

## Role of MyD88 and Toll-Like Receptors 2 and 4 in the Sensing of *Parachlamydia acanthamoebae*<sup>∇</sup>

Thierry Roger,<sup>1</sup># Nicola Casson,<sup>2</sup># Antony Croxatto,<sup>2</sup> José Manuel Entenza,<sup>3</sup> Marc Pusztaszeri,<sup>4</sup> Shizuo Akira,<sup>5</sup> Marlies Knaup Reymond,<sup>1</sup> Didier Le Roy,<sup>1</sup> Thierry Calandra,<sup>1</sup> and Gilbert Greub<sup>2\*</sup>

Infectious Diseases Service, Department of Medicine, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland<sup>1</sup>; Center for Research on Intracellular Bacteria, Institute of Microbiology, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland<sup>2</sup>; Department of Fundamental Microbiology, University of Lausanne, Lausanne, Switzerland<sup>3</sup>; Institute of Pathology, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland<sup>4</sup>; and Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan<sup>5</sup>

Received 20 July 2010/Returned for modification 27 August 2010/Accepted 2 September 2010

*Parachlamydia acanthamoebae* is a *Chlamydia*-related organism whose pathogenic role in pneumonia is supported by serological and molecular clinical studies and an experimental mouse model of lung infection. Toll-like receptors (TLRs) play a seminal role in sensing microbial products and initiating innate immune responses. The aim of this study was to investigate the roles of MyD88, TLR2, and TLR4 in the interaction of *Parachlamydia* with macrophages. Here, we showed that *Parachlamydia* entered bone-marrow derived macrophages (BMDMs) in a TLR-independent manner but did not multiply intracellularly. Interestingly, compared to live bacteria, heat-inactivated *Parachlamydia* induced the production of substantial amounts of tumor necrosis factor alpha (TNF), interleukin-6 (IL-6), and IL-12p40 by BMDMs and of TNF and IL-6 by peritoneal macrophages as well as RAW 264.7 and J774 macrophage cell lines. Cytokine production by BMDMs, which was partially inhibited upon trypsin treatment of *Parachlamydia*, was dependent on MyD88, TLR4, and, to a lesser extent, TLR2. Finally, MyD88<sup>-/-</sup>, TLR4<sup>-/-</sup>, and TLR2<sup>-/-</sup> mice were as resistant as wild-type mice to lung infection following the intratracheal instillation of *Parachlamydia*. Thus, in contrast to *Chlamydia pneumoniae*, *Parachlamydia acanthamoebae* weakly stimulates macrophages, potentially compensating for its low replication capacity in macrophages by escaping the innate immune surveillance.

*Parachlamydia acanthamoebae* is a strict intracellular bacterium which naturally infects free-living amoebae. Like other members of the *Chlamydiales* order, it exhibits a two-stage developmental cycle with infectious elementary bodies and metabolically active replicating reticulate bodies (25). Several pieces of evidence support the role of *P. acanthamoebae* as a new agent of lower respiratory tract infection (reviewed in references 18 and 32). The first hint was provided by the recovery of *P. acanthamoebae* strain Hall's coccus from the water of a humidifier associated with an outbreak of fever and the presence of anti-*Parachlamydia* antibodies among exposed individuals (2). Additional serological studies demonstrated a higher seropositivity rate among patients with pneumonia than among controls (20, 33). Furthermore, parachlamydial DNA was detected by PCR in mononuclear cells of a patient with bronchitis and in sputa and bronchoalveolar lavage samples from patients with lower respiratory tract infections (11, 12, 19, 37). Moreover, *Parachlamydia* infects human pneumocytes and macrophages *in vitro* (7, 23, 24). Finally, we recently developed an experimental model in which mice injected with living *Parachlamydia* showed signs of severe pneumonia and bacte-

rial localization in cells that were likely pneumocytes and macrophages (9, 10).

The sensing of invasive pathogens by innate immune cells relies on their capacity to sense microbial molecular motifs through pattern recognition receptors. Toll-like receptors (TLRs) expressed on the surface or in the endosomes of immune cells allow the detection of microbially derived molecular structures such as lipids, proteins, and nucleic acids. TLR4 is an obligate partner for the host response to bacterial lipopolysaccharide (LPS) (endotoxin) and most Gram-negative bacteria (1). TLR2, which generates heterodimers in combination with either TLR1 or TLR6, has been reported to recognize a broad range of microbial compounds, among which are lipopeptides, lipoproteins, peptidoglycan subcomponents, and  $\beta$ -glucans (29). The activation of the intracellular signaling pathways upon microbial recognition by TLRs engages several adaptor molecules. Myeloid differentiation primary response gene 88 (MyD88) specifies most TLRs and the TLR4 MyD88-dependent signaling pathway, which is involved in the activation of mitogen-activated protein kinases and nuclear factor  $\kappa$ B and in the generation of proinflammatory cytokines and immune-related genes (35).

Although *Parachlamydia* may represent an emerging agent of pneumonia (18, 26), very little is known about its recognition by innate immune cells. Unfortunately, the mechanisms involved in the sensing of *Chlamydia* are most likely irrelevant for *Parachlamydia* given the specificities of this bacterium, such as its ability to replicate within amoebae (22) and its genome size, which is more than twice that of *Chlamydia* (21). In

\* Corresponding author. Mailing address: Center for Research on Intracellular Bacteria, Institute of Microbiology, Centre Hospitalier Universitaire Vaudois and University of Lausanne, 1011 Lausanne, Switzerland. Phone: 41-21-314 49 79. Fax: 41-21-314 40 60. E-mail: gilbert.greub@chuv.ch.

# These authors contributed equally to the work.

<sup>∇</sup> Published ahead of print on 13 September 2010.

addition, the bioinformatics-based annotation of the genome of the *Parachlamydia*-related symbiont UWE25 indicates that it likely possesses a truncated LPS and lacks most immunogenic outer membrane proteins present in the *Chlamydiaceae* (27). Considering the central role played by TLRs in microbial sensing, we studied the contributions of MyD88, TLR2, and TLR4 in the recognition of *P. acanthamoebae* by macrophages *in vitro* and in the outcome of parachlamydial infection in an experimental model of pneumonia in mice. Our results show that living *P. acanthamoebae* enters but does not multiply in macrophages and that heat-inactivated *P. acanthamoebae* stimulates cytokine production by macrophages in a MyD88/TLR4-dependent manner and to a lesser extent through TLR2. Furthermore, MyD88<sup>-/-</sup>, TLR4<sup>-/-</sup>, and TLR2<sup>-/-</sup> mice are resistant to *P. acanthamoebae* infection. Taken together, these data indicate that *P. acanthamoebae* weakly stimulates the innate immune system, which may allow the bacterium to survive in infected cells despite its low replication capacity.

