INFECTION AND IMMUNITY, Jan. 2011, p. 527–541 Vol. 79, No. 1
0019-9567/11/$12.00 doi:10.1128/IAI.00590-10

Interleukin-1 Receptor Signaling Is Required To Overcome the Effects of Pertussis Toxin and for Efficient Infection- or Vaccination-Induced Immunity against *Bordetella pertussis*▽

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Received 2 June 2010/Returned for modification 30 June 2010/Accepted 13 October 2010

Interleukin-1 receptor-deficient (IL-1R$^{-/-}$) mice are healthy despite being colonized by commensal microbes but are defective in defenses against specific pathogens, suggesting that IL-1R-mediated effects contribute to immune responses against specific pathogenic mechanisms. To better define the role of IL-1R in immunity to respiratory infections, we challenged IL-1R$^{-/-}$ mice with *Bordetella pertussis* and *Bordetella parapertussis*, the causative agents of whooping cough. Following inoculation with *B. pertussis*, but not *B. parapertussis*, IL-1R$^{-/-}$ mice showed elevated bacterial numbers and more extensive inflammatory pathology than wild-type mice. Acellular *B. pertussis* vaccines were not efficiently protective against *B. pertussis* in IL-1R$^{-/-}$ mice. *B. pertussis*-stimulated dendritic cells from IL-1R$^{-/-}$ mice produced higher levels of tumor necrosis factor alpha (TNF-α) and IL-6 than wild-type cells. Moreover, elevated levels of gamma interferon (IFN-γ) and TNF-α but lower levels of IL-10 were detected during *B. pertussis* infection in IL-1R$^{-/-}$ mice. Since *B. parapertussis* did not cause severe disease in IL-1R$^{-/-}$ mice, we hypothesized that the extreme requirement for IL-1R involves pertussis toxin (Ptx), which is expressed only by *B. pertussis*. An isogenic Ptx-deficient *B. pertussis* strain had only a modest phenotype in wild-type mice but was completely defective in causing lethal disease in IL-1R$^{-/-}$ mice, indicating that the particular virulence of *B. pertussis* in these mice requires Ptx. Ptx contributes to IL-1β induction by *B. pertussis*, which is involved in IL-10 induction through IL-1R signaling. IL-10 treatment reduced *B. pertussis* numbers in IL-1R$^{-/-}$ mice, suggesting that the lower IL-10 responses partially account for the uncontrolled inflammation and bacterial growth in these mice.

Inflammatory responses effectively combat infection and, when properly controlled, ensure restoration of normal tissue architecture. However, overwhelming inflammation causes damage to host tissue and facilitates pathogen spread. A complex array of cytokines can contribute to the regulation of inflammation under different conditions. By discovering the specific conditions under which key cytokines are required for regulation, we can better define their individual roles. Interleukin-1 (IL-1), a pleiotropic proinflammatory cytokine presented either as IL-1α or IL-1β, is a key player in this regulation. IL-1α is active in both a 31-kDa precursor polypeptide form (pro-IL-1α) and a calpain-cleaved "mature" 17-kDa form (17, 21). Pro-IL-1β is inactive and requires cleavage by caspase-1 to be active and secreted (10, 17, 21, 70). There are two membrane receptors for IL-1, type I IL-1 receptor (IL-1R), which mediates signal transduction, and type II IL-1 receptor, which lacks the cytosolic domains and acts as a decoy receptor (68). Both IL-1α and IL-1β bind to the same receptors and induce indistinguishable responses, including endothelial activation, leukocyte recruitment, T helper (Th) cytokine production, and alterations of the hypothalamic thermoregulatory set point (17).

IL-1 has been implicated in various inflammatory diseases as well as having a role during microbial infections. IL-1 signaling plays pathogenic roles in some autoimmune diseases, such as rheumatoid arthritis and Crohn's disease (17). It is also involved in immunopathology induced by bacterial pathogens, for example, *Yersinia enterocolitica* and *Shigella flexneri* (19, 61, 62). On the other hand, IL-1 signaling also plays beneficial roles in combating microbial infections. For example, exogenous administration of recombinant murine IL-1α (rmIL-1α) enhanced antibacterial resistance to the intracellular pathogen *Listeria monocytogenes* (14). IL-1β-deficient mice challenged with *Staphylococcus aureus*, a Gram-positive bacterium, developed larger lesions associated with decreased neutrophil recruitment (51). IL-1R$^{-/-}$ mice showed increased intestinal damage and lethality following challenge by *Citrobacter rodentium*, a Gram-negative pathogen (40).

IL-1R$^{-/-}$ mice are also defective in host defenses against some respiratory pathogens. For example, mice deficient in IL-1R had higher *Pseudomonas aeruginosa* loads in the lungs (60) and suffered lethal necrotic pneumonia following *Mycobacterium tuberculosis* infection (23). Although IL-1 signaling is required for the control of several pathogens, the critical aspects of host-pathogen interactions that require this pathway

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▽ Published ahead of print on 25 October 2010.

for effective immune responses have not yet been determined. Since IL-1$\alpha^{-/-}$, IL-1$\beta^{-/-}$, and IL-1R$^{-/-}$ mice are viable and healthy despite a complex resident flora, IL-1 signaling is apparently not required for healthy homeostasis or the containment of nonpathogens. There appear to be specific virulence mechanisms of certain pathogens that stress the host responses in ways that reveal important roles of the IL-1 signaling. Here, we examine the role of IL-1R in the immune responses to *Bordetella pertussis* and *Bordetella parapertussis*, identifying a key role in mitigating the virulence associated with pertussis toxin (Ptx) secreted only by *B. pertussis*.

B. pertussis and *B. parapertussis* are Gram-negative coccobacilli and human pathogens that cause whooping cough, an acute and severe respiratory disease (48). Despite high vaccine coverage in developed countries, whooping cough causes approximately 50 million cases and 300,000 deaths annually worldwide (12). Even though a large portion of infections remain unreported (16), whooping cough incidence is on the rise (24, 34, 65, 71). *B. pertussis* utilizes complex strategies to modulate and evade host immune responses by producing various virulence determinants, such as Ptx, adenylate cyclase toxin, tracheal cytotoxin, filamentous hemagglutinin, fimbriae, pertactin, and lipopolysaccharide (48, 52). *B. parapertussis* shares most of these virulence determinants (48) but does not express Ptx (4, 46). Although *B. pertussis* and *B. parapertussis* are closely related (57), the distinct strategies they use to modulate host immune responses result in differential dependence on specific immune functions for their efficient control and clearance.

Ptx is a multisubunit toxin with an AB_5 configuration. The B-oligomer binds to glycoproteins or glycolipids on the surface of target cells (72, 75). The enzymatic activity of Ptx resides in the A subunit, also known as S1. Once in the cell cytosol, S1 mediates ADP-ribosylation of the α-subunit of a subset of Gi proteins in mammalian cells (5, 35). The modification results in the inhibition of Gi protein-coupled signaling pathways, causing a variety of downstream effects. Ptx is known to be the cause of some systemic symptoms associated with whooping cough, such as lymphocytosis, histamine sensitivity, and insulinemia (53). Ptx also exerts multiple modulating effects on the immune system, including targeting airway macrophages to promote infection (9), blocking the early migration of neutrophils into the lungs (2, 37), suppressing the production of anti-*Bordetella* serum antibodies (8, 50), reducing major histocompatibility complex class II on the surface of monocytes (63), and interfering with CD1a expression on dendritic cells (47). A recent study indicates that Ptx contributes to the induction of proinflammatory cytokine production at the peak of infection (3), but whether these cytokines are important for host defenses against *B. pertussis* has not been established.

