

Inefficient Toll-Like Receptor-4 Stimulation Enables *Bordetella parapertussis* to Avoid Host Immunity

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Abstract

The recognition of bacterial lipopolysaccharide (LPS) by host Toll-like receptor (TLR)4 is a crucial step in developing protective immunity against several gram negative bacterial pathogens. *Bordetella bronchiseptica* and *B. pertussis* stimulate robust TLR4 responses that are required to control the infection, but a close relative, *B. parapertussis*, poorly stimulates this receptor, and TLR4 deficiency does not affect its course of infection. This led us to hypothesize that inefficient TLR4 stimulation enables *B. parapertussis* to evade host immunity. In a mouse model of infection, *B. parapertussis* grew rapidly in the lungs, but no measurable increase in TLR4-mediated cytokine, chemokine, or leukocyte responses were observed over the first few days of infection. Delivery of a TLR4 stimulant in the inoculum resulted in a robust inflammatory response and a 10- to 100-fold reduction of *B. parapertussis* numbers. As we have previously shown, *B. parapertussis* grows efficiently during the first week of infection even in animals passively immunized with antibodies. We show that this evasion of antibody-mediated clearance is dependent on the lack of TLR4 stimulation by *B. parapertussis* as co-inoculation with a TLR4 agonist resulted in 10,000-fold lower *B. parapertussis* numbers on day 3 in antibody-treated wild type, but not TLR4-deficient, mice. Together, these results indicate that inefficient TLR4 stimulation by *B. parapertussis* enables it to avoid host immunity and grow to high numbers in the respiratory tract of naïve and immunized hosts.

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Introduction

The ability of a pathogen to persist in its host for an extended period of time requires that it first evades rapid control and clearance by the innate immune response. Lipopolysaccharide (LPS), a major component of the outer membrane of gram negative bacteria, stimulates host Toll-like receptor (TLR)4 to initiate the production of pro-inflammatory cytokines and chemokines that recruit and activate leukocytes [1,2], which is important to protection against many bacterial pathogens [3,4,5,6,7,8]. Interestingly, LPS is not an invariant structure among gram negative bacteria. For example, *Salmonella* deacylates and palmitoylates lipid A in response to the host environment, allowing this bacterium to evade TLR4 responses [9,10]. *Yersinia* and *Pseudomonas* species also modulate their LPS structures, resulting in diminished TLR4 responses to infection [11,12,13,14]. These findings have led to the recent realization that bacteria can modulate pathogen associated molecular patterns, such as LPS, to optimize interactions with the host.

Bordetella bronchiseptica, *B. pertussis* and *B. parapertussis* are three very closely related species that make up the classical bordetellae. *Bordetella bronchiseptica*, infects a wide-range of mammals where it chronically colonizes the nasal cavity [15] and is often observed as a commensal [16,17]. Both *B. pertussis* and *B. parapertussis* are highly infectious pathogens that cause the acute disease whooping cough

in humans [16]. Each of these human-adapted species has independently evolved from a *B. bronchiseptica*-like progenitor [18,19]. The comparative immunobiology of the bordetellae has shed light on some key differences among these bacteria. For example, the LPS structures of each of these bordetellae differs [20,21,22] which results in a wide-range of TLR4 responses and requirements [3]. The LPS of *B. pertussis* and *B. bronchiseptica* are very stimulatory of TLR4 and TLR4 is required for their clearance [3]. In contrast, the LPS of *B. parapertussis* LPS is much less stimulatory of TLR4 and TLR4-deficiency does not render mice more susceptible to *B. parapertussis* [3].

The clearance of these *Bordetella* species by antibodies also differs and appears to relate to their epidemiology [15]. *B. bronchiseptica* is rapidly cleared, three days post-inoculation, by adoptively transferred antibodies [15]. Previous studies have shown that this rapid antibody-mediated clearance is due to TLR4-dependent leukocyte recruitment [23]. *B. bronchiseptica* can persist for years within the nasal cavity of its host, where serum antibodies have no effect, and therefore a strong selection to avoid antibody-mediated clearance does not exist [15]. In contrast to *B. bronchiseptica*, *B. pertussis* and *B. parapertussis* avoid rapid antibody-mediated clearance for the first week of infection until a sufficient T-cell response is generated [15,24]. Therefore, while both *B. pertussis* and *B. parapertussis* are more closely related to *B. bronchiseptica* than they are to each other [18], they share the ability to resist rapid

antibody-mediated clearance from the lower respiratory tract. The high prevalence of detectable antibodies to *B. pertussis* and *B. parapertussis* in human populations, either due to vaccination or previous infection, presents a strong selection for the ability to avoid antibody-mediated clearance, allowing for repeated infection of individuals [15].

While both of these human-adapted species avoid rapid antibody-mediated clearance, they do so by distinct mechanisms. *B. pertussis* avoids rapid antibody-mediated clearance by inhibiting the TLR4-dependent recruitment of leukocytes to the lungs via pertussis toxin (Ptx) [25,26]. A strain lacking Ptx (*B. pertussis*ΔPtx) is rapidly cleared from the lungs upon adoptive transfer of antibodies [25]. Since *B. parapertussis* lacks Ptx [27], this bacterium must avoid antibody-mediated clearance in a Ptx-independent manner.

Since the rapid antibody-mediated clearance of *B. bronchiseptica* is dependent on TLR4 [23] and *B. parapertussis* is a weak stimulator of TLR4 [3], we hypothesized that the inefficient TLR4 stimulation by *B. parapertussis* allows it to avoid the robust inflammatory response required for rapid antibody-mediated clearance. Using a mouse model of infection, we determined that co-inoculation of *B. parapertussis* with a TLR4 stimulant led to enhanced pro-inflammatory cytokine production and leukocyte accumulation as well as more efficient control and rapid antibody-mediated clearance of the bacteria. These results, observed in wild type but not TLR4-deficient animals, explain several characteristics of this important human pathogen and suggest interventions in the disease process. They also demonstrate how very closely related organisms can change complex structural components such as LPS to modulate stimulation of innate immune receptors to optimize their interactions with the host.