#### MATERIALS AND METHODS

**Mice.** Eight- to 12-week-old female BALB/c and C57BL/6 mice were obtained from Charles River Laboratories (L'Arbresle, France) and acclimatized for at least 1 week before experimentation. MyD88<sup>-/-</sup>, TLR2<sup>-/-</sup>, and TLR4<sup>-/-</sup> mice have been described previously (28, 30, 45). All animal procedures were approved by the Office Vétérinaire du Canton de Vaud, Lausanne, Switzerland (authorization no. 876.5, 877.5, and 1860.1), and performed according to our institutional guidelines for animal experiments.

**Cells and reagents.** *P. acanthamoebae* strain Hall's coccus was propagated in *Acanthamoeba castellanii* (strain ATCC 30010; American Type Culture Collection, Manassas, VA) in peptone-yeast extract-glucose broth medium (PYG) at 32°C. After 6 days of incubation, cultures were harvested and the bacteria were purified using a sucrose barrier, as previously described (7). Bacteria were inactivated by heating for 1 h at 95°C. In selected experiments, the supernatant and the insoluble material from heat-inactivated *P. acanthamoebae* were obtained by centrifugation (10 min at 20,800 × g). The insoluble pellet was treated at 37°C for 1 h with trypsin (50 µg/ml) or for 8 h with DNase I (100 µg/ml) (Sigma-Aldrich, St. Louis, MO). *Salmonella enterica* serovar Minnesota ultrapure LPS was from List Biologicals Laboratories (Campbell, CA) and palmitoyl-cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-OH trifluoroacetate salt (Pam<sub>3</sub>CSK<sub>4</sub>) from EMC Microcollections (Tuebingen, Germany).

Bone marrow-derived macrophages (BMDMs) and thioglycolate-elicited peritoneal macrophages were obtained as previously described (15, 41). BMDMs were cultured in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen, Basel, Switzerland) containing 10% fetal calf serum (FCS) (Seromed, Berlin, Germany) and 50 µM 2-mercaptoethanol. Thioglycolate-elicited peritoneal macrophages and RAW 264.7 and J774 macrophage cell lines (ATCC) were cultured in RPMI 1640 medium (Invitrogen) containing 2 mM glutamine and 10% FCS.

**Phagocytosis.** BMDMs (5 × 10<sup>5</sup>) were grown overnight on 12-mm-diameter round coverslips in flat-bottom 24-well cell culture plates (Corning, Lowell, MA). After washing, BMDMs were incubated with living or heat-inactivated bacteria (bacterium-to-cell ratio, 100:1), centrifuged at 1,793 × g for 10 min at room temperature, and incubated for 15 min at 37°C. BMDMs were washed with RPMI medium and further incubated for different periods of time at 37°C in a 5% CO<sub>2</sub> atmosphere. Coverslips were washed with phosphate-buffered saline (PBS). Cells were fixed with 3% paraformaldehyde, washed with PBS, and permeabilized in PBS containing 0.3% Tween (Merck-Schuchardt, Hohenbrunn, Germany). Coverslips were incubated with rabbit anti-*Parachlamydia* antibodies for 30 min at room temperature, washed, and incubated with Alexa 488-coupled anti-rabbit immunoglobulin antibodies (Bio-Rad, Rheinach, Switzerland) and 1% Evans blue (bioMérieux, Marcy-l'Étoile, France) for 30 min at room temperature. Primary and secondary antibodies were diluted in PBS containing 0.3% Tween and 10% FCS. The coverslips were washed successively with PBS-Tween and PBS and mounted onto glass slides. Cells were observed under an epifluorescence microscope (Axioplan 2; Zeiss, Feldbach, Switzerland) and a confocal microscope (Axioplan 2 LSM 510; Zeiss). The number of bacteria per macrophage was determined by counting three times the number of *Parachlamydia* organisms within about 50 macrophages.

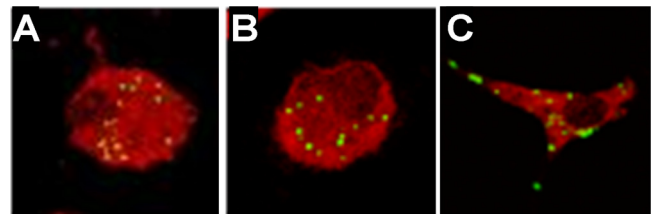


FIG. 1. *Parachlamydia acanthamoebae* is internalized by macrophages. Intracellular detection of *P. acanthamoebae* within wild-type (A), TLR2<sup>-/-</sup> (B), and TLR4<sup>-/-</sup> (C) BMDMs at 4 h postinfection is shown. Macrophages and *P. acanthamoebae* were stained with Evans blue and Alexa 488, respectively, and analyzed by confocal microscopy as described in Materials and Methods.

**Cytokine measurements.** Macrophages were plated at a density of 5 × 10<sup>5</sup> cells per well in 24-well culture plates (Costar). Cells were stimulated for 4 to 24 h with LPS (10 ng/ml), Pam<sub>3</sub>CSK<sub>4</sub> (100 ng/ml), or *P. acanthamoebae*. The concentrations of tumor necrosis factor (TNF) and interleukin-6 (IL-6) in cell culture supernatants were measured using WEHI 164 clone 13 mouse fibrosarcoma cells (TNF) and 7TD-1 mouse hybridoma cells (IL-6) as described previously (40). IL-1β and IL-12p40 were quantified by enzyme-linked immunosorbent assay (ELISA) (RnD, Minneapolis, MN, and Becton Dickinson, San Diego, CA).

**Mouse model of infection.** Mice were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg) and midazolam (1 mg/kg). The trachea was uncovered, and 1.25 × 10<sup>8</sup> to 2.5 × 10<sup>8</sup> *P. acanthamoebae* bacteria were injected intratracheally using a sterile 26-gauge needle. Sham-operated mice were injected with PBS. The incision was closed using surgical staples. The body weight and the survival of mice were followed at least once daily. In selected experiments, blood was collected after 24 h to quantify cytokine levels. At selected time points, mice were euthanized by CO<sub>2</sub> inhalation. Lungs were harvested and snap-frozen in liquid nitrogen. DNA was extracted and the copy number of the *P. acanthamoebae* genome determined by quantitative PCR as described previously (8). Parts of mouse lungs were minced in PBS for cytokine quantification or fixed with 4% paraformaldehyde for histology. Hematoxylin-eosin stained slides were read blindly by M.K.P. and G.G.

