

# Delayed Role of Tumor Necrosis Factor- $\alpha$ in Overcoming the Effects of Pertussis Toxin

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***Bordetella pertussis* causes whooping cough, an endemic respiratory disease that is increasing in prevalence despite vaccination efforts. Although host immunity is modulated by virulence factors of this pathogen, it is unclear what host factors are required to overcome their effects. Here, we investigate an apparent relationship between the effects of pertussis toxin and tumor necrosis factor (TNF)- $\alpha$ . *B. pertussis* grows efficiently and causes moderate pathology in wild-type mice, whereas TNF- $\alpha^{-/-}$  mice have higher numbers of bacteria and leukocytes in lungs, experience more airway resistance, and die of the infection. Interestingly, an isogenic *B. pertussis* strain lacking pertussis toxin did not induce these effects in TNF- $\alpha^{-/-}$  mice and behaved similarly in wild-type and TNF- $\alpha$ -deficient hosts. Together, these results indicate that TNF- $\alpha$  is essential for the host to overcome the effects of pertussis toxin, allowing both control of *B. pertussis* numbers and regulation of the inflammation induced by infection.**

*Bordetella pertussis* is one of the etiologic agents of whooping cough, a severe coughing illness that can progress to become spasmodic and occasionally cause convulsions, coma, and death [1]. In 2002, there were ~50 million cases and >300,000 deaths due to whooping cough worldwide [2]. Although the majority of lethal cases of pertussis are observed in countries lacking vaccination programs, the number of diagnosed cases in immunized populations is on the rise [3, 4], and periodic epidemics are common [5].

Tumor necrosis factor (TNF)- $\alpha$  is a classic proinflammatory cytokine that induces the production of chemokines by a variety of cell types [6, 7]. Endothelial cells and circulating leukocytes exhibit an up-regulation

of adhesion molecules on stimulation by this cytokine [8, 9], facilitating the extravasation of leukocytes from the bloodstream. TNF- $\alpha$  also activates these cells by inducing efficient respiratory bursts, degranulation, and bactericidal activity [10, 11]. This cytokine is required for host control of a wide range of respiratory pathogens, including *Pneumocystis* species, respiratory syncytial virus, and *Mycobacterium*, *Listeria*, *Chlamydia*, *Pseudomonas*, and *Klebsiella* species [12–19].

We have previously shown that TNF- $\alpha$  induced by Toll-like receptor (TLR) 4 signaling is crucial to the early response to the animal pathogen *B. bronchiseptica* [20]. TNF- $\alpha$ -deficient mice are unable to limit *B. bronchiseptica* numbers and pathology, resulting in death within ~72 h [20]. TLR4 signaling is also required for the early TNF- $\alpha$  response to *B. pertussis*, but it is not essential to survival after infection. However, a deficiency in TLR4 results in higher numbers of *B. pertussis* in the respiratory tract after 1 week, which suggests that TNF- $\alpha$  might be important later during infection [21, 22].

Here, we show that TNF- $\alpha$  is essential for protection against an infection of the murine respiratory tract with *B. pertussis*. This cytokine appeared to modulate the immune response by limiting the accumulation of leukocytes in response to *B. pertussis* infection. Blocking

Received 7 December 2006; accepted 20 April 2007; electronically published XX September 2007.

Potential conflicts of interest: none reported.

Financial support: US Department of Agriculture (grant 2002-35204-11684 to E.T.H.); National Institutes of Health (grant AI 053075 to E.T.H.).

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The Journal of Infectious Diseases 2007;196:000–000

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0022-1899/2007/19608-00XX\$15.00

DOI: 10.1086/521303

neutrophil accumulation reduced airway resistance and extended the life span of these mice, which suggests that mortality involves neutrophil-induced damage to the lungs. TNF- $\alpha$  was not required to survive an infection by or to limit the accumulation of leukocytes in response to a mutant of *B. pertussis* lacking pertussis toxin (Ptx) (*Bp $\Delta$ ptx*). Thus, TNF- $\alpha$  is essential only when Ptx is expressed and appears to overcome the effects of Ptx by modulating the neutrophil response to *B. pertussis*.

## MATERIALS AND METHODS

**Bacterial strains and growth.** *B. pertussis* strain 536 is a streptomycin-resistant derivative of Tohama I [23]. BPH101 (*Bp $\Delta$ ptx*) is a Ptx mutant of strain 536 and was a gift from Dr. Drusilla Burns (US Food and Drug Administration, Rockville, MD) [24]. Both were maintained on Bordet-Gengou agar (Difco) that contained 10% defibrinated sheep blood (Hema Resources) and 20  $\mu$ g/mL streptomycin. Liquid-culture bacteria were grown overnight at 37°C on a roller drum to the midlog phase in Stainer-Scholte broth.

**Animal experiments.** C57BL/6 and TNF- $\alpha^{-/-}$  mice were obtained from Jackson Laboratories and bred in our *Bordetella*-free, specific pathogen-free breeding rooms at Pennsylvania State University (University Park, PA). Then, 4–6-week-old mice were lightly sedated with 5% isofluorane (Abbott Laboratories) in oxygen and inoculated by pipetting 50  $\mu$ L of PBS containing  $\sim 5 \times 10^5$  cfu onto the tip of the external nares, as described elsewhere [23]. For coinoculation with the control or TNF- $\alpha$ -expressing adenovirus,  $5 \times 10^8$  pfu of the adenovirus in 50  $\mu$ L of PBS [25] was pipetted onto the external nares immediately after inoculation with *B. pertussis*. For survival curves, once the progression of disease was clear and death was imminent, moribund mice were euthanized to prevent unnecessary suffering. Protocols were approved by the university Institutional Animal Care and Use Committee, and all mice were handled in accordance with institutional guidelines.

