Comparative Toll-Like Receptor 4-Mediated Innate Host Defense to *Bordetella* Infection

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*Bordetella pertussis, B. parapertussis, and B. bronchiseptica are closely related species associated with respiratory disease in humans and other mammals. While *B. bronchiseptica* has a wide host range, *B. pertussis* and *B. parapertussis* evolved separately from a *B. bronchiseptica*-like progenitor to naturally infect only humans. Despite very different doubling times in vitro, all three establish similar levels of infection in the mouse lung within 72 h. Recent work has revealed separate roles for Toll-like receptor 4 (TLR4) in immunity to *B. pertussis* and *B. bronchiseptica*, while no role for TLR4 during *B. parapertussis* infection has been described. Here we compared the requirement for TLR4 in innate host defense to these organisms using the same mouse infection model. While *B. bronchiseptica* causes lethal disease in TLR4-deficient mice, *B. pertussis* and *B. parapertussis* do not. Correspondingly, TLR4 is critical in limiting *B. bronchiseptica* but not *B. pertussis* or *B. parapertussis* bacterial numbers during the first 72 h. Interestingly, *B. bronchiseptica* induces a TLR4-dependent cytokine response that is considerably larger than that induced by *B. pertussis* or *B. parapertussis*. Analysis of their endotoxins using RAW cells suggests that *B. bronchiseptica* lipopolysaccharide (LPS) is 10- and 100-fold more stimulatory than *B. pertussis* or *B. parapertussis* LPS, respectively. The difference in LPS stimulus is more pronounced when using HEK293 cells expressing human TLR4. Thus, it appears that in adapting to infect humans, *B. pertussis* and *B. parapertussis* independently modified their LPS to reduce TLR4-mediated responses, which may compensate for slower growth rates and facilitate host colonization.

The bordetellae are aerobic, gram-negative coccobacilli that infect the respiratory tracts of mammals (38, 46). *Bordetella pertussis* and *B. parapertussis* are pathogens of humans and the etiologic agents of whooping cough, or pertussis, an acute respiratory disease. *B. bronchiseptica*, the likely progenitor of the two human pathogens, has a wide host range and often causes persistent asymptomatic infection (38, 46). These three bacteria are closely related species that share a large number of virulence determinants that facilitate colonization of the host’s respiratory tract (34). Mouse models for studying host-pathogen interactions during *Bordetella* infection are a well-developed system in which to compare the role of specific host defense mechanisms during infection with each of these bacteria (19).

The ability of bacteria to successfully infect the host is dependent on a variety of factors which, in the case of *Bordetella* species, are regulated via the BvgAS two-component system (2, 10). This system regulates a number of adhesins, toxins, and lipopolysaccharide (LPS) modifications, some of which may counter host defenses and aid in colonization of the respiratory tract (34). Previous work suggested that certain *Bordetella* virulence factors synergize to modulate host immunity (42, 45). Interestingly, *B. bronchiseptica*, *B. pertussis*, and *B. parapertussis* express overlapping yet distinct subsets of these factors (38). This suggests that each species has unique ways to modify immune responses in order to optimize its ability to infect the host.

Toll-like receptors (TLR) are a family of germ line-encoded pattern recognition receptors that mediate the host’s ability to detect pathogen-associated molecular patterns (PAMPs) and activation of host defense mechanisms (3, 23, 35, 36). TLR4 is required for the detection of many bacterial LPSs and thus plays an important role in host defense to certain gram-negative bacteria (8, 39). Interestingly, the described role of TLR4 in host defense against a number of gram-negative organisms appears to vary widely, and in some instances it does not appear to be required (5, 6, 13, 17, 21, 31, 32, 37, 40, 43, 44, 47). The apparently different roles of TLR4 may simply be explained by differences in experimental design or, more interestingly, may be related to differences in host-pathogen interactions.