#### RESULTS

***Parachlamydia acanthamoebae* is internalized by macrophages but does not multiply inside macrophages.** Entry of *P. acanthamoebae* into BMDMs was confirmed by confocal microscopy and found to be similar for wild-type, TLR2<sup>-/-</sup>, and TLR4<sup>-/-</sup> BMDMs (Fig. 1). No growth of *Parachlamydia* was observed in BMDMs. The mean numbers of bacteria per macrophage at 15 min after infection were 7.6, 11.0, and 7.9 and those 48 h later were 10, 8.6, and 13.3 in wild-type, TLR2<sup>-/-</sup>, and TLR4<sup>-/-</sup> BMDMs, respectively ( $P > 0.05$  for all conditions).

**MyD88, TLR4, and, to a lesser extent, TLR2 contribute to the sensing of heat-inactivated *Parachlamydia acanthamoebae* by macrophages.** As a first step to investigate whether TLRs play a role in the sensing of *P. acanthamoebae* by innate immune cells, we compared the production of TNF, IL-6, and IL-12p40 by wild-type, TLR2<sup>-/-</sup>, and TLR4<sup>-/-</sup> BMDMs infected with *P. acanthamoebae* for 4, 8, 12, and 24 h. Experiments were performed using both living and heat-inactivated (1 h at 95°C) bacteria (Fig. 2). Overall, TLR2<sup>-/-</sup> BMDMs exposed to heat-inactivated *P. acanthamoebae* produced reduced levels of TNF and IL-6 compared to wild-type BMDMs, whereas TLR4<sup>-/-</sup> BMDMs produced almost no TNF, IL-6, or IL-12p40. Strikingly, living *P. acanthamoebae* slightly stimulated cytokine production by BMDMs. To substantiate this observation, we then tested the cytokine-inducing capacities of

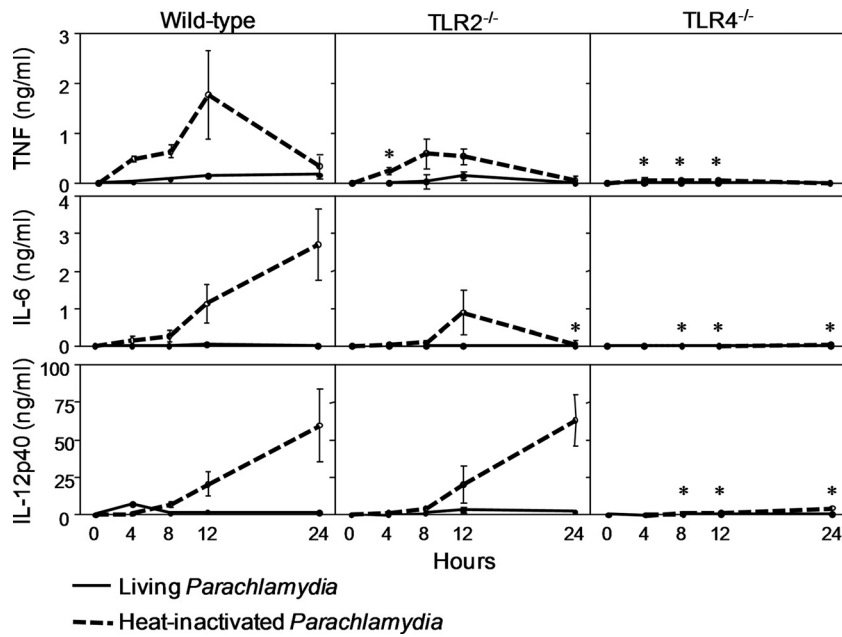


FIG. 2. Heat-inactivated *Parachlamydia acanthamoebae* stimulates the production of proinflammatory cytokines by macrophages through TLR4 and, to a lesser extent, TLR2. The kinetics of TNF, IL-6, and IL-12p40 production by wild-type, TLR2<sup>-/-</sup>, and TLR4<sup>-/-</sup> BMDMs infected with live and heat-inactivated *P. acanthamoebae* (multiplicity of infection [MOI], 100) are shown. Data are means ± standard deviations (SD) for triplicate samples from one experiment and are representative of three independent experiments. \*, *P* < 0.05 versus result for wild-type BMDMs.

living and heat-inactivated *P. acanthamoebae* in peritoneal macrophages and in the RAW 264.7 and J774 macrophage cell lines. Recapitulating the results obtained with BMDMs, living bacteria induced much lower levels of TNF and IL-6 than heat-inactivated bacteria (*P* < 0.001) (Fig. 3).

Considering that MyD88 is required for signaling through TLR2 and TLR4, we then compared the responses of wild-type and MyD88<sup>-/-</sup> peritoneal macrophages and BMDMs to infection with heat-inactivated *P. acanthamoebae* (Fig. 4). As expected, MyD88 deficiency strongly impaired TNF production and fully abrogated IL-6 production by macrophages, indicating that heat-inactivated *P. acanthamoebae* stimulated the release of cytokines in a MyD88-dependent manner.

To gain insight into the nature of the molecular structures involved in the activation of macrophages by *P. acanthamoebae*, the supernatant and the insoluble material obtained after centrifugation of heat-inactivated *P. acanthamoebae* preparations were treated with DNase and trypsin and tested for their ability to stimulate TNF, IL-6, and IL-12p40 release by BMDMs (Fig. 5 and data not shown). Whereas the supernatant of heat-inactivated bacteria did not induce cytokine release by BMDMs, the bacterial pellet recovered in PBS stimulated cytokine production to the same extent as the original bacterial preparation. Trypsin treatment reduced TNF and IL-6 production induced by the bacterial pellet by 2-fold, whereas DNase treatment had no impact.

Altogether, these data suggested that heat inactivation of *P. acanthamoebae* exposed a trypsin-sensitive microbial molecular pattern that stimulated cytokine release by macrophages.