**Bacterial quantification.** Mice were killed on days 0, 3, 7, 10, and/or 14 after inoculation. Lungs, trachea, nasal cavity, heart, kidneys, liver, and spleen were homogenized in 1 mL of PBS and plated onto Bordet-Gengou agar that contained 20  $\mu$ g/mL streptomycin at appropriate dilutions to quantify colony-forming units.

**Lung leukocyte and cytokine quantifications.** Lungs were perfused with sterile PBS, harvested, and placed in 5 mL of Dulbecco's modified Eagle medium (DMEM; HyClone) supplemented with 10% fetal bovine serum. Lungs and medium were then pressed through Collector tissue sieves (Bellco Glass) for homogenization. Lung homogenate was laid over Histo-paque 1119 (Sigma Aldrich) and centrifuged for 30 min at 3000 rpm at room temperature. The leukocyte layer was collected, and the total number of cells was determined by counting at  $\times 40$  magnification on a hemocytometer. Numbers of individ-

ual cell types were quantified by spinning the leukocyte layer onto a glass slide and staining the isolated cells with a modified Giemsa stain (Fisher Scientific). For the quantification of cytokines and chemokines, lung homogenates were examined by cytokine ELISAs (for TNF- $\alpha$ , interferon [IFN]- $\gamma$ , interleukin [IL]-10, IL-4, transforming growth factor [TGF]- $\beta$ , macrophage inflammatory protein [MIP]-1 $\alpha$ , and KC), which were run in accordance with the suppliers' protocols (R&D Systems).

**Splenocyte restimulations.** Splenocytes were isolated by homogenizing spleens, spinning at 1500 rpm for 5 min at 4°C, lysing the red blood cells, and washing the cells with DMEM. Then,  $2 \times 10^6$  cells were resuspended in DMEM supplemented with 10% fetal calf serum (HyClone), 1 mmol/L sodium pyruvate (HyClone), 100  $\mu$ g/mL penicillin and streptomycin (HyClone), and 0.005%  $\beta$ -mercaptoethanol and were placed into wells of a 96-well plate. Splenocytes were stimulated with medium or  $1 \times 10^7$  heat-killed *B. pertussis*. After 3 days, supernatant was collected and analyzed for TNF- $\alpha$  production by ELISA.

**Airway resistance.** Airway resistance was evaluated by whole-body plethysmography [26]. Unrestrained mice were placed into a whole-body plethysmography chamber and allowed to move freely. Airway resistance was estimated by the enhanced respiratory pause (Penh) index, a measurement of the phase shift of thoracic displacement and air flow through the nares.  $\text{Penh} = (\text{Te}/\text{RT} - 1) \times \text{PEF}/\text{PIF}$ , where Te is expiratory time, RT is relaxation time, PEF is peak expiratory flow, and PIF is peak inspiratory flow [26].

**Depletions via neutralizing antibodies.** Neutrophils were depleted by intraperitoneal injections of 1 mg of the antibody from the hybridoma RB6-8C5 [27], 24 h before and 7 days after infection. CD4<sup>+</sup> and CD8<sup>+</sup> T cells were depleted by injections of 1 mg of the antibody from the hybridomas GK1.5 [28, 29] and YTS168.4 [30] respectively, on days 0 and 7. Mice were depleted of TNF- $\alpha$  by injection of 1 mg of the antibody from the hybridoma MP6-XT3 [31] at specified time points.

**Statistical analysis.** The mean  $\pm$  SD (error bars) was determined for each group for colony-forming units, leukocyte numbers, or Penh values. Two-tailed, unpaired Student's *t* tests were used to determine statistical significance between groups. All experiments were performed at least 2 times and achieved similar results.

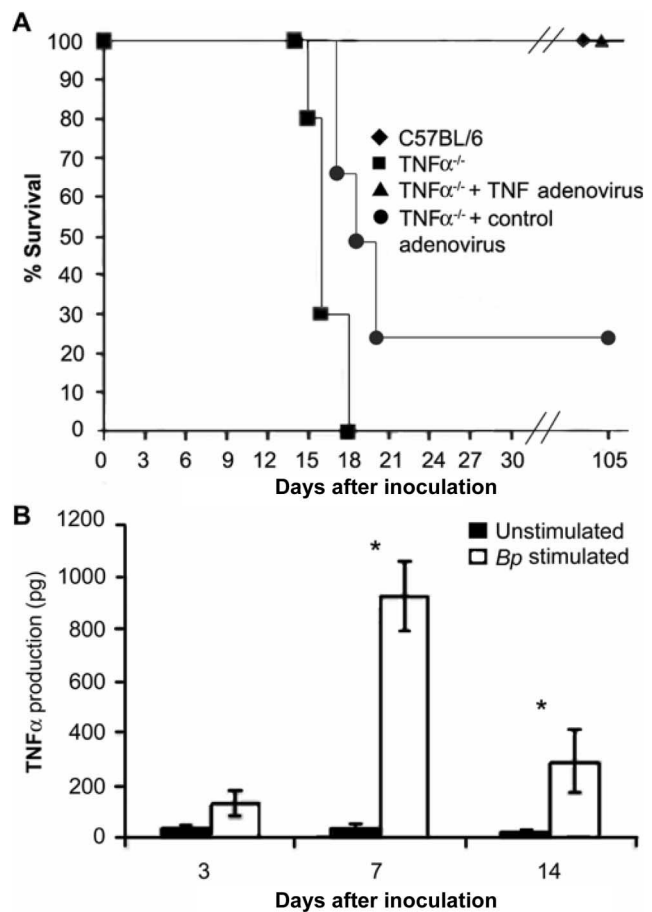
## RESULTS

**TNF- $\alpha$  and survival after infection with *B. pertussis*.** To test whether the production of TNF- $\alpha$  is important for protection against *B. pertussis*, wild-type and TNF- $\alpha^{-/-}$  mice were inoculated with  $5 \times 10^5$  cfu in 50  $\mu$ L of PBS. Wild-type mice inoculated with *B. pertussis* showed no signs of distress for at least 105 days after inoculation [32]. However, TNF- $\alpha^{-/-}$  mice began to show signs of disease  $\sim 2$  weeks after inoculation,