We previously demonstrated that TLR4 is critical for innate immunity during *B. bronchiseptica* infection and that TLR4-deficient mice rapidly die following infection with initial doses as low as 5,000 CFU (32, 33). In this model a critical role for TLR4 is in mediating a protective early elicited tumor necrosis factor alpha (TNF-α) response. Interestingly, Higgins et al. have described a different role for TLR4 during infection with *B. pertussis*. In their model TLR4 appears to be more important for developing adaptive immunity, specifically, interleukin-10 (IL-10)-mediated T regulatory cell responses (21). Currently, there are no published observations on a requirement...
for TLR4 for host defense during B. parapertussis infection. Here, we used the mouse infection model to compare the requirement for TLR4 in innate host defense to each of these closely related bacteria using strains whose genomes were recently sequenced. Our results indicate that TLR4-mediated immunity is vital to innate host defense against B. bronchiseptica; however, it appears less significant against B. pertussis or B. parapertussis.

MATERIALS AND METHODS

Animal infections. Wild-type C57BL/10ScSn, TLR-deficient (TLR4-/-) C3H/HeJ, and TLR4-deleted (TLR4-/-) C57BL/10ScNcr mice were obtained from Jackson Laboratories. Wild-type C3H/HeN mice were obtained from Charles River Laboratories. All mice were maintained in the animal care facility at The Pennsylvania State University in accordance with institutional guidelines. Mice were lightly sedated with isoflurane and inoculated intranasally (i.n.) with the indicated CFU of B. bronchiseptica in 50 μl of phosphate-buffered saline (PBS; Merck) by pipetting the inoculum onto the tip of the external nares. B. bronchiseptica (RB50), B. pertussis (536), and B. parapertussis (12822) were grown as previously described. (18, 20). For animal experiments, groups of at least four mice were sacrificed at the indicated time point, and colonization of the lungs was quantified as previously described (18). For in vivo cytokine measurements, the lungs were homogenized in 5 ml of PBS and stored at −80°C until assayed. The lung homogenate aliquots were subsequently thawed and assayed by enzyme-linked immunosorbent assay (ELISA) using matched antibody sets and appropriate standards (R&D Systems). For TNF-α neutralization experiments, 1 ml of PBS containing 1 mg of anti-TNF-α, from clone MP6-XT3, was injected intraperitoneally at the indicated time postinoculation (22). For neutrophil depletion experiments, 1 ml of PBS containing 0.5 mg of anti-Ly6, from clone RB6-8C5, was injected intraperitoneally at the indicated time postinoculation (9, 22). Determinations of lung leukocyte and neutrophil numbers were performed as previously described (31). For survival studies, mice were observed for increased signs and symptoms of bordetellosis, including ruffled fur, labored breathing, and diminished responsiveness. Moribund mice were euthanized to alleviate suffering.

In vitro LPS experiments. LPS from B. bronchiseptica (RB50), B. pertussis (536), and B. parapertussis (12822) was extracted and purified as previously described (14). Cells were replated in 24-well plates at approximately 10⁶ cells per well in complete Dulbecco’s modified Eagle’s medium and then exposed to 1 μg/ml of LPS in overnight culture. RAW 264.7 cells were obtained from ATCC. HEK293 cells stably transfected with human TLR4/MD2 were previously described (28). LPS stimulations were performed by placing cells in 96-well plates at approximately 10⁵ cells per well in complete Dulbecco’s modified Eagle’s medium. LPS was resuspended in sterile PBS at 1 mg/ml, and 10-fold dilutions were made. Each LPS suspension was sonicated for 5 min prior to diluting or mixing with cell culture media. Cells were exposed to the indicated concentration of LPS in overnight culture. The culture medium was removed and assayed by ELISA.

Statistics. The mean ± standard error was determined for each treatment group in the individual experiments. Statistical significance was calculated using a paired Student’s t-test, with a significance level of P < 0.05 for a single comparison. Animal infection experiments for cytokine assays or counting bacteria were repeated two or three times.