**Roles of MyD88, TLR2, and TLR4 in the host response to *Parachlamydia acanthamoebae* pneumonia.** To analyze *in vivo* the roles of the TLR pathways in modulating the innate im-

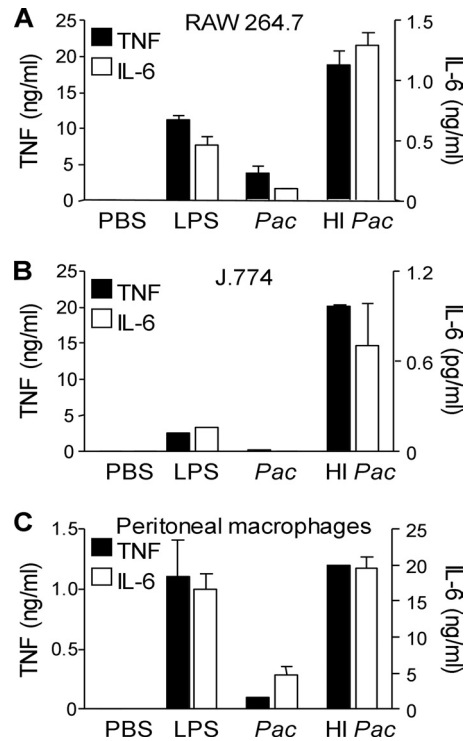


FIG. 3. Heat-inactivated *Parachlamydia acanthamoebae* stimulates cytokine production by RAW 264.7, J774, and peritoneal macrophages. TNF and IL-6 production by RAW 264.7 (A), J774 (B), and thioglycolate-elicited peritoneal (C) macrophages cultured with PBS, LPS, and living (*Pac*) or heat-inactivated (*HI Pac*) *P. acanthamoebae* is shown. Data are means ± SD for triplicates samples from one experiment; *P* < 0.05 for living versus heat-inactivated *P. acanthamoebae* (A to C).



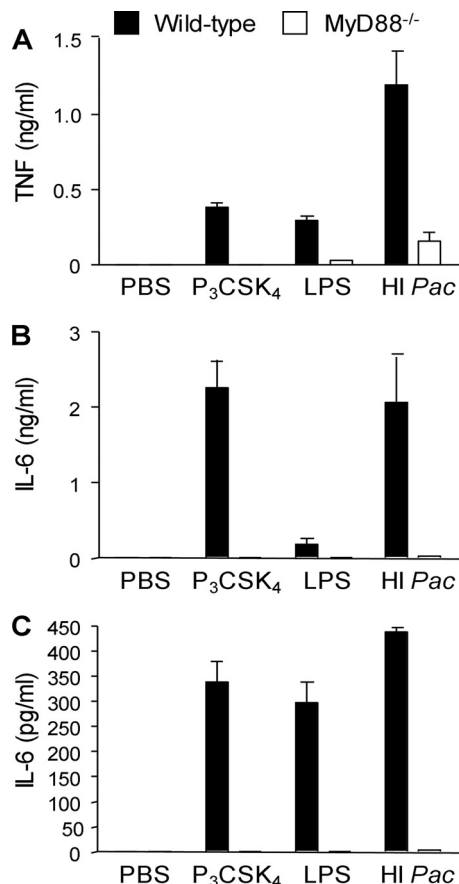


FIG. 4. MyD88 is required for the sensing of heat-inactivated *Parachlamydia acanthamoebae* by macrophages. TNF and IL-6 production by thioglycolate-elicited macrophages (A and B) and BMDMs (C) exposed to PBS, Pam<sub>3</sub>CSK<sub>4</sub> (P<sub>3</sub>CSK<sub>4</sub>), LPS, and heat-inactivated *P. acanthamoebae* (HI Pac) is shown. Data are means  $\pm$  SD for triplicate samples from one experiment and are representative of two independent experiments;  $P < 0.05$  for wild-type versus MyD88<sup>-/-</sup> BMDMs for all conditions.

immune response to *P. acanthamoebae*, we established a model of pneumonia following the intratracheal instillation of  $2.5 \times 10^8$  bacteria in wild-type and MyD88<sup>-/-</sup> animals. Lungs and blood were collected from wild-type and MyD88<sup>-/-</sup> animals at 24 h after infection with  $2.5 \times 10^8$  *P. acanthamoebae* bacteria to quantify cytokines. In line with our *in vitro* observations, larger amounts of IL-1 $\beta$  and IL-6 were detected in the lungs and larger amounts of IL-6 and IL-12p40 were detected in the circulation of wild-type mice, confirming *in vivo* that MyD88 is involved in the host response to *P. acanthamoebae* (Fig. 6A and B).

To analyze whether the reduced inflammatory response impaired host defenses, we then compared the susceptibilities of wild-type and MyD88<sup>-/-</sup> animals to infection with  $2.5 \times 10^8$  bacteria (Fig. 7A). Interestingly, MyD88<sup>-/-</sup> mice were as resistant as wild-type mice to infection with *P. acanthamoebae* (64% survival and 72% survival in wild-type and MyD88<sup>-/-</sup> mice, respectively;  $n = 25$  or  $26$  [ $P > 0.5$ ]). In agreement with these findings, at 8 days after the onset of infection, the copy numbers of the *P. acanthamoebae* genome in the lungs of wild-type and MyD88<sup>-/-</sup> mice were similar (Fig. 7B). Con-

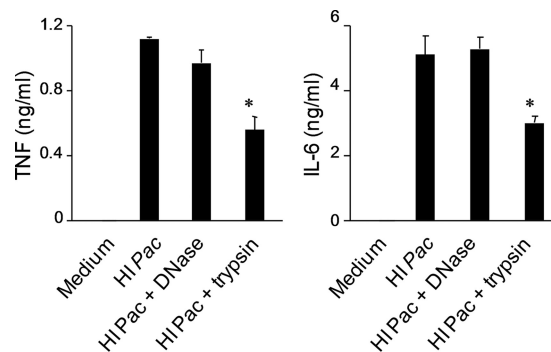


FIG. 5. Heat-inactivated *Parachlamydia acanthamoebae* expresses a trypsin-sensitive determinant that stimulates cytokine production by macrophages. TNF and IL-6 production by BMDMs exposed to heat-inactivated *P. acanthamoebae* insoluble material treated or not with trypsin or DNase is shown. Data are means  $\pm$  SD for triplicate samples from one experiment. \*,  $P < 0.05$ .

firming these observations, deficiency in TLR2 or TLR4 did not increase the susceptibility of mice to infection with *P. acanthamoebae* ( $1.25 \times 10^8$  bacteria) (Fig. 7C). Moreover, histological analyses showed that the natural history was similar in wild-type and MyD88<sup>-/-</sup> mice infected with *P. acanthamoebae*. Thus, at 24 h postinfection, one mouse in each group (three mice per group) presented an inflammatory infiltrate, which was composed mainly of neutrophils (Fig. 8A and B). Moreover, wild-type and MyD88<sup>-/-</sup> mice that died at about 7 to 10 days postinfection exhibited signs of severe pneumonia, with multifocal to confluent lympho-monocytic infiltrates composed primarily of macrophages (Fig. 8C and D).