Materials and Methods

Bacterial strains and growth

B. parapertussis strain 12822 was isolated from German clinical trials [28] and 12822G is a gentamicin-resistant derivative of 12822 [24]. *B. bronchiseptica* strain RB50 was originally isolated from a rabbit [29]. Bacteria were maintained on Bordet-Gengou agar (Difco) containing 10% defibrinated sheep blood (Hema Resources) and appropriate antibiotics. Liquid culture bacteria were grown at 37°C overnight on a roller drum to mid-log phase in Stainer-Scholte broth.

Inoculation of mice

C57BL/6, C3H/HEO_uJ (wild type), and C3H/HEJ (TLR4-deficient) mice were obtained from Jackson Laboratories and bred in our *Bordetella*-free, specific pathogen-free facilities at The Pennsylvania State University. Bacteria grown overnight (to an optical density at 600 nm of approximately 0.3) in liquid culture were diluted in PBS to approximately 10⁷ CFU/ml. 50 μl of the inoculum (5 × 10⁵ CFU) was pipetted on to the external nares of 4–6 week old mice that had been lightly sedated with 5% isoflurane in oxygen. For co-inoculations with *B. parapertussis* and *B. bronchiseptica*, both species were present at 10⁷ CFU/ml in the inoculum and mice were inoculated as above (5 × 10⁵ CFU of each species in 50 μl). For co-inoculation with heat-killed *B. bronchiseptica*, bacteria were grown overnight to an optical density of 0.3 and heat-inactivated by incubating in a water bath at 75°C for 30 minutes. Bacteria in the inoculum were plated before and after heat-inactivation to quantify the number of bacteria present and ensure that the incubation killed *B. bronchiseptica*. Inocula were prepared so that they contained 10⁷ CFU/ml of *B. parapertussis* and 10⁹ CFU/ml of heat-killed *B. bronchiseptica* (5 × 10³ CFU *B. parapertussis* and 5 × 10⁷ CFU of heat-killed *B. bronchiseptica* in

50 μl per mouse). For co-inoculations with LPS, inocula were prepared containing 10⁷ CFU/ml of *B. parapertussis* and 10 μg/ml of purified LPS from *B. bronchiseptica*, *B. parapertussis*, or *E. coli* (5 × 10⁵ CFU *B. parapertussis* and 500 ng LPS in 50 μl per mouse). All protocols were reviewed by the university's Institutional Animal Care and Use Committee and all animals were handled in accordance with institutional guidelines.

Adoptive transfer of serum antibodies

To generate convalescent phase (immune) serum, C57BL/6 mice were inoculated with 5 × 10³ CFU of *B. parapertussis* and allowed to convalesce for 28 days. By this time, these mice have generated high titers of *B. parapertussis*-specific antibodies [24]. Blood was then collected from these mice and the serum portion was isolated and stored at –80°C until use. 200 μl of immune serum was delivered by I.P. injection into mice immediately before inoculation. Serum from uninfected mice (naïve serum) was used as a control.

Quantification of bacteria, leukocytes, and cytokines in the lungs

To quantify bacterial numbers, the lungs were excised on day 0, 0.5, 1, 2, 3, 7, or 14 post-inoculation. Lungs were homogenized in 1 ml of PBS. The lung homogenate was then plated onto Bordet-Gengou agar plates at the appropriate dilutions and CFU were counted 4 days later for *B. parapertussis* and 2 days later for *B. bronchiseptica*. To quantify leukocytes, mice were infected for 0, 0.5, 1, 2, 3, 7, or 14 days, sacrificed, and bronchoalveolar lavage (BAL) fluid was collected. Red blood cells were lysed by treatment with ammonium chloride as previously described [30]. Leukocytes were counted on a hemocytometer to quantify total numbers of leukocytes in the BAL fluid. Aliquots of cells were stained with FITC-labeled anti-Ly-6G (BD Biosciences Pharmingen), and the percentage of Ly-6G positive cells was multiplied by the total number of leukocytes to calculate the number of neutrophils. For the quantification of cytokines and chemokines in the lungs, wild type or TLR4-deficient mice were inoculated with *B. parapertussis*, *B. bronchiseptica*, or both species and sacrificed 2 hours or 1 day later. Lungs were homogenized in 1 mL of PBS and samples were run on ELISAs specific for TNFα, KC, MIP-1α, and/or IL-1β according to the manufacturer's protocols (R&D Systems, Minneapolis, Minnesota, USA).

In vitro growth curves of *B. parapertussis* and *B. bronchiseptica* and enumeration of co-inoculated samples

Both bacteria (RB50 and 12822G) were grown overnight to an optical density of 0.3. They were then diluted in fresh Stainer-Scholte broth to 10⁷ CFU/ml. The liquid cultures were then grown on a roller drum at 37°C and aliquots were plated at the indicated times on Bordet-Gengou agar plates with 20 μg/ml of streptomycin or gentamicin. The streptomycin plates, on which both species could have grown, were counted 2 days later, before *B. parapertussis* colonies became visible. The gentamicin plates, on which only *B. parapertussis* could grow, were counted 4 days later.

Statistical Analysis

The mean ± SD (error bars) was determined for CFU, leukocytes, and cytokines. For experiments quantifying bacterial numbers, either three or four mice were used per group. For all other experiments, four mice were used per group. Two-tailed, unpaired Student's T-tests were used to determine statistical significance between groups. All experiments were performed at

least twice with similar results and P -values < 0.05 were taken to be statistically significant.