q7  
q8

including ruffled fur, hunched posture, and unresponsiveness, and they died of the infection soon after morbidity became apparent (mean survival time, ~16.4 days) (figure 1A). To determine whether this defect could be compensated for by restoring TNF expression in the lungs, TNF- $\alpha^{-/-}$  mice were coinoculated with *B. pertussis* and  $5 \times 10^8$  pfu of either an adenovirus vector expressing TNF- $\alpha$  or a control adenovirus. This dose of the TNF- $\alpha$ -expressing adenovirus produces a peak of ~900 pg of TNF- $\alpha$  around day 4 after inoculation [33] and prevented death in all of the TNF- $\alpha^{-/-}$  mice, whereas 75% of the mice coinoculated with the control adenovirus died of the infection (figure 1A). Because TNF- $\alpha$  appeared to be protective, its production in response to *B. pertussis* was assessed by monitoring splenocytes from *B. pertussis*-infected mice for cytokine production. Splenocytes from infected mice produced low levels of TNF- $\alpha$  on exposure to medium alone (figure 1B). Splenocytes from mice that had been infected for 3 days did not produce significantly more TNF- $\alpha$  on stimulation by *B. pertussis*, but stimulation did result in increased TNF- $\alpha$  production by splenocytes from mice infected for 7 and 14 days (figure 1B). These data indicate that TNF- $\alpha$  is produced in response to *B. pertussis*, peaking after 1 week of infection, and is protective against this pathogen.

**TNF- $\alpha$  and reduction in numbers of *B. pertussis* and limiting leukocyte accumulation in the murine lung.** To determine whether TNF- $\alpha$  is important to limiting bacterial loads in the respiratory tract or systemic spread of the infection, bacterial numbers were quantified in the lungs, trachea, nasal cavity, spleen, liver, heart, kidneys, and blood of wild-type and TNF- $\alpha^{-/-}$  mice. Mice were inoculated with *B. pertussis* and killed on day 0, 3, 7, 10, or 14. No differences in bacterial numbers were observed between C57BL/6 and TNF- $\alpha^{-/-}$  mice for up to 7 days after inoculation. On day 10, *B. pertussis* numbers began to decrease in wild type mice, and, by day 14, *B. pertussis*-specific antibodies were detectable (data not shown) and bacterial numbers were reduced to  $\sim 1 \times 10^4$  cfu in the lungs. However, *B. pertussis* remained at high numbers in the lungs of TNF- $\alpha^{-/-}$  mice ( $\sim 5 \times 10^6$  cfu) on day 14 after inoculation (figure 2A). Similar trends were observed in tracheas and nasal cavities (data not shown). Additionally, depleting TNF- $\alpha$  from wild-type mice led to an inability to reduce bacterial numbers in lungs (data not shown). Bacteria were not detectable in the spleen, liver, heart, kidneys, or blood of wild-type or TNF- $\alpha^{-/-}$  mice, which indicates that *B. pertussis* had not spread systemically by day 14 after inoculation (data not shown). The failure of TNF- $\alpha^{-/-}$  mice to reduce numbers of *B. pertussis* provides a possible explanation for death in these mice. However, *B. pertussis* reaches  $\sim 1 \times 10^7$  cfu in the lungs of other immunodeficient mouse strains and persists at high levels for at least 105 days without causing symptomatic disease [32, 34]. Thus, we speculated that mortality associated with *B.*



**Figure 1.** Production of tumor necrosis factor (TNF)- $\alpha$  in response to *Bordetella pertussis* and survival curve of wild-type vs. TNF- $\alpha^{-/-}$  mice infected with *B. pertussis*. *A*, Groups of 8 C57BL/6 and 12 TNF- $\alpha^{-/-}$  mice inoculated with  $5 \times 10^5$  cfu of *B. pertussis* in 50  $\mu$ L of PBS. TNF- $\alpha^{-/-}$  mice were untreated or coinoculated with  $5 \times 10^8$  pfu of control adenovirus or TNF- $\alpha$ -expressing adenovirus. Survival is represented as the percentage of mice living on the indicated day after inoculation. *B*, Splenocytes collected from *B. pertussis*-infected mice at day 3, 7, or 14 after inoculation and exposed to medium or heat-killed *B. pertussis* (*Bp* stimulated) for 3 days. Levels of TNF- $\alpha$  in the supernatant were quantified by ELISA and are expressed as the mean  $\pm$  SD.

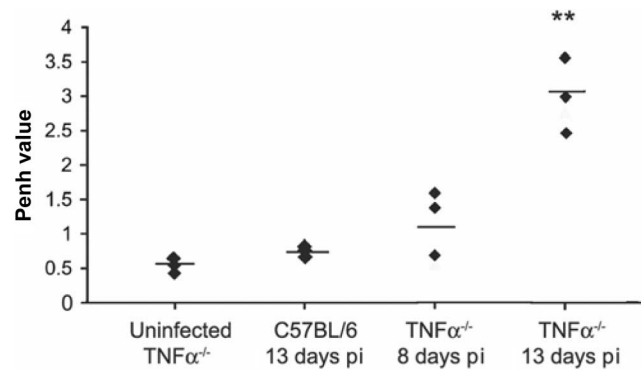
*pertussis* in the absence of TNF- $\alpha$  was not directly due to high bacterial numbers.

