bars) mice. *, $P < 0.05$ compared with wild-type.

required for the generation of protective Th1-type host responses against *Legionella* infection, whereas MIP-2 and KC are major chemokines involved in neutrophil recruitment. Although *L. pneumophila* infection induced an increase in the expression of the cytokines in all groups, the extent and pattern differed in the various mutant and wild-type strains. At early time points (6 and 12 h), a reduced production of MIP-2, KC and IL-6 was found in TLR2-deficient mice compared with that observed in wild-type and TLR4-deficient mice. Conversely, significantly higher levels of IL-12 were observed in TLR2-deficient mice than in the wild-type control at 18 and 24 h after infection. No significant differences in IL-12 levels were noted between wild-type and TLR4-deficient mice.

In addition, we examined MIP-2 levels in BALF. Whilst no detectable levels of MIP-2 were observed in control uninfected mice, there was considerable expression of MIP-2 (approx. 400 pg ml⁻¹) detected in BALF from the lungs of infected wild-type mice. In TLR4-deficient mice, a greater than twofold increase in MIP-2 in BALF was found compared with that detected in wild-type control mice. In contrast, the levels of MIP-2 were only approximately 50 % of that observed in infected wild-type animals, although this difference was not statistically significant. These data were consistent with the MIP-2 results in the lung homogenates and further suggested the importance of TLR2-mediated signalling, including MIP-2 production, in host responses against *L. pneumophila* infection.

Leukocyte accumulation in BALF

To understand further the higher mortality in TLR2-deficient mice in the absence of changes in bacterial

clearance, leukocyte accumulation in the air space was examined in infected mice on day 1 (Table 1). Compared with uninfected animals, *L. pneumophila* administration resulted in an almost tenfold increase in BAL cells within the lungs of wild-type mice, of which > 90 % were neutrophils. In TLR4-deficient mice, no substantial differences were observed in cell numbers and differentials from those of wild-type mice. Importantly, a drastic reduction in inflammatory cells was observed in TLR2-deficient mice. Total cell number and numbers of neutrophils, macrophages and lymphocytes in TLR2-deficient mice were reduced by 24.9, 23.4, 38.0 and 46.5 %, respectively, compared with wild-type mice with *L. pneumophila* infection. These data suggested a reduction in cellular infiltration, especially neutrophils, in the infected lungs of TLR2-deficient mice, but not TLR4-deficient mice, which was associated with a decrease in chemokine responses and with the higher mortality observed in TLR2-deficient mice.

BM-M ϕ responses to *L. pneumophila* and its LPS

To examine the cellular mechanisms accounting for higher mortality in TLR2-deficient mice, BM-M ϕ s from wild-type and TLR2- and TLR4-deficient mice (5×10^4 c.f.u. per well) were infected with *L. pneumophila* (day 0) and bacterial numbers and MIP-2 production were examined at the indicated time points ($n=5$). As shown in Fig. 4, the bacterial number of *L. pneumophila* decreased with time in culture. No differences were observed among wild-type and TLR2- and TLR4-deficient mice when an m.o.i. of 10 was applied to the BM-M ϕ s. Next, we examined MIP-2 production in culture supernatants after stimulation with *L. pneumophila*, LPS isolated from *L. pneumophila* and *E. coli*

Table 1. Cell numbers and differentiation in BALF of mice 1 day after infection

Wild-type and TLR2- and TLR4-deficient mice were intratracheally infected with *L. pneumophila*, as described in Methods. Mice were sacrificed on day 1 and BAL was performed ($n=5$). Total cell numbers of leukocytes were counted with a haemocytometer and differentiation of cells was examined in cytopsin samples after staining with May-Giemsa.

	Total no. of cells per mouse ($\times 10^3$)	Neutrophils (%)	Macrophages (%)	Lymphocytes (%)
Wild-type	19.3 ± 3.5	$17\,538.9 \pm 343.0$ (90.9 ± 1.8)	1544 ± 351.7 (8.0 ± 1.8)	217.1 ± 43.7 (1.1 ± 0.2)
TLR2-deficient	4.8 ± 1.3	$4112.0 \pm 37.5^*$ (85.7 ± 0.8) [*]	$586.7 \pm 33.7^*$ (12.2 ± 0.7) [*]	$101.3 \pm 20.3^*$ (2.1 ± 0.4) [*]
TLR4-deficient	20.3 ± 6.0	$18\,716.6 \pm 207.0$ (92.2 ± 1.0)	1258.6 ± 226.1 (6.2 ± 1.1)	324.8 ± 49.7 (1.6 ± 0.2)

* $P < 0.05$ compared with the corresponding results from wild-type mice.