RESULTS

TLR4 is critical to innate host defense to B. bronchiseptica but not B. pertussis or B. parapertussis. The reported roles for TLR4 in immunity against B. bronchiseptica and B. pertussis appear to differ dramatically (21, 32); however, it is possible that differences in experimental conditions may contribute to these disparate findings. Additionally, the role of TLR4 during B. parapertussis infection has not been reported. In order to examine the relative requirement for TLR4 in innate host defense against the bordetellae using the same experimental model, that of limiting acute infection, we inoculated groups of wild-type (WT) C3H/HeN mice and TLR4-deficient C3H/HeJ mice with a range of doses of B. bronchiseptica, B. pertussis, or B. parapertussis via the i.n. route. The mice were then observed for signs of morbidity associated with severe bordetellosis. As previously observed, TLR4-deficient mice inoculated with approximately 5 × 10⁵ CFU of B. bronchiseptica developed lethal disease within 96 h of inoculation; however, these mice exhibited no outward signs of disease when inoculated with the same number of either B. pertussis or B. parapertussis organisms (Fig. 1A). Additionally, TLR4-deficient mice infected with B. pertussis and B. parapertussis survived and eventually eliminated the bacteria (Fig. 1A and data not shown), whereas TLR4-
deficient mice developed lethal disease following inoculation with as few as $5 \times 10^5$ CFU of *B. bronchiseptica*. Similar mortality patterns were observed in TLR4-deleted C57BL/10ScNCr mice, further supporting a critical requirement for TLR4 in innate host defense against *B. bronchiseptica* but not *B. pertussis* or *B. parapertussis* (Fig. 2A).

To examine the requirement for TLR4 in limiting bacterial growth within the lungs during the first 3 days, we inoculated wild-type and TLR4-deficient mice as previously described and measured bacterial numbers in the lungs at 0, 2, 6, 12, 24, 48, and 72 h postinoculation. There were no significant differences between the CFU of *B. pertussis* or *B. parapertussis* recovered from the lungs of wild-type and TLR4-deficient mice (Fig. 1C and D) at any time point. However, starting at approximately 12 hours postinoculation, there were approximately 10-fold higher numbers of *B. bronchiseptica* recovered from the lungs of TLR4-deficient mice compared to the lungs of wild-type mice (Fig. 1B). Wild-type mice limited *B. bronchiseptica* numbers to between $10^5$ and $10^6$ CFU after this time point. However, the bacterial numbers in TLR4-deficient mice continued to increase to approximately $10^9$ CFU at 72 h postinoculation. These data suggest that TLR4-mediated innate immune responses are critical to limiting infection with *B. bronchiseptica*, but apparently not those with *B. pertussis* or *B. parapertussis*. To further compare the requirement for TLR4 during infection with these three species, we examined bacterial numbers in the lungs of WT C57BL/10ScSn mice and TLR4-deleted C57BL/10ScNCr mice 3 days postinoculation. The lungs of WT mice contained approximately $10^6$ CFU of *B. bronchiseptica*, whereas the lungs of TLR4-deleted mice infected with this bacterium harbored approximately $10^9$ CFU (Fig. 2B). Similar to what was observed in C3H mice, there were no significant differences between the number of CFU of *B. pertussis* or *B. parapertussis* found in the lungs of WT and TLR4-deleted mice. This result further indicates a critical requirement for TLR4 in innate host defense against *B. bronchiseptica* but not *B. pertussis* or *B. parapertussis*.

**FIG. 2. Comparative role of TLR4.** (A) Survival curve of TLR4-deleted C57BL/10ScNCr mice inoculated with the indicated CFU of *B. bronchiseptica* (circles), *B. pertussis* (diamonds), or *B. parapertussis* (squares). (B) CFU of *B. bronchiseptica*, *B. pertussis*, or *B. parapertussis* in the lungs of WT mice (open bars) or TLR4-deleted mice (solid bars) at 72 h after inoculation.