An alternative explanation for the lethality of *B. pertussis* infections in TNF- $\alpha^{-/-}$  mice might be that TNF- $\alpha$  regulates the inflammatory response to this bacterium. Numbers and types of leukocytes in the lungs of wild-type and TNF- $\alpha$ -deficient mice were quantified. There were no significant differences between wild-type and TNF- $\alpha^{-/-}$  mice in the numbers of lymphocytes in lungs throughout the infection (figure 2B). No significant differences were observed between wild-type mice and those lacking TNF- $\alpha$  in the accumulation of macrophages or neutrophils in the lungs up to 7 days after inoculation (figure 2C and 2D). However on day 14, macrophage

and neutrophil numbers were low in the lungs of wild-type mice ( $3 \times 10^5$  and  $1 \times 10^5$ , respectively), whereas numbers in lungs increased dramatically in  $\text{TNF-}\alpha^{-/-}$  mice ( $3 \times 10^6$  and  $5 \times 10^6$ , respectively) (figure 2C and 2D). Despite the differences in leukocyte numbers, many classic pro- and anti-inflammatory cytokines and chemokines (IFN- $\gamma$ , IL-4, IL-10, IL-1 $\beta$ , MIP-1 $\alpha$ , MIP-2, and KC) were produced at similar levels in wild-type and  $\text{TNF-}\alpha^{-/-}$  mice (data not shown). Together, these data suggest that there might be a defect in the resolution of inflammation, rather than an increase in the recruitment of leukocytes.

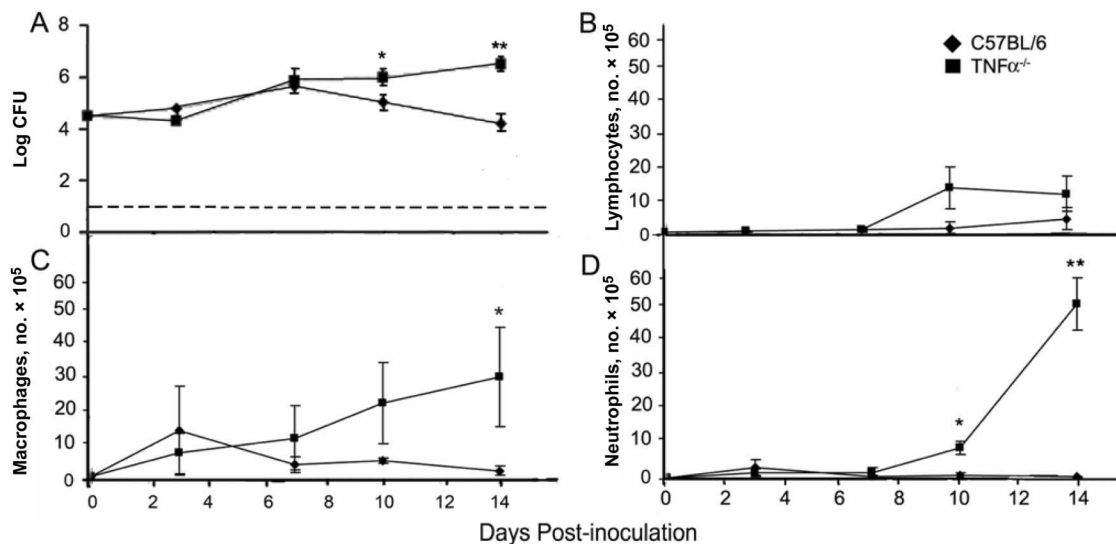
**Increased airway resistance in  $\text{TNF-}\alpha^{-/-}$  mice in response to infection with *B. pertussis*.** It was observed that *B. pertussis*-infected  $\text{TNF-}\alpha^{-/-}$  mice appeared to be having difficulty breathing. Whole-body plethysmography was performed as a measurement of airway resistance. C57BL/6 and  $\text{TNF-}\alpha^{-/-}$  mice were analyzed by unrestrained plethysmography before infection and on days 8 and/or 13 after inoculation. On day 13 after inoculation, *B. pertussis*-infected wild-type mice showed levels of airway resistance similar to those of uninfected mice; Penh values were 0.61 and 0.68, respectively (figure 3).  $\text{TNF-}\alpha^{-/-}$  mice infected with *B. pertussis* for 8 days had Penh values that were apparently higher (1.012) than those of uninfected mice, but this difference was not statistically significant. On day 13 after inoculation, these mice had high levels of airway resistance (Penh value,  $\sim 3.1$ ), compared with infected wild-type mice (figure 3). Thus, *B. pertussis*-induced lethality observed in the absence of  $\text{TNF-}\alpha$  is associated with increased airway resistance.

**Life span of *B. pertussis*-infected  $\text{TNF-}\alpha^{-/-}$  mice extended by depleting neutrophils or  $\text{CD4}^+$  T cells.** Because lethality



**Figure 3.** Measurement of airway resistance by whole body plethysmography. Groups of 3 C57BL/6 and tumor necrosis factor (TNF)- $\alpha^{-/-}$  mice were inoculated with *Bordetella pertussis*. Enhanced respiratory pause (Penh) values were determined by unrestrained plethysmography on the indicated day after inoculation (pi). Penh values are denoted for each individual mouse with the horizontal bars representing the means. \* $P < .01$  vs. wild-type mice infected for 13 days.