Results

Reduction of *B. parapertussis* numbers correlates with an accumulation of leukocytes in the lungs

B. parapertussis grows rapidly over the first few days post-inoculation but does not induce an early recruitment of neutrophils, which are known to be essential to eliminating this pathogen [3,24]. Therefore, we sought to determine if the eventual reduction of *B. parapertussis* numbers in the lungs correlates with a delayed accumulation of neutrophils. C57BL/6 mice were inoculated with *B. parapertussis* and sacrificed on days 0, 3, 7, or 14 post-inoculation to quantify the numbers of bacteria in the lungs. *B. parapertussis* numbers peaked at approximately 4×10^6 CFU on day 3, but began to decline by day 7 and were reduced to 6×10^3 CFU by day 14 post-inoculation (Fig. 1A). Groups of C57BL/6 mice were also sacrificed on days 0, 3, 7, or 14 post-inoculation to quantify the numbers of leukocytes in the BAL fluid. Approximately 6×10^4 leukocytes and less than 1×10^4 neutrophils were recovered from the BAL fluid of uninfected mice. Leukocyte numbers had slightly, but significantly, increased by day 3 post-inoculation (1.6×10^5 leukocytes, 1×10^5 neutrophils), and peaked on day 7 post-inoculation (6×10^5 leukocytes, 2×10^5 neutrophils), declining thereafter (Fig. 1B). Together, these data show that the time when *B. parapertussis* numbers began to decline in murine lungs correlated with peak numbers of neutrophils in the lungs.

***B. parapertussis* does not induce an early, TLR4-mediated leukocyte response**

Although the induction of TLR4 responses is crucial to protection against other *Bordetella* species, *B. parapertussis* LPS does

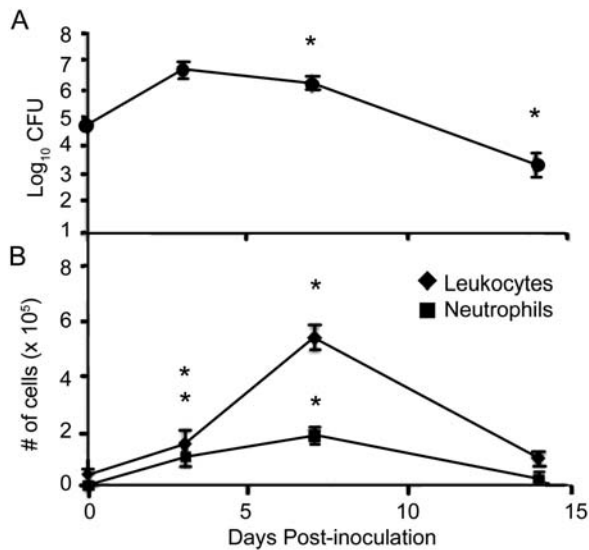


Figure 1. Numbers of *B. parapertussis* and leukocytes in the lungs over time. Groups of C57BL/6 mice were inoculated with *B. parapertussis* and sacrificed on day 0, 3, 7, or 14 post-inoculation. (A) Bacterial numbers in the lungs are represented as the Log₁₀ mean \pm S.D. The dashed line represents the lower limit of detection. (B) Leukocyte and neutrophil numbers in the BAL fluid are represented as the mean \pm S.D. and asterisks denote P -values < 0.05 when compared to numbers at day 3 for CFU (the highest observed numbers) or day 0 (naïve mice) for leukocytes. doi:10.1371/journal.pone.0004280.g001

not efficiently stimulate these responses [3]. We addressed whether or not this pathogen induces any TLR4-dependent recruitment of leukocytes to the lungs over the course of infection. Wild type (C3H/HEOJ) and TLR4-deficient (C3H/HEJ) mice were inoculated with *B. parapertussis* and sacrificed 0, 2 hours, 1, 3, 7, or 14 days later to quantify the numbers of bacteria in the lungs and leukocytes in the BAL fluid. As previously shown, similar bacterial numbers were observed in wild type and TLR4-deficient mice [3] (Fig. 2A). In the lungs of wild type mice, leukocyte numbers were highest on day 7 post-inoculation ($\sim 3 \times 10^5$ cells), and the same was true for TLR4-deficient mice ($\sim 6 \times 10^5$ cells) (Fig. 2B). Fewer than 10^5 neutrophils were found in the lungs of both wild type and TLR4-deficient mice over the first 3 days post-inoculation but peaked on day 7 in both wild type ($\sim 2 \times 10^5$ cells)

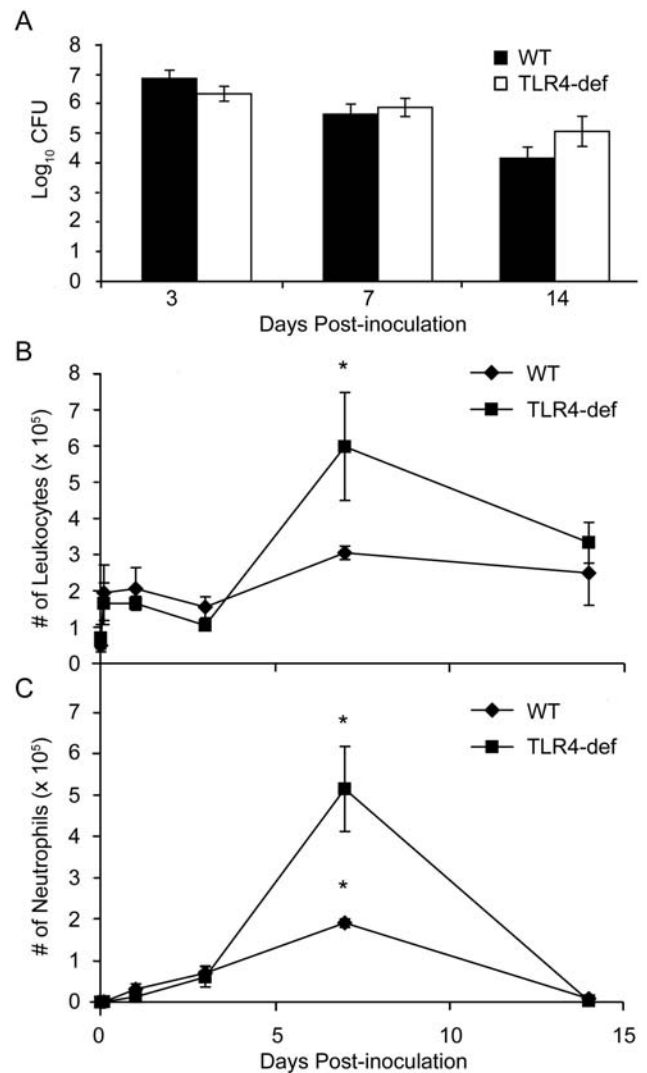


Figure 2. Numbers of leukocytes in the lungs of wild type and TLR4-deficient mice upon *B. parapertussis* infection. Groups of C3H/HEOJ (WT) and C3H/HEJ (TLR4-def) mice were inoculated with *B. parapertussis* and sacrificed 2 hours later or on day 1, 3, 7, or 14 post-inoculation. (A) Bacterial numbers in the lungs were quantified on days 3, 7, and 14 post-inoculation and are expressed as the Log₁₀ mean \pm S.D. (B) Total leukocytes and (C) neutrophils in the BAL fluid were quantified at all time points and are represented as the mean \pm S.D. Asterisks denote P -values < 0.05 when compared to naïve mice. doi:10.1371/journal.pone.0004280.g002