A recent report indicates that adenylate cyclase toxin from *B. pertussis* promotes IL-1β production through the activation of the inflammasome and that IL-1β is involved in the generation of Th17 cells that contribute to protective immunity against *B. pertussis* (20). Both *B. pertussis* and *B. parapertussis* express adenylate cyclase toxin, suggesting that IL-1β should be produced during infection with either pathogen and that its receptor might be important for the host defense against them. We demonstrated here that although IL-1 is induced by either *B. pertussis* or *B. parapertussis*, mice lacking type I IL-1 receptor (IL-1R$^{-/-}$) are defective in controlling *B. pertussis* but not *B. parapertussis* infection. IL-1R$^{-/-}$ mice suffered increased mortality from *B. pertussis* infection associated with increased bacterial burdens throughout the respiratory tract, atypical disseminated disease, increased histopathology, increased leukocyte recruitment, elevated proinflammatory cytokine production, and decreased anti-inflammatory cytokine production. These mice were also less efficiently protected against *B. pertussis* following vaccination with a commercial acellular *B. pertussis* (aP) vaccine. IL-1R signaling was involved in the IL-10 induction by Ptx. IL-10 treatment reduced *B. pertussis* numbers in IL-1R$^{-/-}$ mice to numbers comparable to those of wild-type mice. IL-1R was not required for the control of *B. parapertussis*, which lacks the expression of Ptx, or for the control of a *B. pertussis* strain lacking Ptx. Overall, our study suggests an indispensable protective role for IL-1R signaling in overcoming the effects of Ptx during *B. pertussis* infection.

MATERIALS AND METHODS

Bacterial strains and growth. The *B. pertussis* strain 536, a streptomycin-resistant derivative of Tohama I, and the *B. parapertussis* strain 12822, an isolate from German clinical trials, have been previously described (31, 67). BPH101, a pertussis toxin-deficient derivative of *B. pertussis* strain 536 (the *B. pertussis* Δptx strain), was a gift from Drusilla Burns (U.S. Food and Drug Administration) (30). Bacteria were maintained on Bordet-Gengou agar (Difco) supplemented with 10% defibrinated sheep blood (Hema Resources) and 20 μg/ml streptomycin (Sigma-Aldrich). Liquid cultures were grown overnight in Stainer-Scholte broth at 37°C to mid-log phase (66).

Bone marrow-derived cell assays. Bone marrow-derived macrophages (BMM) and dendritic cells (BMDC) were prepared as previously published, with modifications (42, 64). In brief, bone marrow was isolated from femurs of C57BL/6 or IL-1R$^{-/-}$ mice and cultured for 10 days in Dulbecco's modified Eagle's medium (DMEM) (HyClone) supplemented with 10% fetal calf serum (FCS) (HyClone), 100 μg/ml penicillin-streptomycin (HyClone), and 20 ng/ml macrophage colony-stimulating factor (M-CSF) (PeproTech) for BMM differentiation or in RPMI medium supplemented with 2 mM L-glutamine (HyClone), 10% FCS, 100 μg/ml penicillin-streptomycin, and 40 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech) for BMDC differentiation. For cytokine responses, 10^5 to 2×10^5 BMM or BMDC were seeded into each well of 96-well tissue culture plates in medium without antibiotics and stimulated with medium alone or medium containing *B. pertussis*, *B. parapertussis*, or Ptx. After incubation for the times indicated in the figure legends, the culture supernatant was removed and assayed for IL-1α, IL-1β, tumor necrosis factor alpha (TNF-α), IL-6, or IL-10 via enzyme-linked immunosorbent assays (ELISAs) as per the manufacturer's instructions (R&D Systems). For BMM bactericidal assays, 10^5 cells were seeded into each well of 96-well tissue culture plates and primed with medium without antibiotics and containing 25 ng/ml recombinant murine gamma interferon (rmIFN-γ; R&D Systems) or 1 μg/ml *B. pertussis* lipopolysaccharide (LPS) for 2 h. Early-exponential-phase *B. pertussis* cells were opsonized with 5% convalescent-phase complement-depleted (incubated at 56°C for 30 min) immune serum from C57BL/6 mice for 30 min in a shaking 37°C incubator. Primed BMM were inoculated with opsonized *B. pertussis* at a multiplicity of infection (MOI) of 10. After 2 h or 4 h, 1% ice-cold Triton X-100 in phosphate-buffered saline (PBS) was added to lyse the macrophages. Serial dilutions (1/10) of the lysed macrophages from each well were plated for bacterial enumeration. Bacterial numbers in similarly treated wells without cells were enumerated as total bacterial numbers.

Peritoneal macrophage killing assay. Peritoneal cells were recovered from abdominal cavity lavage fluid of mice, and macrophages were purified by adherence to plastic overnight (>95% F4/80$^+$ macrophages). A total of 10^5 macrophages were seeded in each well of a 96-well plate and stimulated with 1 μg/ml *B. pertussis* LPS for 2 h and then inoculated with *B. pertussis* at an MOI of 10. After 4 h, 1% ice-cold Triton X-100 in PBS was added to lyse the macrophages. Serial dilutions (1/10) of the lysed macrophages from each well were plated for bacterial enumeration. Bacterial numbers in similarly treated wells without cells were enumerated as total bacterial numbers.

Animal experiments. C57BL/6, B6.129S1-*Il1r1*^{tm1Roml}/J (IL-1R$^{-/-}$), B6.129S2-*Igh-6*^{tm1Cgn}/J (μMT), and B6.129P2-*Il10*^{tm1Cgn}/J (IL-10$^{-/-}$) mice were obtained

from Jackson Laboratories (Bar Harbor, ME). IL-17R$^{-/-}$ mice (C57BL/6 background) were kind gifts from Jay K. Kolls (Louisiana State University Health Sciences Center) and have been described previously (76). All mice were bred in *Bordetella*-free, specific-pathogen-free breeding rooms at The Pennsylvania State University. For inoculation, 4- to 6-week-old mice were lightly sedated with 5% isoflurane (Abbott Laboratories) in oxygen and inoculated by pipetting 50 μl of phosphate-buffered saline (PBS) containing 5×10^5 CFU (unless otherwise specified) of bacteria onto the external nares (38). This method reliably distributes the bacteria throughout the respiratory tract (29). Mice were vaccinated by intraperitoneal (i.p.) injection with one-fifth of a human dose of Adacel (Sanofi Pastuer) with Imject Alum (Thermo Scientific) (an aP vaccine) or with only Imject Alum in 200 μl of PBS 14 and 28 days prior to challenge (77, 78). For survival curves or 50% lethal dose (LD_{50}) determination, mice were inoculated with the dose indicated in the figure legends, and the percent survival was monitored over a 100-day period. Mice with lethal bordetellosis, indicated by ruffled fur, labored breathing, and diminished responsiveness, were euthanized to alleviate unnecessary suffering (28, 74). For adoptive transfer of serum antibodies, sera were collected from naïve animals or on days 21 after *B. pertussis* inoculation, and 200 μl of pooled serum was i.p. injected at the time of inoculation (38). These mice were euthanized on day 14 postinoculation (p.i.) for bacterial number quantification in their lungs, by which time the inhibition of antibody-mediated clearance by pertussis toxin is overcome with T cell help in wild-type animals (37) (D. N. Wolfe, unpublished data). For intranasal administration of rmIL-17, C57BL/6 or IL-1R$^{-/-}$ mice were lightly sedated and intranasally inoculated with 50 μl of PBS or PBS containing rmIL-17 (R&D Systems) on days 3 (1.25 μg/mouse) and 6 (1 μg/mouse) p.i.; mice were sacrificed on day 7 p.i. For intranasal administration of rmIL-10, C57BL/6 or IL-1R$^{-/-}$ mice were lightly sedated and intranasally inoculated with 50 μl of PBS or PBS containing 0.1 μg/mouse rmIL-10 (R&D Systems) on days 0 and 3 p.i.; mice were sacrificed on day 7 p.i. For quantification of bacterial numbers, mice were sacrificed via CO_2 inhalation; lung, trachea, nasal cavity, spleen, and liver were excised, and around 750 μl of blood was collected from each mouse by cardiac puncture into tubes with 50 μl of 0.5 M (pH 8) EDTA. Tissues were homogenized in 1 ml of PBS, serially diluted, and plated onto Bordet-Gengou agar plates with 20 μg/ml streptomycin, and colonies were counted after 4 to 5 days of incubation at 37°C (38). The lower limit of detection was 10 CFU. All protocols were reviewed and approved by The Pennsylvania State University Institutional Animal Care and Use Committee (IACUC), and all animals were handled in accordance with institutional guidelines.