## DISCUSSION

Clinical and experimental data suggest that *P. acanthamoebae* represents a new agent of lower respiratory tract infection (18). The characterization of the innate immune receptors engaged by *P. acanthamoebae* is essential for improving our understanding of the pathogenesis of *P. acanthamoebae* infection. Analyses of the response of macrophages to *P. acanthamoebae* revealed a critical role for the MyD88/TLR4 pathway, and to a lesser extent TLR2, in the production of proinflammatory cytokines. Yet, heat-inactivated bacteria were much more immunogenic than live bacteria. In line with these results, mice deficient in either MyD88, TLR4, or TLR2 were resistant to lung infection with *P. acanthamoebae*.

*P. acanthamoebae* quickly entered BMDMs. *P. acanthamoebae* internalization was not affected in macrophages deficient in either TLR2 or TLR4, corroborating the notion that TLRs do not function primarily as phagocytic receptors (46). Of note, the initial rate of infection of BMDMs (around 10 bacteria per BMDM) was similar to that of human macrophages, A549 pneumocytes, and HEL lung fibroblasts (two to four bacteria per infected cell [7, 24]). Nonetheless, upon internalization, *P. acanthamoebae* did not multiply inside BMDMs, whereas it grows poorly in human macrophages, A549, and HEL cells (7, 24). Overall, these data suggest that *P. acanthamoebae* has a rather limited replication capacity in innate immune cells compared to amoebae, where an exponential growth of 2 log units was observed (22).

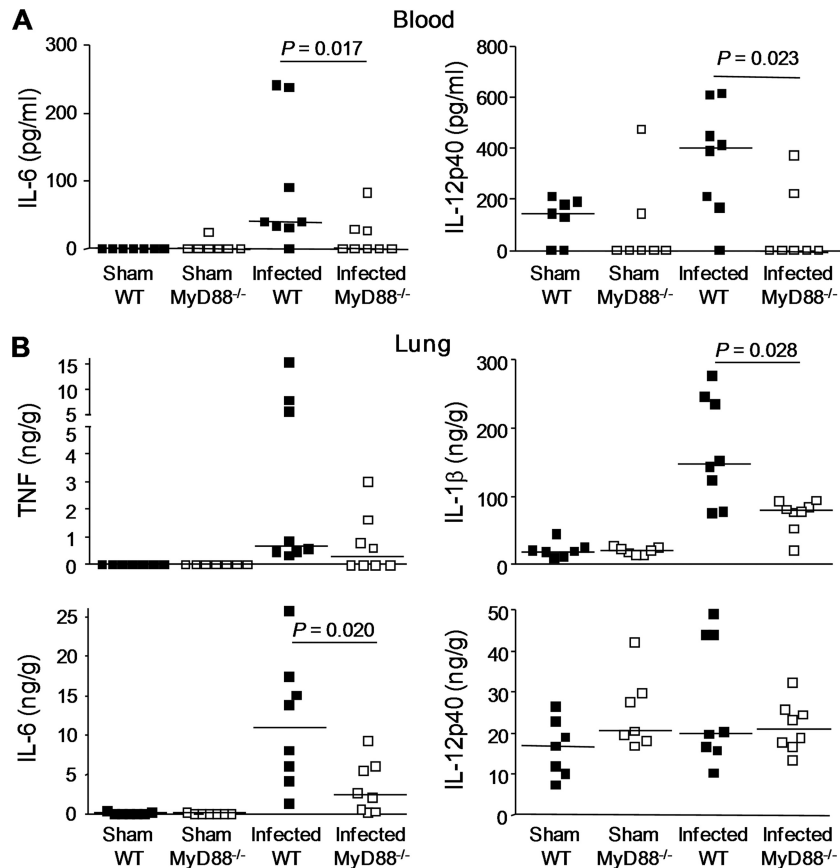


FIG. 6. Reduced cytokine levels in the blood and the lungs of MyD88<sup>-/-</sup> mice infected with *Parachlamydia acanthamoebae*. Wild-type (WT) (black symbols) and MyD88<sup>-/-</sup> (white symbols) C57BL/6 mice ( $n = 8$  mice per group) were either sham operated or infected with  $2.5 \times 10^8$  *P. acanthamoebae*. Blood (A) and lungs (B) were collected after 24 h to quantify TNF, IL-6, IL-1 $\beta$ , and IL-12p40. The horizontal line represents the median value. Of note, TNF and IL-1 $\beta$  were not detected in the blood.

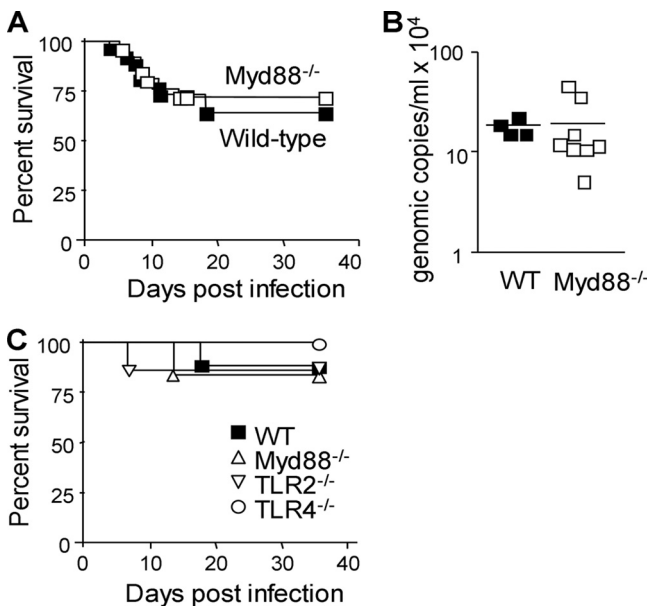


FIG. 7. MyD88<sup>-/-</sup>, TLR2<sup>-/-</sup>, and TLR4<sup>-/-</sup> mice are resistant to *Parachlamydia* infection. Survival (A and C) and *P. acanthamoebae* genomic copy number in the lungs (day 8) (B) of mice infected with  $2.5 \times 10^8$  (A and B) and  $1.25 \times 10^8$  (C) *P. acanthamoebae* bacteria is shown ( $n = 25$  or  $26$  mice per group for panel A and  $n = 6$  to  $8$  mice per group for panel C);  $P > 0.05$  for all conditions.

Unexpectedly, heat-inactivated *P. acanthamoebae* induced the production of substantial levels of cytokines by macrophages, whereas living bacteria only weakly stimulated cytokine production. Most likely, heat inactivation exposed an immunogenic determinant, probably a protein given its partial susceptibility to trypsin, hidden in living bacteria. An obvious candidate is a heat shock protein (HSP). Indeed, the genome of *P. acanthamoebae* contains several genes encoding putative HSPs (21), and HSP60 from *Chlamydia pneumoniae* and *Chlamydia trachomatis* have been reported to strongly stimulate innate immune cells (4, 13, 31). Further investigations will be required to characterize the structure of the microbially associated molecular pattern(s) of *P. acanthamoebae* that stimulates innate immune cells.