and TLR4-deficient (~5×10⁵ cells) mice (Fig. 2C). Interestingly, more leukocytes accumulated in the lungs of TLR4-deficient mice compared to wild type mice. Therefore, TLR4 signaling does not measurably enhance the recruitment of leukocytes or the control of *B. paraptentis* infection, but may affect anti-inflammatory signals in response to this bacterium.

TLR4-mediated cytokine and chemokine responses are not inhibited by *B. paraptentis* during infection of mice

The lack of a measurable TLR4-mediated accumulation of leukocytes in response to *B. paraptentis* (Fig. 2) led us to assess whether or not *B. paraptentis* actively inhibits TLR4-mediated cytokine production. For these experiments, the effects of *B. paraptentis* on TLR4-mediated responses to a respiratory pathogen that is closely related and a potent stimulator of TLR4, *B. bronchiseptica*, were examined. Wild type and TLR4-deficient mice were inoculated with *B. paraptentis*, *B. bronchiseptica*, or both bacteria and sacrificed 2 hours later. *B. paraptentis* did not induce significant levels of TNF-α, KC, or MIP-1α in wild type or TLR4-deficient mice relative to mock-infected controls (Fig. 3A–C). Approximately 1000 pg of IL-1β was produced in the lungs of wild type mice in response to *B. paraptentis*, but this was not significantly different from the amount produced by TLR4-deficient mice (Fig. 3D). *B. bronchiseptica* induced the production of approximately 3000 pg of TNF-α, 3500 pg of KC, 9000 pg of MIP-1α, and 2300 pg of IL-1β in the lungs of wild type mice, but much lower levels in TLR4-deficient mice (250, 125, 200, and 1200 pg respectively) (Fig. 3A–D). Similar to *B. bronchiseptica*, the lungs of wild type mice that were inoculated with both species contained approximately 3500 pg of TNF-α, 4500 pg of KC, 8500 pg of MIP-1α, and 2400 pg of IL-1β, and this production was also dependent on TLR4 (Fig. 3A–D). Together, these data indicate that *B. paraptentis* does not stimulate TLR4 or inhibit the TLR4-mediated cytokine and chemokine responses to *B. bronchiseptica* infection.

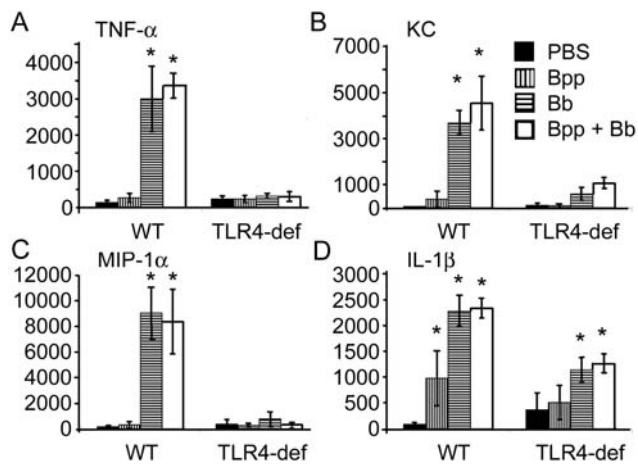


Figure 3. Effect of a co-inoculation with *B. paraptentis* on the TLR4-mediated cytokine response to *B. bronchiseptica*. Groups of wild type C3H/HEOuJ and TLR4-deficient C3H/HEJ mice were inoculated with *B. paraptentis* (Bpp), *B. bronchiseptica* (Bb), or both bacteria (Bpp+Bb) and sacrificed 2 hours later for the quantification of (A) TNF-α, (B) KC, (C) MIP-1α, or (D) IL-1β in lungs homogenized in 1 mL of PBS. Cytokine and chemokine numbers are expressed as the mean±SD. Asterisks represent P-values<0.05 when compared to mock-infected mice.
doi:10.1371/journal.pone.0004280.g003

Co-inoculation with *B. bronchiseptica* allows for more efficient control of *B. paraptentis*

The robust, TLR4-mediated cytokine and chemokine responses to a co-inoculation with *B. paraptentis* and *B. bronchiseptica* led us to examine the effect of the co-inoculation on the accumulation of leukocytes and clearance of these bacteria. Wild type mice were inoculated with *B. paraptentis*, *B. bronchiseptica*, or both species and sacrificed 12 hours, 1, 2, or 3 days later to quantify neutrophils in the BAL fluid. Consistent with Figures 1 and 2, the BAL fluid of *B. paraptentis*-infected mice contained few neutrophils (<10⁵/ml of BAL fluid) over the first three days post-inoculation (Fig. 4A). In contrast, *B. bronchiseptica* induced the accumulation of approximately 1.5×10⁶ neutrophils/ml of BAL fluid over the first two days. This early recruitment of neutrophils to the lungs upon *B. bronchiseptica* infection is dependent on TLR4 [3]. The BAL fluid of co-inoculated mice also contained approximately 1.5×10⁶ neutrophils/ml for the first two days (Fig. 4A), indicating that *B. paraptentis* did not measurably inhibit the TLR4-mediated recruitment of neutrophils to the lungs in response to *B. bronchiseptica* infection.