Lung pathology. For analysis of lung pathology, mice were intranasally inoculated as described above and euthanized on days 3, 7, and 21 p.i. The tracheas and lungs were excised and inflated with approximately 2 ml of 10% formaldehyde. The tissues were then sectioned and stained with hematoxylin and eosin (H&E) at the Animal Diagnostic Laboratories Facility of The Pennsylvania State University. Sections were examined and scored by a veterinarian with training and experience in rodent pathology (M. J. Kennett) who was blinded to experimental treatment (44, 45, 58). A score of 0 indicates no noticeable inflammation or lesions; a score of 1 indicates few or scattered foci affecting less than 10% of the tissue, typically with a few mild perivascular and/or peribronchial lymphoid aggregates; a score of 2 indicates frequent mild perivascular and/or peribronchial lymphoid aggregates, with or without occasional small foci of pneumonia, with overall inflammation affecting no more than 10 to 20% of the tissue; a score of 3 indicates moderate lesions, typically with abundant perivascular and peribronchial lymphoid infiltrates and multiple mild to moderate foci of pneumonia, with inflammation affecting approximately 20 to 30% of the tissue; a score of 4 indicates extensive pneumonia and marked inflammation affecting more than 30% of the tissue; and a score of 5 indicates extensive lesions with >50% of the tissue affected. If a severity falls between categories, 0.5 is added to the pathology score of the lower category.

Splenocyte restimulation. Spleens were excised on days 0, 3, 7, 14, and 21 p.i. from groups of *B. pertussis*-inoculated C57BL/6 or IL-1R$^{-/-}$ mice. Splenocytes were isolated as previously described (59, 73). In brief, spleens were homogenized, and red blood cells were lysed with 0.84% ammonium chloride treatment. A total of 2×10^6 cells were resuspended in 200 μl of DMEM supplemented with 10% FCS and 100 μg/ml penicillin-streptomycin and placed into each well of 96-well tissue culture plates. Splenocytes were stimulated with 10 μl of medium alone or medium containing 10^7 CFU (MOI of 5) of heat-killed *B. pertussis* (59, 73). After 3 days, the supernatants were collected, and TNF-α, IFN-γ, IL-10, and IL-17 concentrations were determined by ELISA (R&D Systems).

Titer ELISAs. Antibody titers were determined as previously described (44). Briefly, an exponential-phase culture of *B. pertussis* was heat killed, diluted to 5×10^7 CFU/ml in a 1:1 mix of 0.2 M sodium carbonate and 0.2 M sodium bicarbonate buffers, and used to coat 96-well plates (Greiner Bio-one) by incubation for 4 h at 37°C in a humidified chamber; the plates were then washed and blocked. A 1:50 (for Ig) or 1:20 (for IgG1 and IgG2a) dilution of serum samples collected from individual C57BL/6 or IL-1R$^{-/-}$ mice on days 7, 14, or 21 p.i. was added to the first well and serially diluted 1:2 across the plates. Plates were incubated for 2 h at 37°C, washed, and probed with a 1:4,000 dilution of goat anti-mouse Ig or a 1:2,000 dilution of goat anti-mouse IgG1 or IgG2a horseradish peroxidase (HRP)-conjugated antibodies (Southern Biotechnology Associates and Pharmingen) for 1 h. The plates were read at an absorbance of 405 nm after incubation with 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt and hydrogen peroxide in phosphate-citrate buffer. Titers were determined via the endpoint method using a cutoff that is 0.1 higher than the optical density of similarly treated wells probed with naïve serum (44).

Quantification of leukocytes and cytokines in the lungs. To quantify leukocytes, lungs were perfused with 2 to 3 ml of sterile PBS, excised, and placed in 4 ml of RPMI 1640 medium (HyClone). Lungs were homogenized, laid over Histopaque 1119 (Sigma Aldrich), and centrifuged for 30 min at $700 \times g$ at 20°C. The leukocyte layer was collected, spun at $300 \times g$ for 5 min, and resuspended in PBS supplemented with 2% FCS. The total number of leukocytes was determined by counting at a ×40 magnification on a hemocytometer. Aliquots of cells were blocked with anti-mouse CD16/CD32 antibodies (BD Biosciences Pharmingen) and stained with fluorescein isothiocyanate (FITC)-labeled anti-mouse Ly6G, F4/80, or CD4 antibodies (BD Biosciences Pharmingen), and the percentages of Ly6G$^+$, F4/80$^+$, or CD4$^+$ cells were determined by flow cytometry. Percentages were multiplied by the total number of leukocytes to calculate the number of Ly6G$^+$, F4/80$^+$, or CD4$^+$ cells, respectively. To measure the cytokine concentration in the lungs, lungs were homogenized in 1 ml of PBS, tissues were spun down at $8{,}000 \times g$ for 10 min at 4°C, and IL-1α, IL-1β, TNF-α, IFN-γ, IL-10, and IL-17 concentrations in the aliquots of the supernatants were examined via ELISAs (R&D Systems).

Enzyme-linked immunospot (ELISPOT) analysis. Lung leukocytes were isolated from lung homogenates using Histopaque 1119 (Sigma Aldrich) gradient density centrifugation. Cells were resuspended in RPMI medium supplemented with 10% FCS and 100 μg/ml penicillin-streptomycin. Either 10^5 or 2×10^4 lung leukocytes in 100 μl of medium were added to each well of nitrocellulose-backed 96-well plates (Millipore) previously coated with anti-mouse IFN-γ (BD Biosciences) or IL-10 (R&D Systems) antibody and blocked with medium. Ten microliters of medium or medium containing 10^6 CFU of heat-killed *B. pertussis* was added to each well. After 24 h, the plates were washed and treated with biotinylated anti-mouse IFN-γ (BD Biosciences) or IL-10 (R&D Systems) antibody overnight. The plates were then washed, and streptavidin-horseradish peroxidase (BD Biosciences) was added. The plates were developed with 3-amino-9-ethylcarbazole (BD Biosciences). The number of *B. pertussis*-specific cytokine-producing cells was determined by subtracting the number of spots in the presence of medium only from the number of spots observed when the leukocytes were incubated with heat-killed *B. pertussis*.

Statistical analysis. The means ± the standard error (error bars in figures) were determined for all appropriate data. Two-tailed, unpaired Student's *t* tests were used to determine statistical significance between groups when variance between groups was unequal. When variance was equal among groups, results were also analyzed by analysis of variance (ANOVA) and a Tukey simultaneous test in Minitab with similar significance values. Pathology scores were analyzed by a nonparametric Mann-Whitney test in Minitab.

RESULTS

***B. pertussis* and *B. parapertussis* induce IL-1 production *in vitro* and *in vivo*.** To determine if *B. pertussis* or *B. parapertussis* stimulates the production of IL-1, we stimulated bone marrow-derived macrophages (BMM) from C57BL/6 mice with medium alone or with medium containing *B. pertussis* or *B. parapertussis* and measured the concentrations of IL-1α or IL-1β in the cell culture supernatants after 48 h. Macrophages incubated with medium alone produced little IL-1 (Fig. 1A and B). Macrophages incubated with *B. pertussis* or *B. parapertussis* for 48 h had produced many times more IL-1α and IL-1β (Fig. 1A and B).