correlated with elevated numbers of leukocytes in the lungs of *B. pertussis*-infected  $\text{TNF-}\alpha^{-/-}$  mice, we tested the effects of depleting certain leukocyte populations on survival. In  $\text{TNF-}\alpha^{-/-}$  mice, a small increase in lymphocyte infiltration on day 10 was followed by high numbers of neutrophils in the lungs on day 14 after inoculation (figure 2B and 2D). To examine the contributions of these cell types to the lethality of *B. pertussis* in the absence of  $\text{TNF-}\alpha$ , we depleted  $\text{CD4}^+$  T cells,  $\text{CD8}^+$  T cells, or neutrophils before *B. pertussis* infection. Mean survival times for untreated and  $\text{CD8}^+$  T cell-depleted  $\text{TNF-}\alpha^{-/-}$  mice were similar (95% confidence intervals [CIs], 15.7–17.7 and



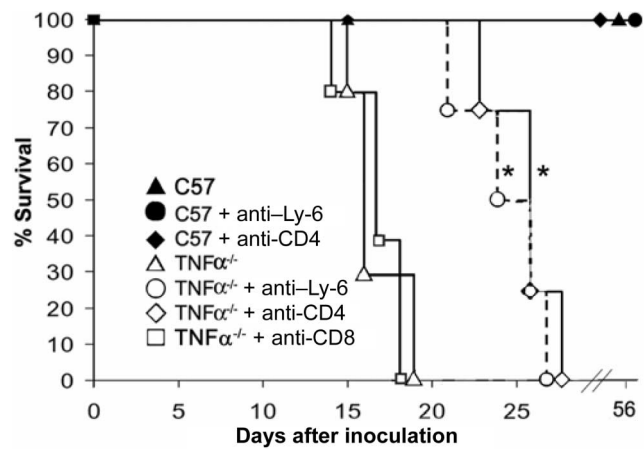
**Figure 2.** Quantification of bacteria and leukocytes in the lungs of wild-type and tumor necrosis factor (TNF)- $\alpha^{-/-}$  mice. Twenty C57BL/6 and 20  $\text{TNF-}\alpha^{-/-}$  mice were inoculated with *Bordetella pertussis*, and 4 of each mouse strain were killed on days 0, 3, 7, 10, and 14 after inoculation. A, No. of bacteria, expressed as  $\log_{10}$  mean cfu. Lymphocytes (B), macrophages (C), and neutrophils (D) were also quantified in the lungs of these mice. Cell counts are means. Error bars represent SDs, and dashed lines represent the lower limit of detection. \* $P < .05$ ; \*\* $P < .01$ .

15.8–17.8 days, respectively). However, CD4<sup>+</sup> T cell–depleted and neutrophil-depleted TNF- $\alpha^{-/-}$  mice infected with *B. pertussis* survived significantly longer (95% CIs, 24.4–26.6 and 22.8–26.7 days, respectively) (figure 4). Thus, CD4<sup>+</sup> T cells and neutrophils appear to contribute to the mortality of *B. pertussis*–infected TNF- $\alpha^{-/-}$  mice.

**No effect on bacterial colonization by limiting neutrophil accumulation in lungs but decreased airway resistance.** Given the positive effects of depleting CD4<sup>+</sup> T cells or neutrophils from *B. pertussis*–infected mice, the effects of these cells on bacterial colonization were assessed. C57BL/6 and TNF- $\alpha^{-/-}$  mice were inoculated with *B. pertussis* and left untreated or depleted of CD4<sup>+</sup> T cells or neutrophils. Untreated wild-type mice harbored  $\sim 1 \times 10^5$  cfu in lungs by day 14 after inoculation, but mice depleted of CD4<sup>+</sup> T cells or neutrophils had  $\sim 1 \times 10^{6.5}$  and  $1 \times 10^7$  cfu, respectively (figure 5A). On day 14 after inoculation, colony-forming units were similar in untreated, CD4<sup>+</sup> T cell–depleted, and neutrophil-depleted TNF- $\alpha^{-/-}$  mice ( $\sim 1 \times 10^7$  cfu) (figure 5A), which indicates that these cells had no measurable effect on bacterial numbers in the absence of TNF- $\alpha$ . These data suggest that TNF- $\alpha$  is required for CD4<sup>+</sup> T cell– and neutrophil-mediated clearance of *B. pertussis* and that the extended life span of CD4<sup>+</sup> T cell– or neutrophil-depleted TNF- $\alpha^{-/-}$  mice infected with *B. pertussis* was not due to reduced bacterial numbers.

To investigate the contribution of neutrophils and CD4<sup>+</sup> T cells to the inflammatory response of *B. pertussis*–infected TNF- $\alpha^{-/-}$  mice, the effects of cell depletions on the leukocyte populations in lungs and on airway resistance were analyzed. Although high numbers of neutrophils were observed in untreated *B. pertussis*–infected TNF- $\alpha^{-/-}$  mice ( $\sim 5 \times 10^6$ ), depletion of neutrophils resulted in very low numbers of these cells ( $< 1 \times 10^5$  cells) on day 14 but did not significantly affect the recruitment of macrophages or lymphocytes (figure 5B–5D). Depletion of CD4<sup>+</sup> T cells decreased the numbers of lymphocytes in lungs on day 14 but also significantly lowered the number of neutrophils ( $\sim 1 \times 10^5$  cells) (figure 5B–5D). Additionally, depleting CD4<sup>+</sup> T cells or neutrophils resulted in large decreases in airway resistance, for Penh values of  $\sim 2.7$ –1.1 and  $-0.95$ , respectively (figure 5E). These data suggest that increased airway resistance and mortality might be due to elevated numbers of neutrophils.