Groups of C57BL/6 mice were then inoculated with *B. paraptentis*, *B. bronchiseptica*, or both bacteria and sacrificed 12 hours, 1, 2, or 3 days later to quantify bacterial numbers in the lungs. *B. bronchiseptica* numbers were not affected by a co-inoculation with *B. paraptentis* (Fig. 4B). When inoculated alone, *B. paraptentis* numbers rose over the first three days, peaking at approximately 3×10⁶ CFU on day 3 post-inoculation. When co-inoculated with *B. bronchiseptica*, however, *B. paraptentis* numbers began to decline after one day and were reduced to approximately 5×10⁴ CFU by day 3 post-inoculation, a 99% reduction from numbers of *B. paraptentis* alone (Fig. 4C). Together, these data indicate that a co-infection with *B. bronchiseptica* results in increased neutrophil recruitment and more efficient control of *B. paraptentis*.

To determine if *B. paraptentis* and *B. bronchiseptica* directly affected the growth of one another, they were grown together in liquid culture. *B. bronchiseptica* grew from approximately 10⁷ CFU/ml to 10¹¹ CFU/ml in 24 hours and its growth was not affected by a co-inoculation with *B. paraptentis* (data not shown). *B. paraptentis*, which grows slower than *B. bronchiseptica*, grew from approximately 10⁷ CFU/ml to 10¹⁰ CFU/ml in 24 hours and its growth rate was not affected by a co-inoculation with *B. bronchiseptica* (data not shown). Thus, *B. paraptentis* and *B. bronchiseptica* do not directly affect each other's growth, even when grown to high density *in vitro*.

Co-inoculation with *B. bronchiseptica* results in rapid antibody-mediated clearance of *B. paraptentis*

B. bronchiseptica is cleared by antibodies within about three days via a TLR4-dependent mechanism [23]. In contrast, antibodies have no effect on the course of *B. paraptentis* infection during the first week but eliminate the infection during the second week [15,24] (Fig. 5A), after significant numbers of neutrophils have accumulated in the lungs. Thus, we hypothesized that the lack of an early TLR4-mediated neutrophil recruitment allows *B. paraptentis* to delay antibody-mediated clearance. To test this, we examined the effect of stimulating TLR4 responses on the rapid antibody-mediated clearance of *B. paraptentis* by inoculating mice with one species or both species and giving an I.P. injection of naïve serum or convalescent phase (immune) serum. Groups of mice were then sacrificed on day 3 or 7 post-inoculation for the enumeration of bacteria in the lungs. While immune serum alone had no measurable effect on the numbers

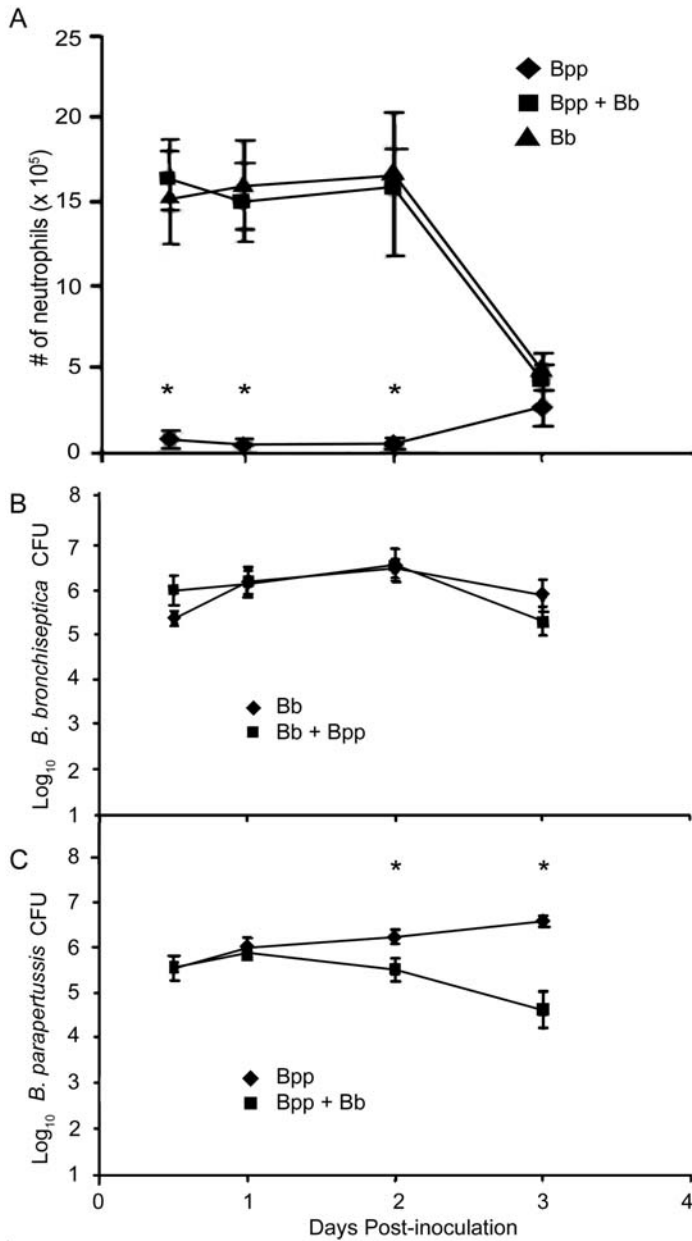


Figure 4. In vivo growth of *B. bronchiseptica* on *B. paraptussis* upon a co-inoculation. (A) Groups of C57BL/6 mice were inoculated with *B. paraptussis* (Bpp), *B. bronchiseptica* (Bb), or both bacteria (Bpp+Bb) and sacrificed 0.5, 1, 2, or 3 days later to quantify neutrophils in the BAL fluid. Groups of mice were also sacrificed at these times to quantify (B) Bb numbers and (C) Bpp numbers in the lungs. Neutrophil numbers are expressed as the mean \pm SD and bacterial numbers are expressed as the Log₁₀ mean \pm SD. Asterisks represent P-values<0.05 when comparing *B. paraptussis*-infected mice to co-infected mice. doi:10.1371/journal.pone.0004280.g004

of *B. paraptussis* [15] (Fig. 5B), immune serum with a co-inoculation of *B. bronchiseptica* rapidly reduced *B. paraptussis* numbers >99%, to approximately 100 CFU by day 3 and to undetectable levels by day 7 post-inoculation (Fig. 5B). These data indicate that a co-inoculation with *B. bronchiseptica* results in rapid antibody-mediated clearance of *B. paraptussis*. The co-inoculation did not affect the ability of *B. bronchiseptica* to colonize the lungs of mice treated with naive serum, but *B. bronchiseptica* numbers were approximately 500-fold lower in the lungs of mice treated with immune serum (Fig. 5C). This was likely due to *B. paraptussis*-induced antibodies being cross reactive with *B. bronchiseptica* antigens.