LPS at various concentrations. *L. pneumophila* infection induced MIP-2 production from BM-M ϕ s of wild-type and TLR4-deficient mice in an m.o.i.-dependent manner, whereas significantly lower production levels of MIP-2 were noted in BM-M ϕ s of TLR2-deficient mice (Fig. 5a). To try to determine the molecules responsible for this effect, purified LPS from *L. pneumophila* Suzuki strain was used to stimulate macrophages. As shown in Fig. 5(b), high levels of MIP-2 were produced by BM-M ϕ s isolated from wild-type and TLR4-deficient mice, whereas significantly less MIP-2 was produced from BM-M ϕ s isolated from TLR2-deficient mice in response to *L. pneumophila* LPS. As expected, TLR4-deficient macrophages did not respond to *E. coli* LPS, whereas TLR2-deficient macrophages produced amounts of MIP-2 that were comparable to that of wild-type macrophages when similarly stimulated. These data suggested that BM-M ϕ s from TLR2-deficient mice produced considerably

less MIP-2 in response to *L. pneumophila* and its LPS compared with that produced in response to *E. coli* LPS. These data further support the importance of TLR2-mediated signalling in response to *L. pneumophila* LPS.

DISCUSSION

The present study is the first to demonstrate that TLR2- but not TLR4-deficient mice are more susceptible to pulmonary infection with *L. pneumophila* than wild-type mice. Our data demonstrated an impairment in chemokine production and recruitment of inflammatory cells in the lungs of TLR2-deficient mice infected with *L. pneumophila*. In addition, it was shown that macrophages lacking the TLR2 molecule produced less MIP-2 in response to *L. pneumophila* LPS, but not *E. coli* LPS. These data are consistent with previous reports that LPS of *L. pneumophila* is recognized by the host-cell receptor TLR2 but not TLR4 (Braedel-Ruoff *et al.*, 2005; Girard *et al.*, 2003), and further defined a crucial role for TLR2 in inflammatory cell responses and pathogenesis of *L. pneumophila* pneumonia.

Our data indicated that TLR2-deficient mice exhibited a higher mortality but no differences in lung bacterial burden. On the other hand, cytokine and chemokine responses in the lungs of TLR2-deficient mice were altered compared with those of TLR4-deficient and wild-type mice. These data suggest that the lethal sensitivity of TLR2-deficient mice to *L. pneumophila* pneumonia may be governed by host inflammatory responses, rather than by bacterial numbers in the lungs. In this regard, we have reported previously that neutrophil depletion by administration of granulocyte-specific monoclonal antibody RB6-8C5 sensitized mice to enhanced mortality as a result of *L. pneumophila* infection, which was associated with a shifting of the cytokine balance from Th1 (IFN- γ , IL-12) towards Th2-type (IL-4, IL-10) host responses, but with no change in bacterial burden in the lungs (Tateda *et al.*, 2001a). Although we did not observe a reduction in the Th1 cytokine IL-12 or skewing of the

Fig. 4. Bacterial numbers in BM-M ϕ s infected with *L. pneumophila*. BM-M ϕ s of wild-type (■), TLR2-deficient (▲) and TLR4-deficient (●) mice were infected with *L. pneumophila* at an m.o.i. of 10. At the indicated time points, viable bacterial numbers were examined as described in Methods ($n=5$).

Fig. 5. MIP-2 production by BM-M ϕ s in response to *L. pneumophila* and its LPS. (a) BM-M ϕ s of wild-type (WT) and TLR2- (T2) and TLR4- (T4) deficient mice were infected with *L. pneumophila* at an m.o.i. of 1 (hatched bars) or 10 (filled bars), or were left uninfected as a control (open bars). (b) BM-M ϕ s of wild-type (WT) and TLR2- (T2) and TLR4- (T4) deficient mice were incubated with *L. pneumophila* LPS at concentrations of 1000 (filled bars), 100 (hatched bars) or 10 (shaded bars) EU ml⁻¹, or were incubated without LPS as a control (open bars). (c) BM-M ϕ s of wild-type (WT) and TLR2- (T2) and TLR4- (T4) deficient mice were incubated with *E. coli* LPS at concentrations of 1000 (filled bars), 100 (hatched bars) or 10 (shaded bars) ng ml⁻¹ or were incubated without LPS as a control (open bars). Culture supernatants were collected 1 day after incubation and the concentrations of MIP-2 were determined ($n=5$).

Th2-type host response (data not shown), lower levels of chemokines and severely reduced inflammatory cell accumulation were demonstrated in the lungs of TLR2-deficient mice. These findings may be associated with exaggerated mortality in TLR2-deficient mice with *L. pneumophila* pneumonia, as a blockade of CXC chemokine receptor-2, a receptor for CXC chemokines including MIP-2 and KC, strikingly enhances the mortality of infected animals (Tateda *et al.*, 2001b). More recently, impairment of chemokine production and inflammatory cell recruitment, especially neutrophils, was reported in the lungs of TLR2-deficient mice in an aerosolized model of *L. pneumophila* pneumonia (Hawn *et al.*, 2006).