**Necessity of pertussis toxin for *B. pertussis*–induced mortality of TNF- $\alpha^{-/-}$  mice.** Despite the high numbers of neutrophils in the lungs of *B. pertussis*–infected TNF- $\alpha^{-/-}$  mice (figure 2D), these cells are ineffective at reducing bacterial numbers (figure 2A). Because Ptx is known to block the function of neutrophils [35, 36], we tested the effect of Ptx on the outcome of *B. pertussis* infection in TNF- $\alpha^{-/-}$  mice. C57BL/6 and TNF- $\alpha^{-/-}$  mice were inoculated with *Bp* $\Delta$ ptx and monitored for survival. Whereas wild-type *B. pertussis* kills TNF- $\alpha^{-/-}$



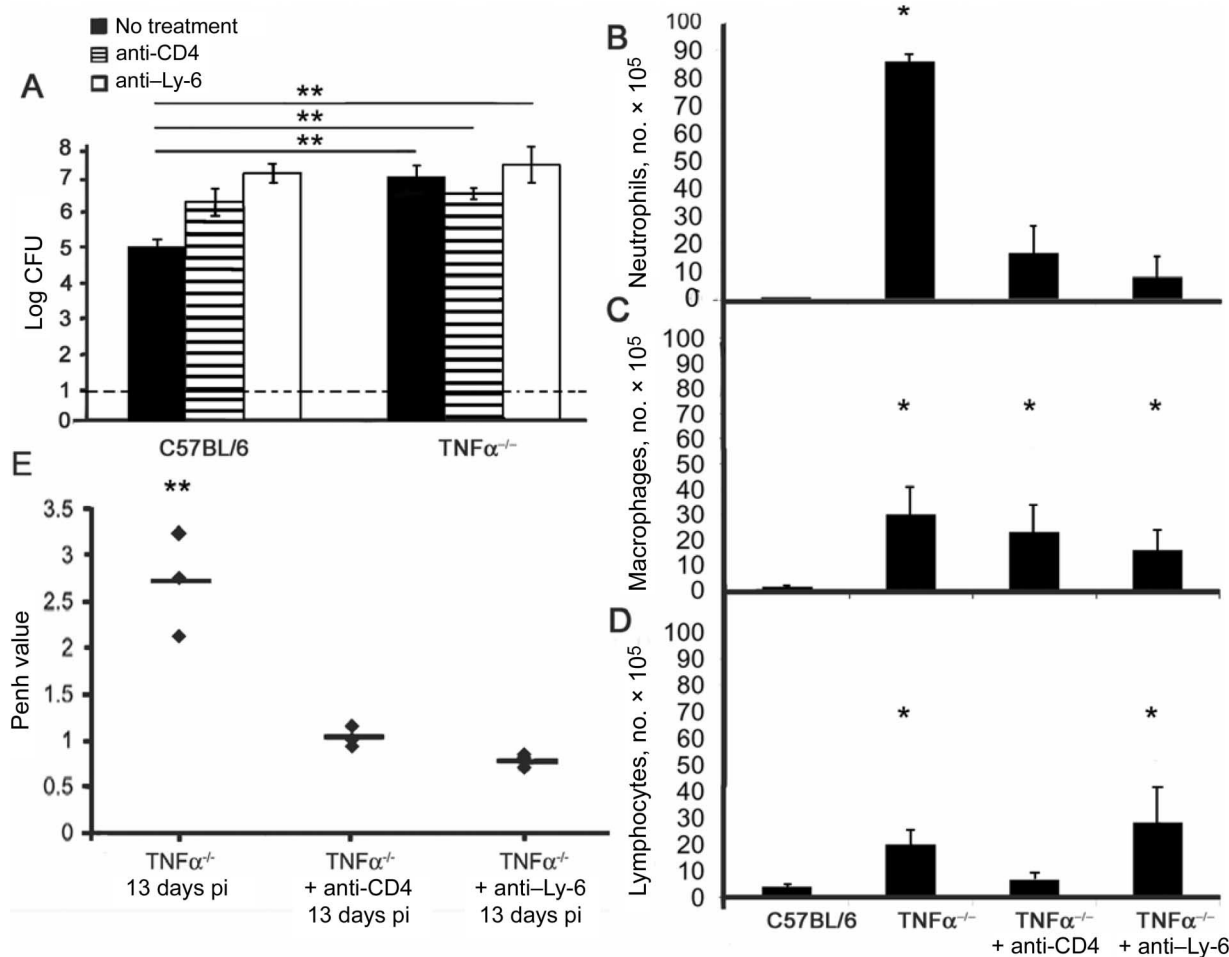
**Figure 4.** Survival curve of *Bordetella pertussis*–infected tumor necrosis factor (TNF)- $\alpha^{-/-}$  mice depleted of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, or neutrophils. Groups of 8 C57BL/6 and 8 TNF- $\alpha^{-/-}$  mice were inoculated with *B. pertussis*. CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells, or neutrophils were depleted on days 0, 7, and 14 after inoculation. As controls, groups of 8 C57BL/6 and TNF- $\alpha^{-/-}$  mice were left untreated. Survival curves are presented as the percentage of mice living on the indicated day after inoculation. \* $P < .01$  vs. the mean survival time of untreated *B. pertussis*–infected TNF- $\alpha^{-/-}$  mice.

mice, these mice did not die of infections with *Bp* $\Delta$ ptx (figure 6), which suggests that TNF- $\alpha$  is required only when Ptx is expressed. In support of this suggestion, *B. parapertussis*, which causes the same disease as *B. pertussis* but does not express Ptx, does not cause lethal disease in TNF- $\alpha^{-/-}$  mice (data not shown).