To examine if IL-1 is produced at the site of *B. pertussis* or *B. parapertussis* infection *in vivo*, C57BL/6 mice were inoculated with 5×10^5 CFU of *B. pertussis* or *B. parapertussis* in 50

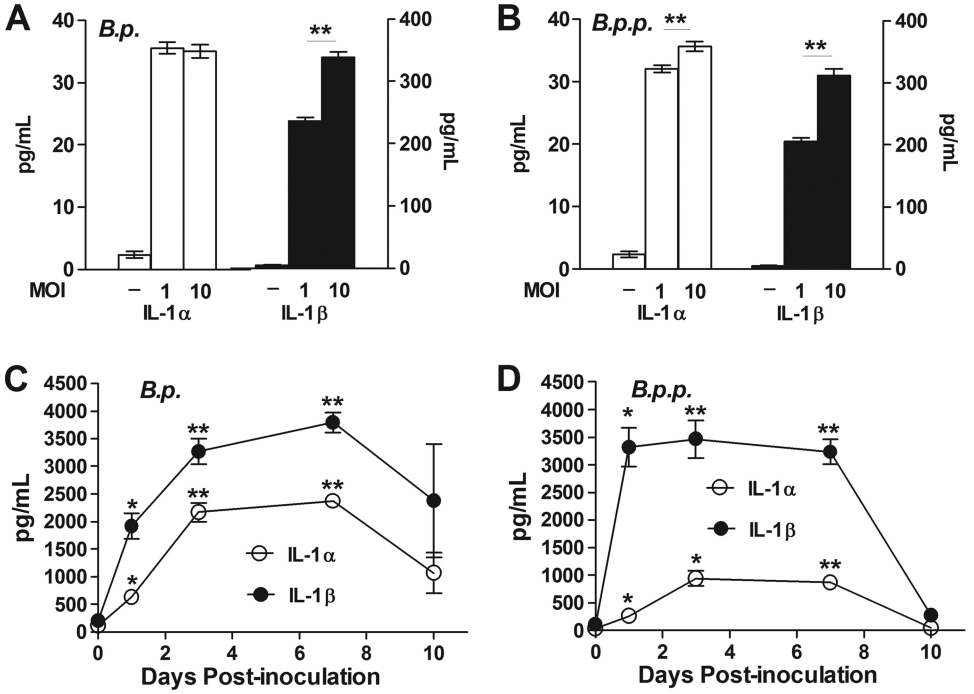

FIG. 1. IL-1 is induced by *B. pertussis* and *B. parapertussis*. BMM were incubated with medium alone (−) or with medium containing live *B. pertussis* (*B.p.*) (A) or *B. parapertussis* (*B.p.p.*) (B) at an MOI of 1 or 10 for 48 h. The IL-1α or IL-1β concentration in culture supernatant is expressed as mean ± standard error for four similarly treated cultures. **, $P \leq 0.01$. Groups of three to four C57BL/6 mice were inoculated with 5×10^5 CFU of *B. pertussis* (C) or *B. parapertussis* (D) and sacrificed on days 0, 1, 3, 7, and 10 p.i. IL-1α or IL-1β concentration in the lungs is expressed as mean ± standard error. *, $P \leq 0.05$; **, $P \leq 0.01$ (compared to the level on day 0 p.i.). Results are representative of two independent experiments.

μl of PBS, and IL-1α and IL-1β concentrations in the lungs were examined on days 0, 1, 3, 7, and 10 p.i. IL-1α levels were elevated within 24 h after *B. pertussis* or *B. parapertussis* inoculation, peaked by day 3 to day 7 p.i., and declined thereafter (Fig. 1C and D). IL-1β levels showed a similar trend, increasing by 24 h and remaining elevated for at least 1 week p.i. (Fig. 1C and D). Together, these data indicate that *B. pertussis* and *B. parapertussis* induce IL-1α and IL-1β production *in vitro* by macrophages and *in vivo* in the lungs.

Increased mortality and elevated respiratory tract and systemic colonization in *B. pertussis*-infected IL-1R$^{-/-}$ mice. To determine whether IL-1R signaling is an important aspect of the immune response to *B. parapertussis* or *B. pertussis* infection, we monitored survival after challenging wild-type C57BL/6 or IL-1R$^{-/-}$ mice with 10^6 CFU of *B. parapertussis* or *B. pertussis*. Challenged C57BL/6 mice showed no signs of disease and were euthanized at the end of the experiment (100 days p.i.) (data not shown). Similarly, IL-1R$^{-/-}$ mice challenged with *B. parapertussis* did not show any sign of disease throughout the 100-day period (Fig. 2A, square). However, IL-1R$^{-/-}$ mice challenged with *B. pertussis* showed signs of disease during the second week p.i., including ruffled fur, hunched posture, decreased activity, and labored breathing, and died or were euthanized when irreversible morbidity became apparent. All IL-1R$^{-/-}$ mice inoculated with *B. pertussis* succumbed to lethal bordetellosis by day 17 p.i. (Fig. 2A, triangle). These data indicate that IL-1R deficiency greatly increases sensitivity to lethal *B. pertussis* infection.

Groups of C57BL/6 or IL-1R$^{-/-}$ mice were challenged with 5×10^5 CFU of *B. parapertussis* and euthanized on day 0, 3, 7, 14, or 28 p.i. to determine whether IL-1R contributes to the control of bacterial numbers following *B. parapertussis* infection. Other than small and transient differences in bacterial numbers in the tracheas, *B. parapertussis* numbers followed similar kinetics throughout the respiratory tracts of both C57BL/6 and IL-1R$^{-/-}$ mice (Fig. 2B), indicating that IL-1R signaling is not required for the control and clearance of *B. parapertussis*.

To test if the increased mortality of IL-1R$^{-/-}$ mice following *B. pertussis* inoculation is associated with higher bacterial loads in the respiratory tract, mice were inoculated with 5×10^5 CFU of *B. pertussis* and sacrificed on days 0, 1, 3, 7, 10, 14, and 26 p.i. In wild-type mice, *B. pertussis* grew rapidly throughout the respiratory tract during the first week p.i. and decreased thereafter (Fig. 2C, solid line). In IL-1R$^{-/-}$ mice, *B. pertussis* similarly grew to high numbers during the first week p.i., but these mice failed to reduce bacterial numbers throughout the respiratory tract thereafter (Fig. 2C, dashed line). We originally planned a day 28 p.i. time point, but two out of five IL-1R$^{-/-}$ mice showed signs of severe disease on day 24 p.i. and were euthanized. The surviving IL-1R$^{-/-}$ mice displayed signs of morbidity on day 26 p.i. and were sacrificed along with the C57BL/6 controls for bacterial number quantification. The IL-1R$^{-/-}$ mice harbored 100-fold more *B. pertussis* bacteria in the nasal cavity and 1,000-fold more *B. pertussis* bacteria in the lower respiratory tract (LRT) than C57BL/6 mice (Fig. 2C). In a separate experiment, groups of C57BL/6 and IL-1R$^{-/-}$ mice were challenged with 5×10^5 CFU *B. pertussis* and sacrificed on