Rapid clearance of *B. paraptussis* upon co-inoculation with *B. bronchiseptica* is dependent on TLR4

We hypothesized that the protective effects of adding *B. bronchiseptica* to the *B. paraptussis* inoculum were due to the robust TLR4-mediated inflammatory response to *B. bronchiseptica*. To test this, groups of wild type and TLR4-deficient mice were inoculated with *B. paraptussis* alone or *B. paraptussis* with heat-killed *B. bronchiseptica*. Heat-killed *B. bronchiseptica* was used because live *B. bronchiseptica* is lethal to TLR4-deficient mice within approximately 3 days [3]. This also allowed us to address whether or not the effect on *B. paraptussis* numbers required live *B. bronchiseptica*, or if stimulation of the immune response by heat-inactivated compo-

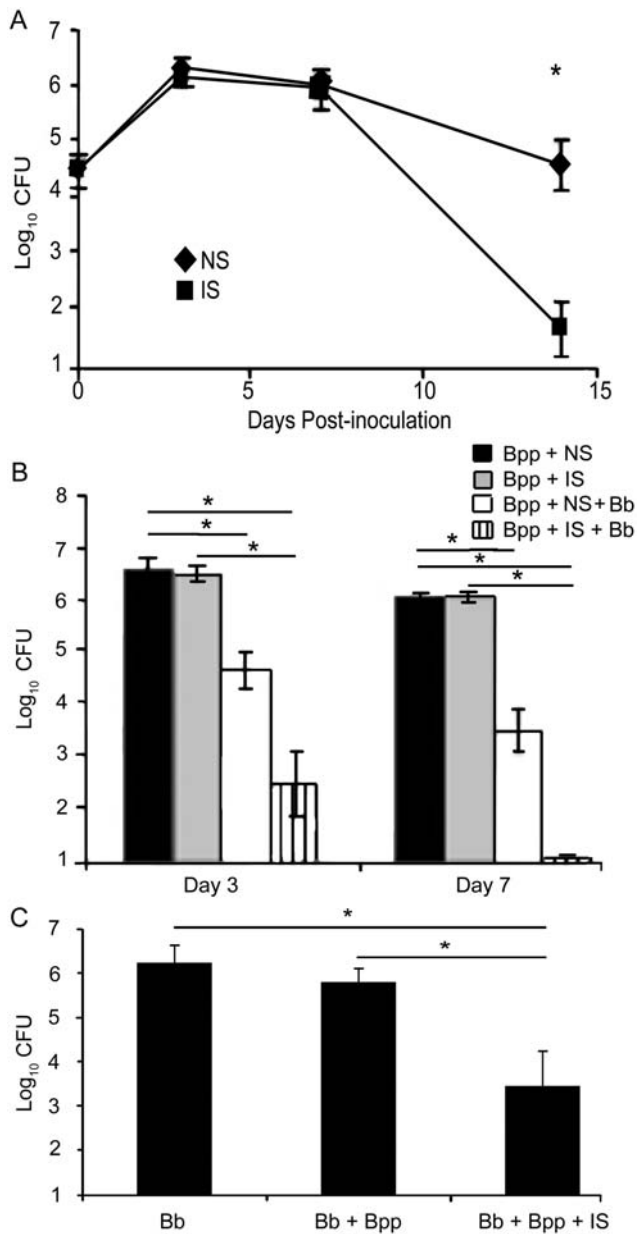


Figure 5. Antibody-mediated clearance of *B. paraptentis* upon a co-inoculation with *B. bronchiseptica*. (A) C57BL/6 mice were inoculated with *B. paraptentis*, given an adoptive transfer of naïve serum (NS) or immune serum (IS), and sacrificed 0, 3, 7, or 14 days later to quantify bacterial numbers in the lungs. (B–C) C57BL/6 mice were inoculated with *B. paraptentis* (Bpp), *B. bronchiseptica* (Bb), or both bacteria (Bpp+Bb), given an adoptive transfer of naïve serum (NS) or immune serum (IS), and sacrificed 3 or 7 days later. Numbers of (B) *B. paraptentis* and (C) *B. bronchiseptica* in the lungs were quantified. Bacterial numbers are expressed as the Log₁₀ mean ± SD. Asterisks represent P-values < 0.05. doi:10.1371/journal.pone.0004280.g005

nents was sufficient to reduce bacterial numbers. The cytokine and leukocyte responses were measured 1 day post-inoculation with *B. paraptentis* alone versus *B. paraptentis* with heat-killed *B. bronchiseptica*. Inoculation with *B. paraptentis* did not induce levels of TNF α and KC in the BAL fluid of wild type or TLR4-deficient mice that were measurably different from mock-infected lungs (Fig. 6A–B). In contrast, co-inoculation with *B. paraptentis* and heat-killed *B. bronchiseptica* resulted in high levels of TLR4-