**Dependence of elevated numbers of *B. pertussis* and leukocytes on Ptx.** Because infections of TNF- $\alpha^{-/-}$  mice with *Bp* $\Delta$ ptx were not lethal, the ability of these mice to control bacterial numbers and inflammation in response to this strain was examined. C57BL/6 and TNF- $\alpha^{-/-}$  mice were inoculated with *Bp* $\Delta$ ptx and killed on day 7 or 14 to determine whether the accumulation of neutrophils in the absence of TNF- $\alpha$  was dependent on Ptx. C57BL/6 and TNF- $\alpha^{-/-}$  mice were both able to reduce *Bp* $\Delta$ ptx numbers to  $\sim 1 \times 10^{2.5}$  cfu in lungs by day 14 after inoculation (figure 7A). Additionally, there were no significant differences in leukocyte numbers between wild-type and TNF- $\alpha^{-/-}$  mice infected with *Bp* $\Delta$ ptx (figure 7B–7D). Thus, TNF- $\alpha$  is important only when Ptx is expressed, and it appears to overcome the effects of Ptx, limiting *B. pertussis* colonization and preventing excessive accumulation of leukocytes in the lungs.

## DISCUSSION

The present study identifies defective TNF- $\alpha$  responses as a potential risk factor for *B. pertussis* disease. TNF- $\alpha^{-/-}$  mice are unable to reduce bacterial numbers, control leukocyte numbers in the lungs, or survive *B. pertussis* infection. Because TNF- $\alpha$



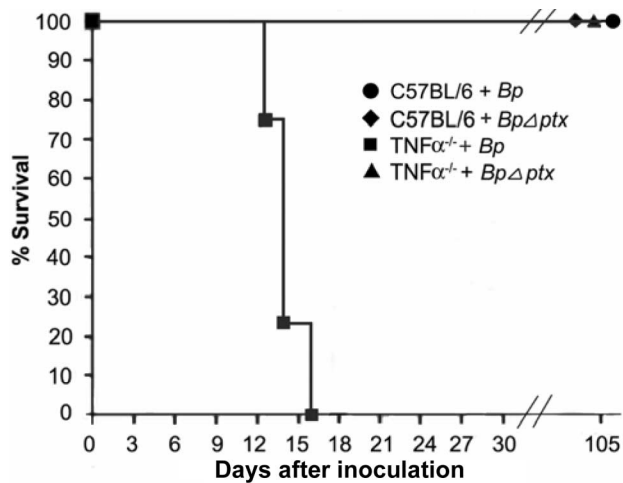
**Figure 5.** Effects of depleting CD4<sup>+</sup> T cells or neutrophils on bacterial numbers, leukocyte accumulation, and airway resistance. Groups of 12 C57BL/6 and tumor necrosis factor (TNF)- $\alpha^{-/-}$  mice were inoculated with *Bordetella pertussis*, and lungs were excised for the quantification of bacteria (A), neutrophils (B), macrophages (C), and lymphocytes (D) on day 14 after inoculation (pi). One group was left untreated, one was depleted of CD4<sup>+</sup> T cells, and one was depleted of neutrophils. Colony-forming units are given as the log<sub>10</sub> mean  $\pm$  SD (error bars) no., with the dashed line representing the lower limit of detection, and cell counts are represented as the mean  $\pm$  SD (error bars). E, Groups of 3 TNF- $\alpha^{-/-}$  mice were inoculated with *B. pertussis* and were left untreated, depleted of CD4<sup>+</sup> T cells, or depleted of neutrophils. Enhanced respiratory pause (Penh) values were determined by whole-body plethysmography on day 13 pi. Penh values are denoted for each individual mouse, with the horizontal bars representing means. \* $P < .05$ ; \*\* $P < .01$ .

had no measurable early effect on infection and TNF- $\alpha$  responses peaked after 1 week, it is likely that this later production of TNF- $\alpha$  is crucial to protection. The life span of *B. pertussis*-infected TNF- $\alpha^{-/-}$  mice was extended by the inhibition of cellular influx to the lungs by depletions of CD4<sup>+</sup> T cells or neutrophils, both of which limited neutrophil accumulation and airway resistance without affecting bacterial numbers. *Bp* $\Delta$ *ptx* numbers were reduced in the respiratory tract without TNF- $\alpha$ , and this cytokine was not essential to regulating the inflammatory response to the isogenic strain. Thus, TNF- $\alpha$  is required only when Ptx is expressed, and it appears to overcome some of the Ptx-mediated effects of *B. pertussis* infection.

The role of TNF- $\alpha$  in counteracting the effects of Ptx was examined in part because of the apparent Ptx-dependent influx

of leukocytes after the first few days of infection [37, 38]. Ptx also affects the function of neutrophils by inhibiting phospholipase C stimulation, lysosomal enzyme secretion, and the increase in intracellular calcium levels after chemokine stimulation [35, 36]. Therefore, it is likely that Ptx expression results in a decrease in the ability of neutrophils to phagocytose and kill *B. pertussis*. Activation by TNF- $\alpha$  might be required to overcome the inhibitory effects of Ptx on neutrophils, perhaps by inducing signaling molecules that act on Ptx-insensitive receptors and activate leukocytes for more efficient phagocytosis.