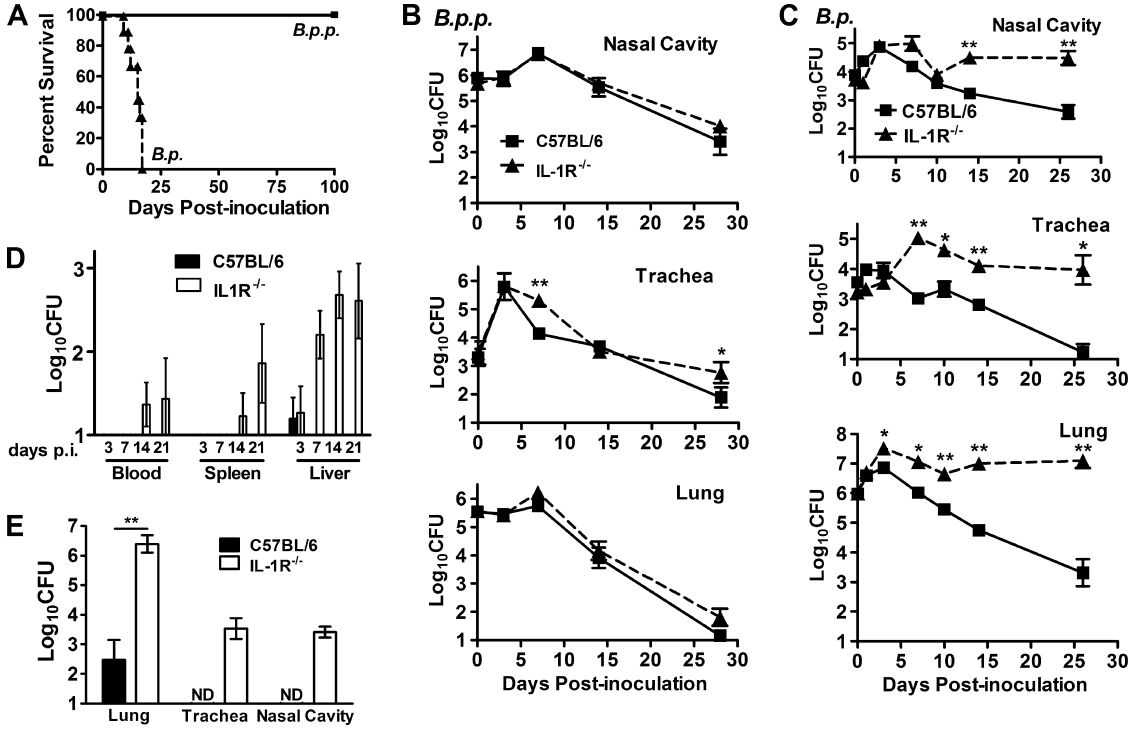

FIG. 2. Increased mortality and morbidity of *B. pertussis*-infected but not *B. parapertussis*-infected IL-1R$^{-/-}$ mice. (A) Groups of IL-1R$^{-/-}$ (n = 9) mice were inoculated with 10^6 CFU of *B. parapertussis* (square) or *B. pertussis* (triangle) and monitored for survival. Groups of three to four C57BL/6 or IL-1R$^{-/-}$ mice were inoculated with 5×10^5 CFU of *B. parapertussis* (B) or *B. pertussis* (C). Bacterial loads in the nasal cavity, trachea, or lung on days 0, 3, 7, 14, and 28 p.i. (B) or days 0, 1, 3, 7, 10, 14, and 26 p.i. (C) are expressed as mean $\log_{10}$ CFU ± the standard error. (D) Groups of three C57BL/6 or IL-1R$^{-/-}$ mice were inoculated with 5×10^5 CFU of *B. pertussis*, and bacterial loads in the blood, spleen, or liver on days 3, 7, 14, or 21 p.i. are expressed as mean $\log_{10}$ CFU ± the standard error. (E) Groups of three to four C57BL/6 or IL-1R$^{-/-}$ mice were inoculated with 10^3 CFU of *B. pertussis*, and bacterial loads in the lungs on day 28 p.i. are expressed as mean $\log_{10}$ CFU ± the standard error. *, $P \leq 0.05$; **, $P \leq 0.01$ (for C57BL/6 versus IL-1R$^{-/-}$ mice). The limit of detection is indicated by the y axis. Results are representative of two or three independent experiments.

day 21 p.i. to avoid the loss of sick animals. At this time point C57BL/6 mice harbored hundreds ($10^{2.70 \pm 0.22}$) of *B. pertussis* bacteria in their lungs. In contrast, tens of millions ($10^{7.14 \pm 0.27}$) of *B. pertussis* bacteria were recovered from the lungs of IL-1R$^{-/-}$ mice (data not shown). Together, these data indicate that IL-1R is required for the clearance of *B. pertussis*, but not *B. parapertussis*, from the respiratory tract.

B. pertussis does not colonize systemic organs of wild-type mice but causes atypical disseminated infection in mice lacking certain immune functions (7, 43). To test if *B. pertussis* colonizes systemic organs of IL-1R$^{-/-}$ mice, in a separate experiment, C57BL/6 and IL-1R$^{-/-}$ mice were challenged with 5×10^5 CFU *B. pertussis*, and the bacterial loads in their blood, spleen, and liver were enumerated on days 3, 7, 14, and 21 p.i. *B. pertussis* was not recovered from the blood or spleen of C57BL/6 mice at any time point. However, *B. pertussis* was recovered from the blood and spleen of IL-1R$^{-/-}$ mice on days 14 and 21 p.i. (Fig. 2D). In the livers of both C57BL/6 and IL-1R$^{-/-}$ mice, *B. pertussis* colonized at a low level in one out of three mice on day 3 p.i. but was not recovered from the livers of C57BL/6 mice at any of the later time points. Interestingly, *B. pertussis* consistently colonized the livers of IL-1R$^{-/-}$ mice on days 7, 14, and 21 p.i. (Fig. 2D). These data indicate that in the absence of IL-1R signaling, *B. pertussis* causes systemic infection.

To test if IL-1R is required for the control of relatively low numbers of *B. pertussis*, separate groups of mice were challenged with 10^3 CFU of *B. pertussis*. Twenty-eight days following this low-dose inoculation, *B. pertussis* burdens were more than 4 orders of magnitude higher in the lungs and approximately 2 orders of magnitude higher in the tracheas and nasal cavities of IL-1R$^{-/-}$ mice than in wild-type mice (Fig. 2E). By day 56, although *B. pertussis* was completely cleared from the respiratory tract of wild-type mice, half of IL-1R$^{-/-}$ mice became morbid. The surviving IL-1R$^{-/-}$ mice still harbored ~300 CFU of *B. pertussis* in their lungs at this time point (data not shown). These data also indicate that the LD_{50} of *B. pertussis* in IL-1R$^{-/-}$ mice was approximately 10^3 CFU, whereas in C57BL/6 mice the LD_{50} was greater than 5×10^7 CFU.

IL-1R contributes to the generation of efficient vaccine-induced immunity against *B. pertussis*. To address whether IL-1R contributes to the generation of vaccine-induced immunity against *B. pertussis*, C57BL/6 and IL-1R$^{-/-}$ mice were vaccinated with Adacel, an acellular *B. pertussis* vaccine (aP) containing Ptx, pertactin, filamentous hemagglutinin, fimbriae 2, and fimbriae 3. Naïve or vaccinated mice were challenged with *B. pertussis* and sacrificed 3 or 7 days later for bacterial number quantification. Adjuvant-only-vaccinated C57BL/6 and IL-1R$^{-/-}$ mice had similar bacterial loads in their lungs on

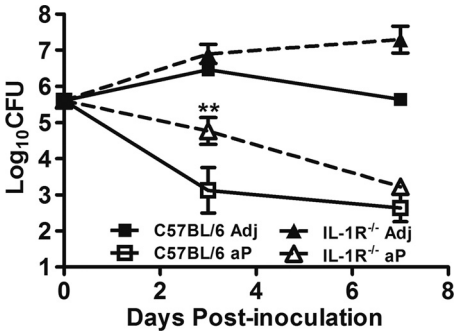

FIG. 3. Inefficient *B. pertussis* vaccine-induced immunity in IL-1R$^{-/-}$ mice. Groups of four C57BL/6 and IL-1R$^{-/-}$ mice were vaccinated with aP. Control mice were vaccinated with PBS containing only adjuvant (Adj). Vaccinated or control mice were challenged with 5×10^5 CFU of *B. pertussis*. Bacterial numbers in the lungs on days 0, 3, and 7 p.i. are expressed as mean $\log_{10}$ CFU ± the standard error. The limit of detection is indicated by the *y* axis. **, $P \leq 0.01$, for vaccinated wild-type versus IL-1R$^{-/-}$ mice. Results are representative of two independent experiments.

day 3 postchallenge (Fig. 3). In comparison, both aP-vaccinated wild-type and IL-1R-deficient mice significantly reduced *B. pertussis* numbers. However, vaccinated IL-1R$^{-/-}$ mice harbored >40-fold more *B. pertussis* bacteria in their lungs than aP-vaccinated wild-type mice, indicating that IL-1R is required for the generation of efficient aP-induced protection against *B. pertussis*. By day 7 postchallenge, similar numbers of *B. pertussis* bacteria were recovered from the lungs of aP-vaccinated C57BL/6 and IL-1R$^{-/-}$ mice, suggesting that the defect in IL-1R$^{-/-}$ mice is overcome with time.