The recognition of heat-inactivated *P. acanthamoebae* by macrophages occurred mainly through MyD88/TLR4. Yet, TNF and IL-6 production by TLR2<sup>-/-</sup> BMDMs was also reduced to some extent. Interestingly, the MyD88/TLR4 pathway has previously been reported to be involved in the sensing of *C. pneumoniae* and *C. pneumoniae* HSP60 (4, 42, 43) and to mediate the acute pulmonary inflammation in mice following the intratracheal instillation of *C. pneumoniae* HSP60 (5). Furthermore, the TLR4 pathway was implicated in *C. trachomatis* HSP60-induced trophoblast apoptosis (16). Besides TLR4,

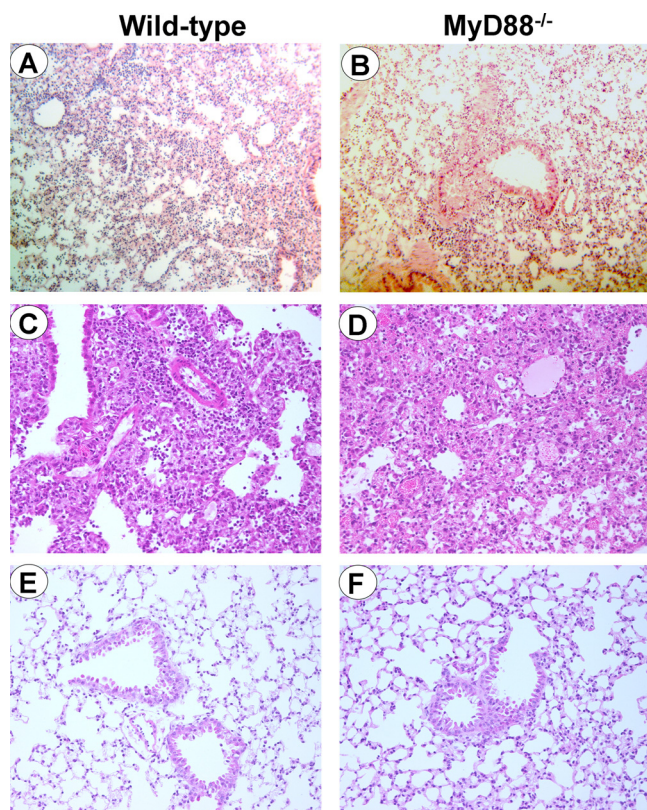


FIG. 8. Histology of lungs from C57BL/6 wild-type (A, C, and E) and MyD88<sup>-/-</sup> (B, D, and F) mice either infected with  $2.5 \times 10^8$  *P. acanthamoebae* bacteria (A to D) or sham operated (E and F). Lungs were collected after 24 h (A, B, E, and F) or at the time of death (C and D). Note the presence of focal inflammation with the presence of neutrophils at 24 h postinfection of both wild-type (A) and MyD88<sup>-/-</sup> (B) mice and the presence of multifocal lympho-monocytic infiltrates, with a predominance of macrophages, in both wild-type mice (C) and MyD88<sup>-/-</sup> (D) mice at time of death. No inflammatory infiltrates were observed in sham operated wild-type (E) and MyD88<sup>-/-</sup> (F) mice. Hematoxylin-eosin staining was used; magnification,  $\times 200$  (A to F).

TLR2 was shown to play a primary role in the activation of dendritic cells by *C. pneumoniae* and *C. pneumoniae* HSP60 (13, 38) and to mediate *C. pneumoniae*-induced macrophage foam cell formation (6). Moreover, TLR2 was implicated in oviduct pathology resulting from genital tract infection with *C. trachomatis* (14) and triggered the early immune response to *C. pneumoniae* pulmonary infection (39). Further substantiating a role for TLRs in the sensing of *Chlamydiaceae*, *C. pneumoniae*-infected MyD88<sup>-/-</sup> mice showed impaired production of cytokines and chemokines and delayed recruitment of T cells, and they ultimately died from severe chronic lung infection (34). Altogether, these data point toward a fundamental role of MyD88, TLR2, and/or TLR4 in detecting *Chlamydia* spp., in particular *Chlamydia* HSPs. In the future, it might be interesting to study the role of intracellular sensors of bacteria such as nucleotide-binding and oligomerization domain-like receptors (NLRs), which have been shown to mediate a cell response to *C. pneumoniae*, *C. trachomatis*, and *Chlamydia muridarum* (3, 36, 44, 47).

Models of lung infection have underscored the vital role of the early inflammatory response in mediating bacterial clear-

ance and conditioning survival. As mentioned above, mice deficient in MyD88, TLR4, and TLR2 ultimately developed a severe and chronic lung inflammation involved in morbidity and mortality after *C. pneumoniae* infection (34, 39, 44). These observations demonstrate that TLRs exert a two-sided role in *Chlamydia* infections, acting on the one hand as key initiators of the host anti-*Chlamydia* defense responses and on the other hand as important contributors to tissue damage and *Chlamydia* pathogenicity. Astonishingly at first sight, MyD88, TLR4, and TLR2 deficient mice were fully resistant to infection with *P. acanthamoebae*. Whereas these data could argue for a minor pathogenic role of *P. acanthamoebae* in healthy individuals, it is worthwhile to remember that most documented cases of probable infection with *P. acanthamoebae* were reported in immunocompromised HIV-infected or grafted patients (19, 33). Thus, it would be of interest to further analyze the pathogenicity of *P. acanthamoebae* in severely immunocompromised mice. Taking into account that *P. acanthamoebae* modestly replicated in infected mammalian cells *in vitro* (7, 24) and that the bacterial load decreased with time (10-fold in 2 days and 1,000-fold in 10 days) in mice infected with *P. acanthamoebae* (9), *P. acanthamoebae* may compensate for its low replication capacity by escaping innate immune surveillance. Importantly, there are significant differences in the replication capacities of *Parachlamydia* in murine and human macrophages (no versus limited replication capacity) as well as in cytokine production by these cells following exposure to heat-inactivated bacteria (presence and absence of proinflammatory cytokine secretion). Thus, the mouse model may partially reflect what occurs in human infections.