*B. pertussis* infections in the absence of TNF- $\alpha$  appear to become fatal because of increased airway resistance, which correlates with large numbers of leukocytes in the lungs. Despite the large number of neutrophils in the lungs of *B. pertussis*-



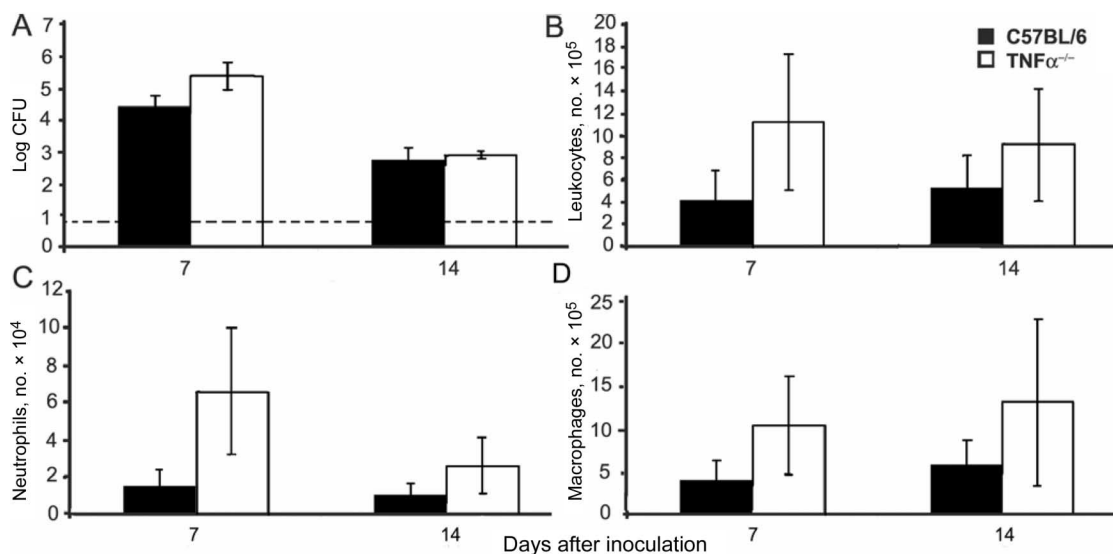
**Figure 6.** Survival of tumor necrosis factor (TNF)- $\alpha^{-/-}$  mice infected with a pertussis toxin (Ptx)-deficient strain of *Bordetella pertussis* (*Bp* $\Delta$ *ptx*). Groups of 8 C57BL/6 and TNF- $\alpha^{-/-}$  mice were inoculated with *B. pertussis* or *Bp* $\Delta$ *ptx* and monitored for survival. Survival curves are presented as the percentage of mice living on the indicated day after inoculation.

infected TNF- $\alpha^{-/-}$  mice 14 days after inoculation (figure 2D), these cells are ineffective at reducing bacterial numbers (figure 2A). Diminished TNF- $\alpha$  levels have been shown to correlate with impaired phagocytosis in several models [18, 39, 40], and this cytokine has been shown to facilitate the phagocytosis of *B. pertussis* in vitro [41]. Furthermore, it has been suggested that, on phagocytosis of bacteria or bacterial products, altered

transcriptional regulation and/or proapoptotic effects of reactive oxygen species cause apoptosis of tissue neutrophils [42–44]. These apoptotic neutrophils are subsequently phagocytosed by macrophages [45], resolving the inflammatory response. Inefficient phagocytosis of *B. pertussis* by TNF- $\alpha$ -deficient neutrophils, combined with the large number of these cells recruited to the lungs, could result in the observed cellular accumulation and airway resistance. In this model, TNF- $\alpha$  might be negatively regulating inflammation by facilitating the phagocytosis of *B. pertussis* by, and consequently the apoptosis of, neutrophils.

The extensive inflammation of *B. pertussis*-infected lungs in the absence of TNF- $\alpha$  could also be more directly mediated by Ptx. This toxin significantly affects the T cell response to *B. pertussis* infection by activating CD4<sup>+</sup> T cells independently of major histocompatibility complex-T cell receptor interactions [46] and skewing them toward a Th1 phenotype and IFN- $\gamma$  production [46, 47]. Although Ptx inhibits the early migration of neutrophils to the lungs [35, 36], the stimulation of pro-inflammatory Th1 responses might result in leukocyte accumulation. Ineffective phagocytosis, apoptosis, and subsequent anti-inflammatory signals might occur in the absence of TNF- $\alpha$ . This is consistent with the fact that the depletion of CD4<sup>+</sup> T cells, as well as of neutrophils, was protective in *B. pertussis*-infected TNF- $\alpha$ -deficient mice. Thus, TNF- $\alpha$  limits the accumulation of leukocytes in response to *B. pertussis* infection, which might be mediated in part by the effects of Ptx.

This study highlights an interesting relationship between



**Figure 7.** Quantification of bacterial numbers and leukocytes in the lungs of wild-type and tumor necrosis factor (TNF)- $\alpha$ -deficient mice infected with a pertussis toxin (Ptx)-deficient strain of *Bordetella pertussis* (*Bp* $\Delta$ *ptx*). Groups of 8 C57BL/6 and TNF- $\alpha^{-/-}$  mice were inoculated with *Bp* $\Delta$ *ptx*. Groups of 4 mice were killed on day 7 or 14 after inoculation for the quantification of bacterial numbers (A), leukocytes (B), macrophages (C), and neutrophils (D) in the lungs. Colony-forming units are given as the log<sub>10</sub> mean  $\pm$  SD (error bars) no., with the dashed line representing the lower limit of detection, and cell counts are represented as the mean  $\pm$  SD (error bars).

TNF- $\alpha$  and a specific bacterial virulence factor, Ptx. It is important that patients who are infected with *B. pertussis* often have secondary infections with other pathogens. Some microbes, such as *Pseudomonas aeruginosa*, utilize mechanisms to inhibit TNF- $\alpha$  signaling [48, 49], which could hinder the host's ability to resolve *B. pertussis*-induced inflammatory responses. The observations that eliminating *B. pertussis* and resolving the inflammation that is induced by this pathogen depend on TNF- $\alpha$  add to our understanding of the pathogenesis and resolution of *B. pertussis* infections and might contribute to intervention strategies.

## Acknowledgments

We thank Drs. Mylisa Parette and Hardy Kornfeld for critical reading of the manuscript and scientific discussions, Dr. Gary Huffnagle for supplying materials, and Drs. Pam Correll and Avery August for the use of laboratory equipment.

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