IL-1R$^{-/-}$ mice showed elevated *B. pertussis* infection-induced inflammatory pathology. Since IL-1R$^{-/-}$ mice have been reported to show increased inflammation during infection by other bacteria (23, 40) and since we visually observed that the lungs of *B. pertussis*-inoculated IL-1R$^{-/-}$ mice were consistently more inflamed than C57BL/6 lungs in multiple experiments, we examined the role of IL-1R signaling in the control of inflammatory pathology post-*B. pertussis* inoculation. Sham (PBS)-inoculated C57BL/6 or IL-1R$^{-/-}$ mice showed little sign of inflammation (Fig. 4A). On day 3 p.i. with 5×10^5 CFU of *B. pertussis*, C57BL/6 mice showed pulmonary edema and scattered mild neutrophil infiltration in the parenchyma and bronchi, whereas IL-1R$^{-/-}$ mice showed severe diffuse suppurative pneumonia with fibrin, areas of consolidation (microabcesses), and some hemorrhage. On day 7 p.i., lung tissues from both C57BL/6 and IL-1R$^{-/-}$ mice showed severe diffuse suppurative pneumonia with fibrin and areas of consolidation, necrosis, and hemorrhage, and the overall af-

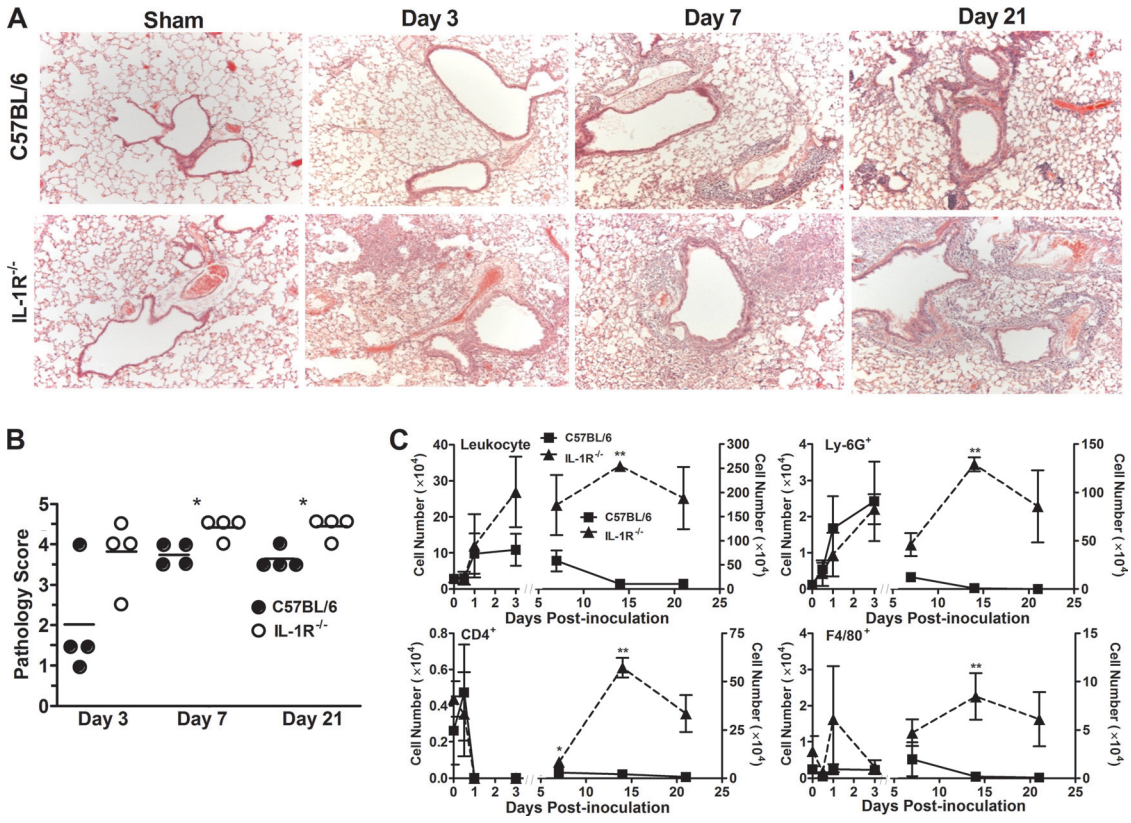

FIG. 4. Increased inflammatory pathology and leukocyte recruitment of *B. pertussis*-infected IL-1R$^{-/-}$ mice. Representative field (×10 magnification) (A) and pathology scores (B) of H&E-stained lung sections of *B. pertussis*-inoculated (5×10^5 CFU) C57BL/6 or IL-1R$^{-/-}$ mice on the indicated days p.i. The horizontal line represents the mean pathology score. (C) Groups of four C57BL/6 or IL-1R$^{-/-}$ mice were inoculated with 5×10^5 CFU of *B. pertussis*, and total leukocyte, Ly6G$^+$ neutrophil, F4/80$^+$ macrophage, and CD4$^+$ T cell numbers in the lungs are expressed as means ± standard errors. *, $P \leq 0.05$; **, $P \leq 0.01$ (for wild-type versus IL-1R$^{-/-}$ mice). Results are representative of two independent experiments.

fected area of IL-1R$^{-/-}$ mouse lungs (average pathology score, 4.4) was significantly larger than that of C57BL/6 mouse lungs (average pathology score, 3.8) (Fig. 4B). On day 21 p.i., lungs of C57BL/6 mice showed marked peribronchiolar, perivascular, lymphoid aggregates, and patchy to diffuse pneumonia with areas of consolidation and lymphocytic infiltrates. IL-1R$^{-/-}$ lungs, however, showed severe diffuse pneumonia with mixed inflammatory cell infiltrates (primarily neutrophils), fibrin, and large areas of consolidation and no lymphocytic cuffing (Fig. 4A), indicating poor resolution of inflammation.

To quantify the numbers of different cell types infiltrated into the lungs, C57BL/6 or IL-1R$^{-/-}$ mice were inoculated with *B. pertussis*, and total leukocytes were separated from lung homogenates and evaluated by flow cytometry after cell surface staining. On 1 and 3 days after *B. pertussis* challenge, wild-type and IL-1R$^{-/-}$ mice contained similar numbers of total leukocytes, Ly6G$^+$ neutrophils, F4/80$^+$ macrophages, and CD4$^+$ T cells in their lungs (Fig. 4C), suggesting that IL-1R$^{-/-}$ mice are not defective in cell recruitment during the early stages of *B. pertussis* infection. Consistent with results from histopathology examination (Fig. 4A and B), more total leukocytes, neutrophils, macrophages, and CD4$^+$ T cells were recovered from IL-1R$^{-/-}$ mouse lungs on day 7 p.i. than from the wild-type mouse lungs (Fig. 4C). By day 14 p.i., total leukocyte, neutrophil, macrophage, and CD4$^+$ T cell numbers had declined in C57BL/6 mice, but these numbers were ~20-, 150-, 50-, and 30-fold higher, respectively, in IL-1R$^{-/-}$ mice. On day 21 p.i., total leukocyte, neutrophil, macrophage, and CD4$^+$ T cell numbers in the lungs of IL-1R$^{-/-}$ mice were ~20-, 90-, 80-, and 60-fold higher, respectively, than those in wild-type mice (Fig. 4C). Bone marrow-derived macrophages or peritoneal macrophages from wild-type or IL-1R$^{-/-}$ mice are similarly efficient in killing *B. pertussis in vitro* (data not shown), suggesting that IL-1R deficiency does not directly impact bactericidal efficiency of phagocytes. Together, these data indicate that IL-1R signaling deficiency results in failure to control *B. pertussis*-induced inflammation, marked by increased cell recruitment and tissue architecture destruction.