Yoshida *et al.* (1991) reported that peritoneal macrophages from C3H/HeJ mice, which carry a non-functional gene encoding TLR4, are permissive for *L. pneumophila* growth, whereas macrophages from control C3H/HeN mice are resistant. In contrast, Lettinga *et al.* (2002) demonstrated that, in pulmonary *L. pneumophila* infection, C3H/HeJ mice were indistinguishable from control C3H/HeN mice in bacterial growth in infected lungs. Others have reported that intracellular growth of *L. pneumophila* was enhanced within TLR2-deficient macrophages compared with wild-type and TLR4-deficient mice (Akamine *et al.*, 2005). In a mouse pneumonia model, genetic deletion of myeloid differentiation primary response gene-88 (MyD88), a major adaptor protein in TLR signalling, resulted in severe impairment of eradication of *L. pneumophila* from the lungs, whereas TLR2-deficient mice displayed only a modest reduction in the clearance of bacteria (Archer & Roy, 2006; Hawn *et al.*, 2006). In our hands, no substantial differences in growth of *L. pneumophila* were observed between TLR2-deficient and wild-type mice in macrophages *in vitro* or in the *in vivo* pneumonia model. These data suggest that TLR2-mediated

signalling and additional MyD88-dependent, TLR2-independent pathways may be essential for full protection against the growth of *L. pneumophila* in the lungs.

Several lines of evidence have demonstrated that TLRs are a critical sensor for pathogen-associated molecular patterns, such as LPS, peptidoglycan and flagella (Aderem & Ulevitch, 2000; Akira & Takeda, 2004; Beutler, 2004; Janeway & Medzhitov, 2002). Although TLR4 was originally recognized as a receptor for LPS of Gram-negative bacteria, a more comprehensive analysis demonstrated an expanded repertoire of TLRs that function in LPS recognition by host immune cells. The endotoxins of several pathogens, including *Leptospira*, *Porphyromonas*, *Chlamydia* and *Rhizobium*, have been reported to stimulate host cells through activation of TLR2 (Erridge *et al.*, 2004; Girard *et al.*, 2003; Hirschfeld *et al.*, 2001; Werts *et al.*, 2001). Girard *et al.* (2003) reported that LPS of *L. pneumophila* required TLR2 rather than TLR4 to elicit the expression of CD14 in bone marrow granulocytes. In addition, Braedel-Ruoff *et al.* (2005) reported that purified *L. pneumophila* LPS, as well as *L. pneumophila* (either viable or formalin killed), was able to activate bone marrow dendritic cells from TLR4-deficient mice, but failed to activate bone marrow dendritic cells from TLR2-deficient mice. Our data in BM-M ϕ s stimulated with *L. pneumophila* LPS are in line with these reports and further demonstrate TLR2 as a recognition molecule of *L. pneumophila* LPS for induction of MIP-2. The present results also demonstrate that higher concentrations of LPS are able to overcome the lack of TLR2, again suggesting the involvement of other factors. Recently, Hawn *et al.* (2003) reported that TLR5 plays an essential role in governing host cytokine responses and susceptibility to *Legionella* infection in humans through the sensing mechanism of flagellin of this organism. Moreover, several investigators, including us, have reported

that Naip5- and caspase-1-dependent cytosolic recognition of flagellin and subsequent host responses may be a critical factor for restriction of *L. pneumophila* in macrophages and in the lungs (Molofsky *et al.*, 2006; Ren *et al.*, 2006; Zamboni *et al.*, 2006). It is likely that cell-surface sensing by TLR(s) and cytosolic recognition by Naip5 may be collaborating and interacting for maximal induction of host innate immune responses, which is an exciting area for future *Legionella* research.

Probably associated with the above results, reduction of MIP-2, KC and IL-6 production was observed in the *L. pneumophila*-infected lungs of TLR2-deficient mice at early time points (6 and 12 h). These data were consistent with a recent report in which complete and partial impairment of chemokine/cytokine responses was demonstrated in the *L. pneumophila*-infected lungs of MyD88- and TLR2-deficient mice, respectively (Hawn *et al.*, 2006). Conversely, we observed an increase in IL-12 at relatively later time points (18 and 24 h) after infection. Although the reason for this result is unknown, a plausible explanation is that exaggerated IL-12 production may be due to a feedback mechanism to compensate for the lack of adequate host responses. A better understanding of inter- and counter-regulation of related cytokines/chemokines in the setting of TLR deficiency is required.

Neumeister *et al.* (1998) demonstrated that *L. pneumophila* LPS was about 1000 times less potent in its ability to induce pro-inflammatory cytokines from Mono Mac 6 cells than LPS of members of the *Enterobacteriaceae*. Several investigators have suggested a unique structure of *L. pneumophila* LPS, such as lack of negatively charged groups, the presence of a hydrophobic outer core and the length of the fatty acid chains (Erridge *et al.*, 2004; Girard *et al.*, 2003; Hirschfeld *et al.*, 2001; Knirel *et al.*, 1996; Moll *et al.*, 1997). From the viewpoint of evolutionary advantage, it will be of interest to consider the reasons for TLR2-mediated recognition and the relatively weak biological activity of *L. pneumophila* LPS. Future studies assessing the molecular mechanisms of receptor–ligand interaction between TLR2 and *L. pneumophila* LPS, and defining the disparity and functional significance of TLR2 or TLR4 signalling in the pathogenesis of a variety of infectious diseases, are warranted.

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