**FIG. 3.** Role of TLR4 during *B. pertussis* and *B. parapertussis* infection. *B. pertussis* (A) or *B. parapertussis* (B) CFU were recovered from the lungs of mice at the indicated times postinoculation (closed symbols) or from TLR4-deficient mice (open symbols).
for cytokine responses to the LPS from all three *Bordetella* species and suggest that the lack of a critical requirement for TLR4 during innate host defense to *B. pertussis* or *B. parapertussis* is not the result of LPS stimulating via an alternative TLR.

**TNF-α and neutrophils are critical to innate host defense against *B. bronchiseptica* but not *B. pertussis* or *B. parapertussis.**

We have previously observed that during *B. bronchiseptica* infection TLR4-deficient mice have impaired early elicited TNF-α responses (32). These findings suggest that TLR4 mediates the early TNF-α responses to *Bordetella* infection. Additionally, deletion of early elicited TNF-α using anti-TNF-α monoclonal antibodies results in the rapid development of lethal disease during *B. bronchiseptica* infection. To examine the role of this innate host defense factor during infection with each *Bordetella* species, we inoculated WT C3H/HeN mice that were depleted of TNF-α with $5 \times 10^5$ CFU of either *B. bronchiseptica*, *B. pertussis*, or *B. parapertussis* and observed for signs of severe disease. As previously described, TNF-α-depleted mice developed lethal bordetellosis within 4 days of inoculation with *B. bronchiseptica* (Fig. 5A). However, TNF-α-depleted mice infected with *B. pertussis* or *B. parapertussis* did not display symptoms of disease. These findings suggest that early elicited TNF-α is critical to innate host defense against *B. bronchiseptica* but not *B. pertussis* or *B. parapertussis*.

To compare the role of TNF-α in limiting the growth of these bacteria within the respiratory tract, we excised the lungs of control and TNF-α-depleted mice and measured the bacterial numbers on day 3 postinoculation. The lungs of control mice infected with *B. bronchiseptica* harbored between $10^5$ and $10^6$ CFU, whereas the lungs of TNF-α-depleted mice contained approximately $10^5$ CFU (Fig. 5C), suggesting that TNF-α is critical to limiting bacterial numbers during *B. bronchiseptica* infection. Interestingly, the lungs of control mice and TNF-α-depleted mice all contained approximately $10^7$ CFU of *B. pertussis* or *B. parapertussis*, suggesting that, unlike infection with *B. bronchiseptica*, early elicited TNF-α is not critical for innate host defense to *B. pertussis* or *B. parapertussis*.

We have previously observed that during *B. bronchiseptica* infection TLR4-deficient mice have impaired early neutrophil migration to the lungs; Higgins et al. demonstrated a similar result during *B. pertussis* infection of TLR4-deficient mice (21). These findings suggest that TLR4 mediates early neutrophil recruitment to the lungs in response to *Bordetella* infection. To examine the role of these cells during infection with each *Bordetella* species, we inoculated WT C3H/HeN mice and neutrophil-depleted mice with $5 \times 10^5$ CFU of either *B. bronchiseptica*, *B. pertussis*, or *B. parapertussis* and observed for signs of severe disease. Neutrophil-depleted mice developed lethal bordetellosis within 3 days of inoculation with *B. bronchiseptica* (Fig. 5B). However, these same mice infected with *B. pertussis* or *B. parapertussis* did not display increased signs of disease. These findings suggest that neutrophils are critical to innate host defense against *B. bronchiseptica* but not *B. pertussis* or *B. parapertussis*.