Bone marrow-derived dendritic cells from IL-1R$^{-/-}$ mice produce higher levels of proinflammatory cytokines in response to *B. pertussis*. Since *B. pertussis*-inoculated IL-1R$^{-/-}$ mice harbored more bacteria by the first week p.i. (Fig. 2C), we hypothesized that innate immune functions might be improperly regulated in these mice. We first examined the production of proinflammatory cytokines, TNF-α and IL-6, and the anti-inflammatory cytokine IL-10 by bone marrow-derived dendritic cells (BMDC) from C57BL/6 or IL-1R$^{-/-}$ mice after stimulation with live *B. pertussis* at an MOI of 10. Incubation in medium alone resulted in little cytokine production from either type of BMDC (Fig. 5). *B. pertussis*-stimulated BMDC from C57BL/6 mice had produced ~350 pg/ml TNF-α by 2 h, but these levels decreased to ~250 pg/ml by 12 h and plateaued at this level. TNF-α production by *B. pertussis*-stimulated IL-1R$^{-/-}$ mouse BMDC was similar to that of wild-type cells by 2 h poststimulation but increased thereafter and was significantly higher than that of wild-type cells at all later time points (Fig. 5A). IL-6 production by IL-1R-deficient BMDC was somewhat higher than that of wild-type cells, with statistical significance reached only at an earlier time point (2 h) (Fig. 5B). IL-10 production by wild-type BMDC increased to

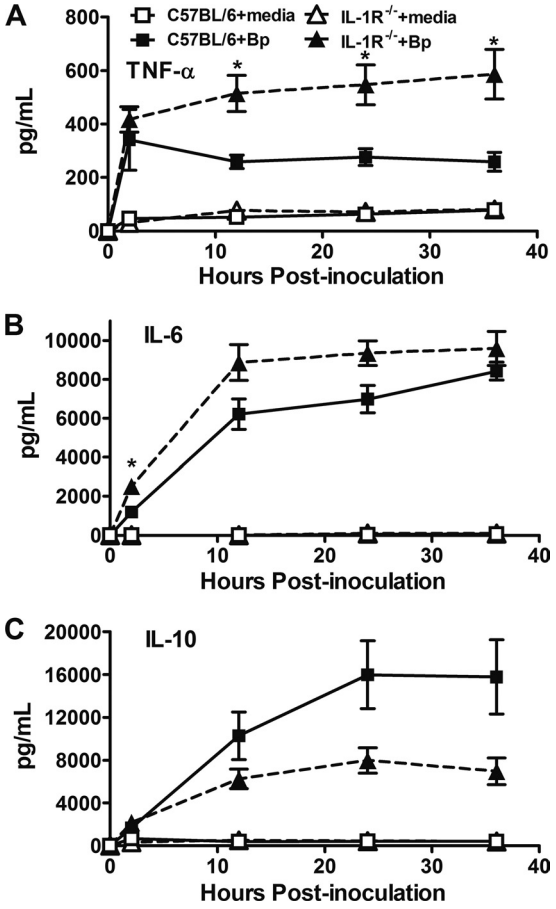

FIG. 5. IL-1R deficiency leads to increased proinflammatory cytokine production by BMDC. BMDC from groups of four C57BL/6 or IL-1R$^{-/-}$ mice were stimulated with medium alone or with medium containing live *B. pertussis* at an MOI of 10. TNF-α, IL-6, or IL-10 concentrations after 0, 2, 12, 24, or 36 h of incubation are expressed as means ± standard errors. *, $P \leq 0.05$, for the wild-type versus IL-1R-deficient cells stimulated with *B. pertussis*. Results are representative of two independent experiments.

~16,000 pg/ml by 24 h after *B. pertussis* stimulation and was still at this level by 36 h poststimulation (Fig. 5C). Interestingly, IL-1R-deficient BMDC produced ~50% less IL-10 than wild-type cells at 12, 24, and 36 h poststimulation although this difference did not reach statistical significance (Fig. 5C). Thus, BMDC lacking IL-1R produced higher levels of the proinflammatory cytokines TNF-α and IL-6 in response to *B. pertussis*, suggesting a substantial contribution of IL-1R signaling to regulation of cytokine responses to *B. pertussis*.

IL-1R signaling is not required for efficient antibody responses against *B. pertussis*. To test if the failure of IL-1R$^{-/-}$ mice to control large numbers of *B. pertussis* is associated with decreased antibody generation, *B. pertussis*-specific antibody titers of serum from C57BL/6 or IL-1R$^{-/-}$ mice collected on days 7, 14, and 21 p.i. were measured. The titers of sera collected on day 7 p.i. from both strains of mice were under the limit of detection (Fig. 6A). On day 14 p.i., Ig titers of sera from wild-type and IL-1R$^{-/-}$ mice were comparable. On day 21 p.i. the *B. pertussis*-specific Ig titers of sera from IL-1R$^{-/-}$ mice were significantly higher than those of sera from wild-type

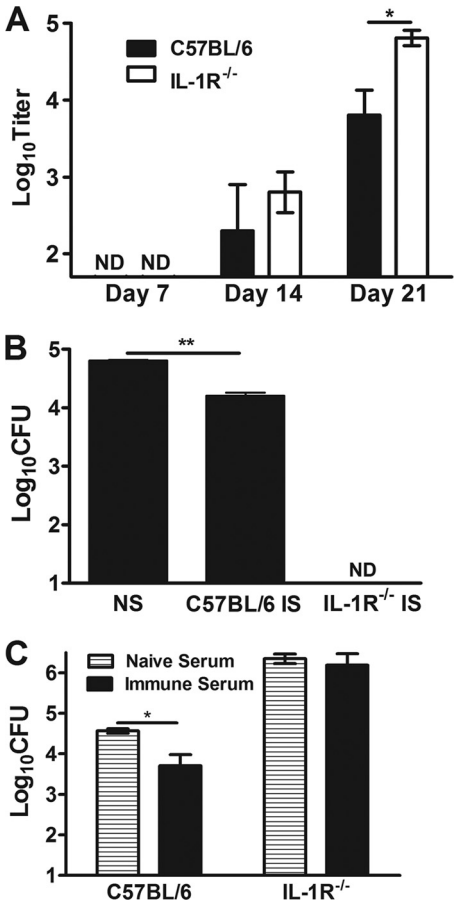

FIG. 6. Antibody responses are not defective in *B. pertussis*-challenged IL-1R$^{-/-}$ mice. (A) *B. pertussis*-specific Ig titers of serum from groups of three to four C57BL/6 or IL-1R$^{-/-}$ mice are expressed as mean $\log_{10}$ titer ± standard error. (B) Naïve serum (NS) or immune serum (IS) collected from C57BL/6 or IL-1R$^{-/-}$ mice on day 21 p.i. was adoptively transferred to groups of four μMT mice. *B. pertussis* numbers in the lungs on day 14 p.i. are expressed as mean $\log_{10}$ CFU ± the standard error. (C) Naïve or convalescent-phase (day 21 p.i.; immune) serum from C57BL/6 mice was adoptively transferred to groups of four C57BL/6 or IL-1R$^{-/-}$ mice. *B. pertussis* numbers in the lungs on day 14 p.i. are expressed as mean $\log_{10}$ CFU ± the standard error. *, $P \leq 0.05$; **, $P \leq 0.01$. ND, not detectable. The limit of detection is indicated by the *y* axis. Results are representative of two or three experiments.

mice. The higher titer of IL-1R$^{-/-}$ mouse serum is likely due to higher bacterial loads later during infection. These data indicate that IL-1R signaling is not required for the generation of *B. pertussis*-specific antibodies. The ratio of *B. pertussis*-specific IgG2a/IgG1 titers of serum collected on day 21 p.i., an indication of T helper 1 (Th1)/Th2 skewing, was higher in serum from IL-1R$^{-/-}$ mice than in that from C57BL/6 mice (data not shown), indicating that T cell responses in IL-1R$^{-/-}$ mice may be more polarized toward Th1 responses.