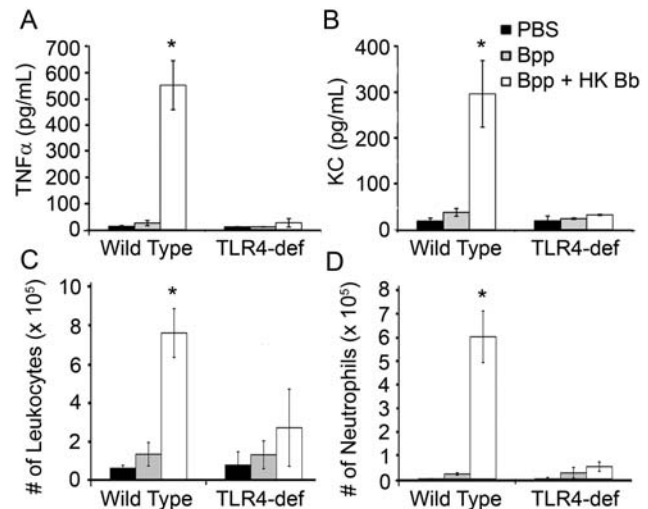


Figure 6. Cytokine and leukocyte levels in the lungs of mice infected with *B. paraptentis* and heat-killed *B. bronchiseptica*. Groups of wild type (C3H/HEOu) and TLR4-deficient (C3H/HEJ) mice were inoculated with PBS, *B. paraptentis* (Bpp) or Bpp and heat-killed *B. bronchiseptica* (HK Bb). (A) TNF α , (B) KC, (C) leukocyte and (D) neutrophil levels were quantified in the BAL fluid one day later. Cytokine levels and cell numbers are represented as the mean ± SD. Asterisks represent P-values < 0.05 when compared to mock-infected mice. doi:10.1371/journal.pone.0004280.g006

dependent TNF α and KC production (approximately 550 and 300 pg respectively). When leukocyte numbers were examined, *B. paraptentis* alone did not induce significant levels of leukocyte accumulation ($\sim 1 \times 10^5$ leukocytes, $\sim 2 \times 10^4$ neutrophils) relative to mock-infected mice (Fig. 6C–D). Co-inoculation with heat-killed *B. bronchiseptica*, however, resulted in the accumulation of 8×10^5 leukocytes and 6×10^5 neutrophils in the lungs of wild type mice by this time, while the lungs of TLR4-deficient mice contained only 3×10^5 leukocytes and 4×10^4 neutrophils (Fig. 6C–D). Thus, heat-killed *B. bronchiseptica* induced robust, TLR4-mediated cytokine and leukocyte responses.

To address the effect on bacterial numbers, wild type and TLR4-deficient mice were then inoculated with *B. paraptentis* alone or *B. paraptentis* with heat-killed *B. bronchiseptica*. These mice were also given an I.P. injection of naïve serum or immune serum and sacrificed 3 days later. In wild type mice that were treated with naïve serum, co-inoculation with heat-killed *B. bronchiseptica* resulted in a 10-fold reduction of *B. paraptentis* numbers. In wild type mice that were treated with immune serum, the co-inoculation resulted in *B. paraptentis* numbers being reduced to nearly undetectable levels within 3 days (Fig. 7A). In TLR4-deficient mice, however, the co-inoculation had no effect on *B. paraptentis* numbers in either naïve serum treated or immune serum treated mice (Fig. 7A). Wild type and TLR4-deficient mice were then inoculated with *B. paraptentis* and purified LPS from *B. bronchiseptica*, *E. coli*, or *B. paraptentis* to determine if the addition of TLR4 stimulatory LPS was the key to rapid clearance of *B. paraptentis*. Co-inoculation with *B. bronchiseptica* LPS resulted in a 10,000-fold reduction in bacterial numbers in the lungs of wild type mice treated with immune serum, but did not affect bacterial numbers in TLR4-deficient mice (Fig. 7B). Similar results were observed when mice were co-inoculated with LPS from *E. coli* (Fig. 7C). In contrast, co-inoculation with purified LPS from *B. paraptentis*, a weak TLR4-stimulant [3], had no effect on *B. paraptentis* numbers in the lungs of wild type or TLR4-deficient

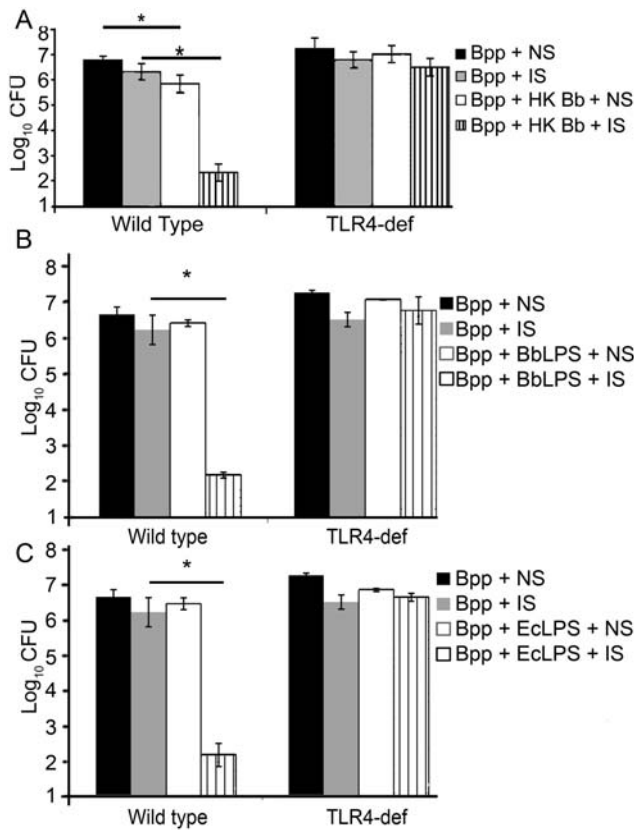


Figure 7. Effect of TLR4 stimulation on the rapid antibody-mediated clearance of *B. paraptussis*. (A) Groups of wild type (C3H/HEOuJ) and TLR4-deficient (C3H/HEJ) mice were inoculated with *B. paraptussis* (Bpp) or Bpp and heat-killed *B. bronchiseptica* (HK Bb) and given I.P. injections of naïve serum (NS) or immune serum (IS). Groups of mice were also inoculated with Bpp and (B) Bb LPS (BbLPS) or (C) *E. coli* LPS (EcLPS) and treated with naïve or immune serum. Bacterial numbers were quantified 3 days later and are expressed as the Log₁₀ mean ± SD. Asterisks denote P-values < 0.05. doi:10.1371/journal.pone.0004280.g007

mice (data not shown). Thus, TLR4 was required for the enhanced clearance of *B. paraptussis* upon co-inoculation with strong TLR4 stimulants (Fig. 7B–C). Combined, these data suggest that a lack of TLR4 stimulation enables *B. paraptussis* to avoid immune clearance and grow to higher numbers within the host.