To examine the role of neutrophils in limiting the growth of these bacteria within the respiratory tract, we excised the lungs of control and neutrophil-depleted mice and measured bacterial numbers on day 3 postinoculation. The lungs of control mice infected with *B. bronchiseptica* harbored between $10^5$ and
10⁶ CFU, whereas the lungs of neutrophil-depleted mice contained approximately 10⁷ CFU (Fig. 5C), suggesting that neutrophils are critical to limiting bacterial numbers during *B. bronchiseptica* infection. Interestingly, the lungs of control mice and neutrophil-depleted mice all contained approximately 10⁷ CFU of *B. pertussis* or *B. parapertussis*, suggesting that, unlike infection with *B. bronchiseptica*, neutrophils are not critical for innate host defense to *B. pertussis* or *B. parapertussis*.

Infection with *B. bronchiseptica* induces greater TLR4-dependent early elicited inflammatory responses in mouse lungs compared to infection with *B. pertussis* or *B. parapertussis*. We have previously observed that protective early elicited cytokine responses to *B. bronchiseptica* are mediated via TLR4. Whereas our in vitro observations indicated that the host response to the LPS of each *Bordetella* species was TLR4 dependent, our in vivo observations raise the possibility that during infection *B. pertussis* or *B. parapertussis*, neutrophils are not critical for innate host defense to *B. pertussis* or *B. parapertussis*.

Infection with *B. bronchiseptica* induces a significantly higher cytokine response compared to infection with *B. pertussis* and *B. parapertussis*.

Secretion of cytokines and chemokines is often associated with a rapid leukocyte migration to the site of infection. Additionally, gram-negative bacteria often induce the rapid migration of neutrophils to the site of infection. Recently, LPS stimulation of TLR4 has been demonstrated to regulate neutrophil chemotaxis (12). We examined whether the differences in cytokine responses observed during infection with the three *Bordetella* resulted in differences in the number of leukocytes present in the lungs shortly after infection. WT and TLR4-deficient mice were infected as described above, and the number of lung leukocytes was measured at 6 h postinoculation.
FIG. 7. Differential TLR4-mediated polymorphonuclear leukocyte recruitment in vivo. Lung leukocyte numbers were determined at 6 h postinoculation with B. bronchiseptica (Bb), B. pertussis (Bp), or B. parapertussis (Bpp) in WT mice (open bars) or TLR4-deficient mice (solid bars). (A) Total leukocytes; (B) PMNs. Sham, PBS inoculated.

The lungs of WT mice inoculated with B. bronchiseptica contained approximately $25 \times 10^5$ lung leukocytes, whereas the lungs of TLR4-deficient mice inoculated with this bacterium contained approximately $5 \times 10^5$ lung leukocytes (Fig. 7A). Additionally, the predominant leukocyte type present in the lungs of TLR4-deficient mice infected with B. bronchiseptica was neutrophils (Fig. 7B). Interestingly, neither WT nor TLR4-deficient mice exhibited an increase in lung neutrophils upon inoculation with B. pertussis or B. parapertussis. These results suggest that TLR4 mediates the recruitment of neutrophils to the lungs during B. bronchiseptica infection and that B. pertussis and B. parapertussis induce very little neutrophil recruitment in the first 6 hours of infection.

B. bronchiseptica LPS induces greater inflammatory responses in vitro than LPSs from B. pertussis and B. parapertussis. Previous studies have reported differences in the biological and immunological properties of LPSs of the three Bordetella species (1, 48, 49), and the results of our infection experiments suggest that B. bronchiseptica induces a greater inflammatory response via TLR4 than B. pertussis or B. parapertussis. These results suggest that there may be differences in the ability of each LPS to induce TLR4-dependent responses. To investigate this, we exposed RAW 264.7 murine macrophages to increasing concentrations of purified LPS from the three Bordetella species in overnight culture. The supernatants were then assayed for TNF-α as an indicator of LPS responsiveness. B. bronchiseptica LPS induced a measurable increase in TNF-α at a concentration of 0.1 ng/ml, whereas similar increases in this cytokine required exposure to 10 times as much B. pertussis LPS or 100 times as much B. parapertussis LPS (Fig. 8A). Similar results were observed by measuring IL-6 and nitric oxide levels in the cell supernatant (data not shown). Using a modified purpald assay (30) and the available literature regarding LPS structures of the bordetellae (4), B. parapertussis was calculated to have 1.15 times as many molecules per μg of LPS as B. bronchiseptica (data not shown). This indicates that a difference in the number of LPS molecules used in the assay does not account for the differences observed in the stimulation of the RAW264.7 cells by each LPS. Exposure to 100 ng/ml of each LPS resulted in similar levels of cytokine production. These results suggest that at concentrations in the physiologically relevant range, B. bronchiseptica LPS is considerably more stimulatory than LPS from B. pertussis or B. parapertussis.