In summary, we report the first insight into the mechanisms of recognition of *P. acanthamoebae* by the innate immune system. MyD88 or TLR4 deficiencies do not impair mouse survival after *P. acanthamoebae* infection, whereas they did so after *C. pneumoniae* infection, indicating that this *Chlamydia*-related bacterium has evolved a different strategy to interact with the host. It will be interesting to investigate the innate immune response to other *Chlamydia*-related bacteria such as *Waddlia chondrophila*, a potential agent of miscarriage and lower respiratory tract infection which was shown to replicate in human macrophages (17). Finally, further work will be devoted to the identification of the molecular pattern(s) of *P. acanthamoebae* that stimulates innate immune cells, as this may help in development of improved serologic diagnostic tests for infection and better understanding of the pathogenesis of *Parachlamydia* infection.

#### ACKNOWLEDGMENTS

This study was supported by Swiss National Science Foundation grants 310000-1140 (T.R.), 310000-118266 (T.C.), 310030-124843 (G.G.), and 3200BO-116445 (G.G.) and by the Leenaards Foundation (T.R., T.C., and G.G.). T.C. is supported by the Bristol-Myers Squibb and the Santos-Suarez Foundations. Gilbert Greub is supported by the Leenaards Foundation through a career award (Bourse Leenaards pour la Relève Académique en Médecine Clinique à Lausanne).

None of the authors has a conflict of interest.

#### REFERENCES

1. Beutler, B., and E. T. Rietschel. 2003. Innate immune sensing and its roots: the story of endotoxin. *Nat. Rev. Immunol.* **3**:169–176.
2. Birtles, R. J., T. J. Rowbotham, C. Storey, T. J. Marrie, and D. Raoult. 1997. *Chlamydia*-like obligate parasite of free-living amoebae. *Lancet* **349**:925–926.