Discussion

B. paraptussis is able to delay clearance by avoiding the induction of a robust innate immune response. Here, we show that its slow clearance from murine lungs correlates with the accumulation of neutrophils in these lungs, which is delayed in comparison to the neutrophil responses to other closely related bacteria (Mann, Harvill, unpublished data). We predicted that inefficient TLR4 stimulation by *B. paraptussis* LPS may result in the low level of neutrophil accumulation in response to infection over the first few days and may allow this pathogen to grow rapidly during this time, even in animals given a passive transfer of immune serum. In support of this, co-inoculation with a potent stimulator of TLR4 resulted in enhanced control and rapid antibody-mediated clearance of *B. paraptussis* from wild type, but not TLR4-deficient mice. This study provides an example of how expressing an LPS that is a poor stimulator of TLR4 can facilitate persistence of a gram negative bacterium within its host.

LPS modulation is often utilized by gram negative bacterial pathogens to optimize interactions with host immunity. For example, *Yersinia pestis* produces a TLR4-stimulatory LPS at 26°C, but an unstimulatory LPS at 37°C, the body temperature of its typical mammalian host [11,12,13]. Montminy et.al. genetically modified *Y. pestis* so that it would produce the stimulatory 26°C LPS at all times [31]. While wild type *Y. pestis* causes sepsis and mortality in a mouse model of infection, the expression of TLR4-stimulatory LPS resulted in containment of the infection by the innate immune response and less efficient systemic spread of the infection [31]. Similarly, co-inoculation of *B. paraptussis* with a TLR4 agonist resulted in an attenuated course of infection (Fig. 4, Fig. 7). The expression of LPS molecules that poorly stimulate TLR4 appears to hinder the generation of effective immunity against *Y. pestis* [31] and *B. paraptussis*, and may be a stealth strategy shared by other gram negative bacteria as well [32,33,34,35].

TLR4 stimulation by LPS results in a branched downstream signaling pathway consisting of a Mal/MyD88 branch and a TRIF/TRAM branch that leads to the production of several different pro-inflammatory cytokines [36]. However, each branch is crucial to the production of a different subset of cytokines. For example, TNF-α and CCL3 are MyD88-dependent cytokines while IFN-β is a TRIF-dependent cytokine [37,38]. Although our *in vivo* data presented above suggested that *B. paraptussis* does not induce measurable amounts of TLR4-mediated cytokine production or leukocyte recruitment (Fig.3, Fig. 6), the higher numbers of leukocytes in TLR4-deficient mice suggests that leukocyte responses to *B. paraptussis* infection may be limited by a TLR4-dependent mechanism. *B. paraptussis* may upregulate the TRIF/TRAM branch of TLR4 signaling, as this branch appears to play a role in endotoxin tolerance [39]. We have also recently observed that IL-10 dampens the inflammatory response to *B. paraptussis* *in vivo* and induces the production of IL-10 *in vitro* (Wolfe and Hester, unpublished data). Since IL-10 production can be induced by TLR4 stimulation [4], it is reasonable to suggest that the anti-inflammatory effect of TLR4 in Figure 2 may be mediated by IL-10.

In contrast to *B. bronchiseptica*, passively transferred antibodies have no effect on colonization by the human pathogens *B. paraptussis* and *B. pertussis* over the first week of infection [15,24,25]. This is likely important to the success of these pathogens, as they are able to re-infect the same host multiple times despite a measurable antibody response [40]. Our lab previously showed that Ptx delays antibody-mediated clearance of *B. pertussis* by inhibiting the migration of neutrophils to the lungs [25]. Although *B. paraptussis* does not express Ptx, poor induction of TLR4 signaling appears to be an alternative method for limiting the neutrophil response and delaying antibody-mediated clearance. Limiting and/or inhibiting pro-inflammatory TLR4 stimulation may be crucial to the ability of *B. paraptussis* to remain endemic in human populations.

In addition to the inefficient stimulation of pro-inflammatory TLR4 responses [3], likely due to its lipid A structure, the O-antigen of *B. paraptussis* LPS also appears to allow it to avoid rapid clearance by antibodies induce by *B. pertussis* infection or vaccination [24]. O-antigen prevents the binding of *B. pertussis*-induced antibodies to the surface of *B. paraptussis*, allowing the latter to colonize hosts that had been previously immunized against the former. This provides an example of a single molecule, LPS, providing multiple, non-overlapping mechanisms to protect a bacterium against the effects of antibodies.

Despite excellent vaccine coverage, whooping cough has been re-emerging in vaccinated populations [41,42,43,44,45], but it is

unclear what the relative roles of *B. pertussis* and *B. paraptussis* are in this resurgence [46]. Importantly, immunity induced by current vaccines protects against *B. pertussis* disease, but is largely ineffective against *B. paraptussis* disease [47,48,49,50,51]. The widespread use of vaccines appears to have resulted in a higher incidence of *B. paraptussis* as the causative agent of whooping cough in vaccinated individuals relative to unvaccinated individuals [49]. Current acellular vaccines induce a T cell response that is Th2-skewed [52]. Given that our data shows that the clearance of *B. paraptussis* by antibodies is enhanced by pro-inflammatory responses, a vaccine that generates a strong Th1-skewed response to *B. paraptussis*, as opposed to a partially cross-reactive Th2 type

response, could potentially provide more efficient protection against this pathogen.

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Author Contributions

Conceived and designed the experiments: DNW AMB ETH. Performed the experiments: DNW AMB. Analyzed the data: DNW AMB ETH. Wrote the paper: DNW AMB ETH.

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