Since B. pertussis and B. parapertussis are human pathogens, we sought to compare the ability of Bordetella LPS to stimulate human TLR4. To examine this, we exposed HEK293 cells expressing human TLR4/MD2 to increasing concentrations of purified LPS from the three Bordetella species in overnight culture. The supernatants were then assayed for IL-8 as an indicator of LPS responsiveness. B. bronchiseptica LPS induced a measurable increase in IL-8 at concentrations of 1 ng/ml, whereas similar increases in this cytokine required exposure to 10 times as much B. pertussis LPS or 100 times as much B. parapertussis LPS (Fig. 8B). Interestingly, exposure to 1,000 ng/ml of each LPS resulted in significantly different levels of IL-8 production. These results indicate that B. bronchiseptica LPS stimulates human TLR4 more than LPS from B. pertussis or B. parapertussis.

**DISCUSSION**

The ability of a pathogen to infect and colonize the host is dependent on the pathogen’s ability to subvert or avoid host defense mechanisms. Here we show that three closely related species of Bordetella are able to efficiently colonize the mouse lung and establish similar bacterial burdens by day 3 postinfection. The ability of the host to limit these bacteria during this period is independent of adaptive immunity. It is now well established that Toll-like receptors are an important component of innate host defenses, and TLR4 is recognized as playing a central role in limiting certain gram-negative bacterial infections (3, 40). However, recent findings suggest that this receptor is not universally important for innate host defense against all gram-negative bacteria (16, 28). The reasons why a single TLR would be critical for protection against some pathogens but not others which belong to the same class of microorganisms is not understood but may be related to dif-
that TLR4 is not required to limit pertussis numbers after day 7. However, our results indicate with ing bacterial numbers within the first 3 days during infection differences in pathogen virulence pattern and PAMP expression structures in several gram-negative bacteria. Hajjar et al. demonstrated that this organism to primarily infect humans, both B. pertussis and B. parapertussis have reduced their ability to stimulate either mouse or human TLR4. It is possible that this is a common strategy that facilitates infection of a new host or reflects a switch from a nasal commensal to an acute pathogen of the lower respiratory tract. There is significant evidence that adaptation to humans provides selective pressure to modify LPS structures in several gram-negative bacteria. Hajjar et al. demonstrated that in adapting to the human lung, P. aeruginosa is
able to alter its LPS acylation to modulate the response of human TL4R but not murine TL4R (16). Subsequent studies determined that several species of *Salmonella* and *Yersinia* also were able to modify LPS acylation and that this was associated with altered TL4R-mediated cytokine responses (25, 41). Although *B. bronchiseptica* rarely infects humans, there is evidence that human-adapted isolates of *B. bronchiseptica* have undergone LPS modification (15, 29). These findings support a hypothesis that LPS modifications by *B. pertussis* and *B. parapertussis* facilitated adaptation to humans. Determining the LPS and lipid A structures of the *Bordetella* species used in this study, as well as the molecular mechanisms by which the bacteria modify LPS, should provide insight into how organisms are able to alter a highly conserved molecule in order to adapt to a new host. Additionally, using purified LPS from specific *Bordetella* strains with mutations in the LPS synthesis pathway to examine the response mediated by human versus murine TL4R may also elucidate molecular mechanisms involved in host adaptation.

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