3. Buchholz, K. R., and R. S. Stephens. 2006. Activation of the host cell proinflammatory interleukin-8 response by *Chlamydia trachomatis*. *Cell. Microbiol.* **8**:1768–1779.
4. Bulut, Y., E. Faure, L. Thomas, H. Karahashi, K. S. Michelsen, O. Equils, S. G. Morrison, R. P. Morrison, and M. Arditì. 2002. Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway. *J. Immunol.* **168**:1435–1440.
5. Bulut, Y., K. Shimada, M. H. Wong, S. Chen, P. Gray, R. Alsabeh, T. M. Doherty, T. R. Crother, and M. Arditì. 2009. Chlamydial heat shock protein 60 induces acute pulmonary inflammation in mice via the Toll-like receptor 4- and MyD88-dependent pathway. *Infect. Immun.* **77**:2683–2690.
6. Cao, F., A. Castrillo, P. Tontonoz, F. Re, and G. I. Byrne. 2007. *Chlamydia pneumoniae*-induced macrophage foam cell formation is mediated by Toll-like receptor 2. *Infect. Immun.* **75**:753–759.
7. Casson, N., N. Medico, J. Bille, and G. Greub. 2006. *Parachlamydia acanthamoebae* enters and multiplies within pneumocytes and lung fibroblasts. *Microbes Infect.* **8**:1294–1300.
8. Casson, N., K. M. Posfay-Barbe, A. Gervais, and G. Greub. 2008. New diagnostic real-time PCR for specific detection of *Parachlamydia acanthamoebae* DNA in clinical samples. *J. Clin. Microbiol.* **46**:1491–1493.
9. Casson, N., J. M. Entenza, N. Borel, A. Pospischil, and G. Greub. 2008. Murine model of pneumonia caused by *Parachlamydia acanthamoebae*. *Microb. Pathog.* **45**:92–97.
10. Casson, N., K. Sommer, A. Klos, J. C. Stehle, M. Pusztaszeri, and G. Greub. 2008. Intranasal murine model of infection by *Parachlamydia acanthamoebae*, p. 17. *Proc. Annu. Meet. Swiss Soc. Infect. Dis.*
11. Corsaro, D., D. Venditti, A. Le Faou, P. Guglielmetti, and M. Valassina. 2001. A new chlamydia-like 16S rDNA sequence from a clinical sample. *Microbiology* **147**:515–516.
12. Corsaro, D., D. Venditti, and M. Valassina. 2002. New parachlamydial 16S rDNA phylotypes detected in human clinical samples. *Res. Microbiol.* **153**:563–567.
13. Costa, C. P., C. J. Kirschning, D. Busch, S. Durr, L. Jennen, U. Heinzmann, S. Prebeck, H. Wagner, and T. Miethke. 2002. Role of chlamydial heat shock protein 60 in the stimulation of innate immune cells by *Chlamydia pneumoniae*. *Eur. J. Immunol.* **32**:2460–2470.
14. Darville, T., J. M. O'Neill, C. W. Andrews, Jr., U. M. Nagarajan, L. Stahl, and D. M. Ojcius. 2003. Toll-like receptor-2, but not Toll-like receptor-4, is essential for development of oviduct pathology in chlamydial genital tract infection. *J. Immunol.* **171**:6187–6197.
15. Delaloye, J., T. Roger, G. G. Steiner-Tardivel, D. Le Roy, M. Knaup Raymond, S. Akira, V. Petrilli, C. E. Gomez, B. Perdigueron, J. Tschopp, G. Pantaleo, M. Esteban, and T. Calandra. 2009. Innate immune sensing of modified vaccinia virus Ankara (MVA) is mediated by TLR2-TLR6, MDA-5 and the NALP3 inflammasome. *PLoS Pathog.* **5**:e1000480.
16. Equils, O., D. Lu, M. Gatter, S. S. Witkin, C. Bertolotto, M. Arditì, J. A. McGregor, C. F. Simmons, and C. J. Hobel. 2006. Chlamydia heat shock protein 60 induces trophoblast apoptosis through TLR4. *J. Immunol.* **177**:1257–1263.
17. Goy, G., A. Croxatto, and G. Greub. 2008. *Waddlia chondrophila* enters and multiplies within human macrophages. *Microbes Infect.* **10**:556–562.
18. Greub, G. 2009. *Parachlamydia acanthamoebae*, an emerging agent of pneumonia. *Clin. Microbiol. Infect.* **15**:18–28.
19. Greub, G., P. Berger, L. Papazian, and D. Raoult. 2003. *Parachlamydiaceae* as rare agents of pneumonia. *Emerg. Infect. Dis.* **9**:755–756.
20. Greub, G., I. Boyadjiev, B. La Scola, D. Raoult, and C. Martin. 2003. Serological hint suggesting that *Parachlamydiaceae* are agents of pneumonia in polytraumatized intensive care patients. *Ann. N. Y. Acad. Sci.* **990**:311–319.
21. Greub, G., C. Kebbi-Beghdadi, C. Bertelli, F. Collyn, B. M. Riederer, C. Yersin, A. Croxatto, and D. Raoult. 2009. High throughput sequencing and proteomics to identify immunogenic proteins of a new pathogen: the dirty genome approach. *PLoS One* **4**:e8423.
22. Greub, G., B. La Scola, and D. Raoult. 2003. *Parachlamydia acanthamoeba* is endosymbiotic or lytic for *Acanthamoeba polyphaga* depending on the incubation temperature. *Ann. N. Y. Acad. Sci.* **990**:628–634.
23. Greub, G., J. L. Mege, J. P. Gorvel, D. Raoult, and S. Meresse. 2005. Intracellular trafficking of *Parachlamydia acanthamoebae*. *Cell. Microbiol.* **7**:581–589.
24. Greub, G., J. L. Mege, and D. Raoult. 2003. *Parachlamydia acanthamoebae* enters and multiplies within human macrophages and induces their apoptosis. *Infect. Immun.* **71**:5979–5985.
25. Greub, G., and D. Raoult. 2002. Crescent bodies of *Parachlamydia acanthamoeba* and its life cycle within *Acanthamoeba polyphaga*: an electron micrograph study. *Appl. Environ. Microbiol.* **68**:3076–3084.
26. Greub, G., and D. Raoult. 2002. *Parachlamydiaceae*: potential emerging pathogens. *Emerg. Infect. Dis.* **8**:625–630.
27. Horn, M., A. Collingro, S. Schmitz-Esser, C. L. Beier, U. Purkhold, B. Fartmann, P. Brandt, G. J. Nyakatura, M. Droege, D. Frishman, T. Rattei, H. W. Mewes, and M. Wagner. 2004. Illuminating the evolutionary history of chlamydiae. *Science* **304**:728–730.
28. Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.* **162**:3749–3752.
29. Ishii, K. J., S. Koyama, A. Nakagawa, C. Coban, and S. Akira. 2008. Host innate immune receptors and beyond: making sense of microbial infections. *Cell Host Microbe* **3**:352–363.
30. Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* **11**:115–122.
31. Kol, A., T. Bourcier, A. H. Lichtman, and P. Libby. 1999. Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages. *J. Clin. Invest.* **103**:571–577.
32. Lamoth, F., and G. Greub. 2010. Amoebal pathogens as emerging causal agents of pneumonia. *FEMS Microbiol. Rev.* **34**:260–280.
33. Marrie, T. J., D. Raoult, B. La Scola, R. J. Birtles, and E. de Carolis. 2001. *Legionella*-like and other amoebal pathogens as agents of community-acquired pneumonia. *Emerg. Infect. Dis.* **7**:1026–1029.
34. Naiki, Y., K. S. Michelsen, N. W. Schroder, R. Alsabeh, A. Slepkin, W. Zhang, S. Chen, B. Wei, Y. Bulut, M. H. Wong, E. M. Peterson, and M. Arditì. 2005. MyD88 is pivotal for the early inflammatory response and subsequent bacterial clearance and survival in a mouse model of *Chlamydia pneumoniae* pneumonia. *J. Biol. Chem.* **280**:29242–29249.
35. O'Neill, L. A., and A. G. Bowie. 2007. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat. Rev. Immunol.* **7**:353–364.
36. Opitz, B., S. Forster, A. C. Hocke, M. Maass, B. Schmeck, S. Hippenstiel, N. Suttorp, and M. Krull. 2005. Nod1-mediated endothelial cell activation by *Chlamydia pneumoniae*. *Circ. Res.* **96**:319–326.
37. Ossewaarde, J. M., and A. Meijer. 1999. Molecular evidence for the existence of additional members of the order *Chlamydiales*. *Microbiology* **145**:411–417.
38. Prebeck, S., C. Kirschning, S. Durr, C. da Costa, B. Donath, K. Brand, V. Redecke, H. Wagner, and T. Miethke. 2001. Predominant role of Toll-like receptor 2 versus 4 in *Chlamydia pneumoniae*-induced activation of dendritic cells. *J. Immunol.* **167**:3316–3323.
39. Rodriguez, N., N. Wantia, F. Fend, S. Durr, H. Wagner, and T. Miethke. 2006. Differential involvement of TLR2 and TLR4 in host survival during pulmonary infection with *Chlamydia pneumoniae*. *Eur. J. Immunol.* **36**:1145–1155.
40. Roger, T., J. David, M. P. Glauser, and T. Calandra. 2001. MIF regulates innate immune responses through modulation of Toll-like receptor 4. *Nature* **414**:920–924.
41. Roger, T., C. Froidevaux, D. Le Roy, M. K. Raymond, A. L. Chanson, D. Mauri, K. Burns, B. M. Riederer, S. Akira, and T. Calandra. 2009. Protection from lethal gram-negative bacterial sepsis by targeting Toll-like receptor 4. *Proc. Natl. Acad. Sci. U. S. A.* **106**:2348–2352.
42. Romano Carratelli, C., N. Mazzola, R. Paolillo, S. Sorrentino, and A. Rizzo. 2009. Toll-like receptor-4 (TLR4) mediates human beta-defensin-2 (HBD-2) induction in response to *Chlamydia pneumoniae* in mononuclear cells. *FEMS Immunol. Med. Microbiol.* **57**:116–124.
43. Sasu, S., D. LaVerda, N. Qureshi, D. T. Golenbock, and D. Beasley. 2001. *Chlamydia pneumoniae* and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via Toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation. *Circ. Res.* **89**:244–250.
44. Shimada, K., S. Chen, P. W. Dempsey, R. Sorrentino, R. Alsabeh, A. V. Slepkin, E. Peterson, T. M. Doherty, D. Underhill, T. R. Crother, and M. Arditì. 2009. The NOD/RIP2 pathway is essential for host defenses against *Chlamydia pneumoniae* lung infection. *PLoS Pathog.* **5**:e1000379.
45. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* **11**:443–451.
46. Underhill, D. M., and B. Gantner. 2004. Integration of Toll-like receptor and phagocytic signaling for tailored immunity. *Microbes Infect.* **6**:1368–1373.
47. Welter-Stahl, L., D. M. Ojcius, J. Viala, S. Girardin, W. Liu, C. Delarbre, D. Philpott, K. A. Kelly, and T. Darville. 2006. Stimulation of the cytosolic receptor for peptidoglycan, Nod1, by infection with *Chlamydia trachomatis* or *Chlamydia muridarum*. *Cell. Microbiol.* **8**:1047–1057.