To test the efficiency of serum antibodies collected from wild-type versus IL-1R$^{-/-}$ mice in antibody-mediated clearance of *B. pertussis in vivo*, B cell-deficient mice (μMT) were adoptively transferred naïve serum or immune serum from C57BL/6 or IL-1R$^{-/-}$ mice collected on day 21 p.i., and mice were sacrificed to enumerate bacterial numbers in the lungs 14 days later. Compared to the naïve-serum-treated mice, C57BL/6 immune serum treatment reduced *B. pertussis* numbers from μMT mouse lungs by 75% (Fig. 6B). Immune serum from IL-1R$^{-/-}$ mice completely cleared *B. pertussis* from the lungs of μMT mice (Fig. 6B), indicating that the higher titer and Th1-skewed immune serum from IL-1R$^{-/-}$ mice are more effective in antibody-mediated clearance *in vivo*.

Although antibodies from IL-1R$^{-/-}$ mice efficiently cleared *B. pertussis* from B cell-deficient mice (Fig. 6B), these antibodies did not protect the IL-1R$^{-/-}$ mice themselves from disease (Fig. 2A, C, and D), suggesting a defect in antibody function in the absence of IL-1R. To determine whether immune serum from C57BL/6 could protect IL-1R$^{-/-}$ mice, naïve or immune serum from wild-type mice, collected on day 21 p.i., was adoptively transferred to C57BL/6 or IL-1R$^{-/-}$ mice, and *B. pertussis* numbers in the lungs were determined on day 14 p.i. Immune serum treatment decreased *B. pertussis* numbers in C57BL/6 mice by more than 85% (Fig. 6C). However, adoptively transferred serum antibodies failed to reduce *B. pertussis* numbers below 10^7 CFU in IL-1R$^{-/-}$ mice, indicating that some immune functions not ameliorated by the addition of antibodies are improperly regulated in IL-1R$^{-/-}$ mice.

Higher Th1 cytokine and lower Th17 cytokine responses in *B. pertussis*-infected IL-1R$^{-/-}$ mice. Since *B. pertussis*-stimulated BMDC from IL-1R$^{-/-}$ mice produced higher levels of TNF-α *in vitro* (Fig. 5A), we examined TNF-α levels at the site of infection in IL-1R$^{-/-}$ mice. TNF-α levels in the lungs of *B. pertussis*-inoculated C57BL/6 mice peaked on day 7 p.i. and declined thereafter, whereas in the lungs of IL-1R$^{-/-}$ mice, TNF-α levels continued to increase throughout the course of infection and were about 7-fold higher than the levels in the wild-type mouse lungs on day 21 p.i. (Fig. 7A). Since Th1 skewing was observed, we also examined IFN-γ, IL-10, and IL-17 concentrations, as representative of Th1, Th2, and Th17 cytokines, in the lungs during *B. pertussis* infection to examine whether increased inflammatory responses in IL-1R$^{-/-}$ mice correlate with altered T cell cytokine production. The IFN-γ concentrations were higher in the lungs of IL-1R$^{-/-}$ mice than in the lungs of C57BL/6 mice by day 7 p.i. and at later time points although the concentrations followed similar patterns, peaking on day 7 p.i. and declining thereafter (Fig. 7B). IL-10 levels in the lungs of both C57BL/6 and IL-1R$^{-/-}$ mice increased modestly, ~2-fold compared to the basal level, on day 3 p.i. and plateaued thereafter (Fig. 7C). A dramatic peak in IL-17 concentrations at ~2,000 pg/ml on day 7 p.i. was observed in wild-type mouse lungs but not in IL-1R$^{-/-}$ mice (Fig. 7D).

To measure systemic cytokine responses, which might not be affected as much as lung cytokine concentrations by differential bacterial loads in the lungs, splenocytes from the same groups of mice described above were stimulated *in vitro* with medium alone or with medium containing heat-killed *B. pertussis*, and TNF-α, IFN-γ, IL-10, and IL-17 concentrations in the cell culture supernatants were determined. Incubation in medium alone resulted in little production of any tested cytokine (Fig. 7E to H, open symbols). Consistent with the findings of lung TNF-α concentrations (Fig. 7A), splenic TNF-α responses from both C57BL/6 and IL-1R$^{-/-}$ mice increased to over 3,000 pg/ml by day 7 p.i. (Fig. 7E). Interestingly, the splenic TNF-α response declined thereafter in C57BL/6 mice but remained

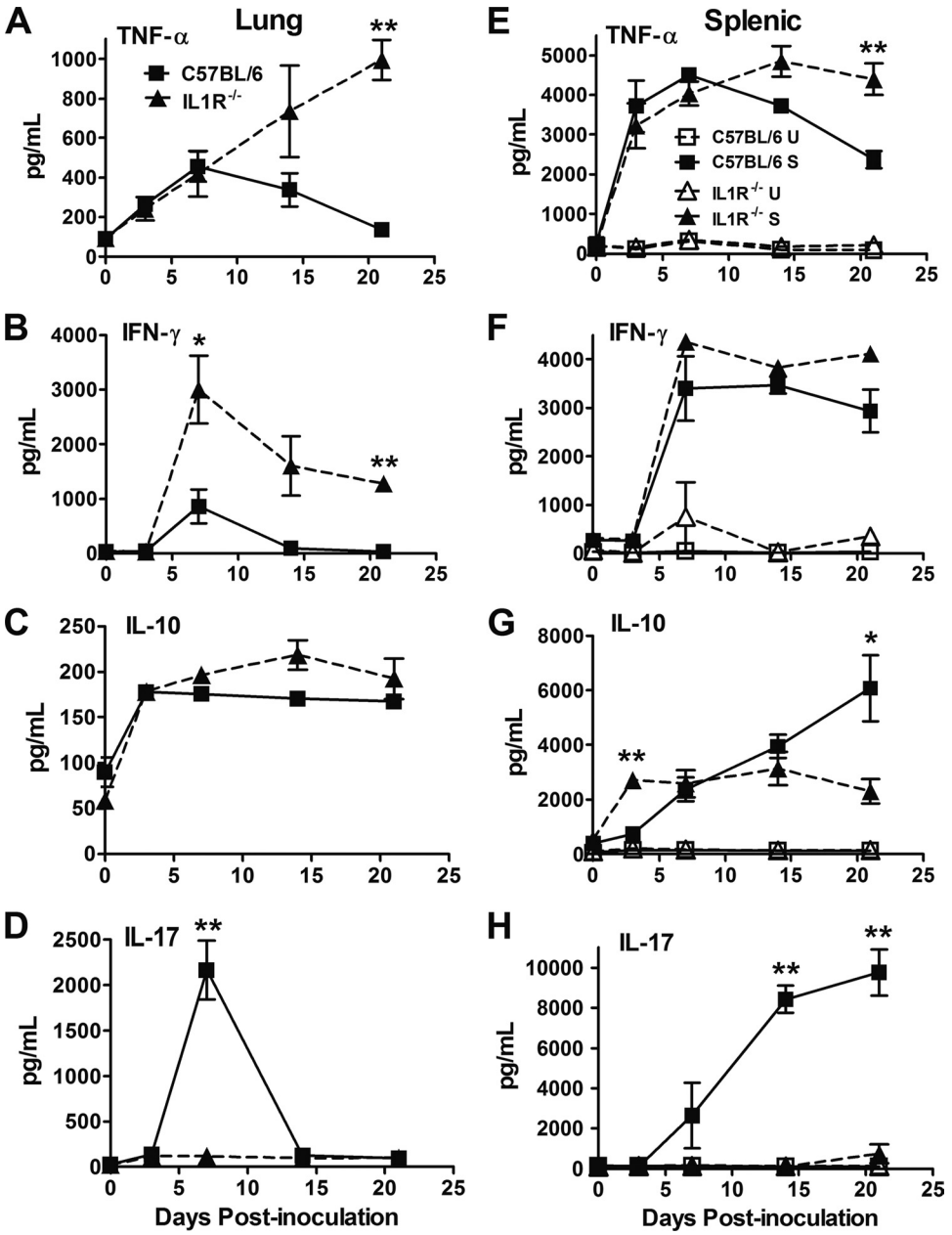

FIG. 7. Increased TNF-α and IFN-γ and decreased IL-17 responses in *B. pertussis*-infected IL-1R$^{-/-}$ mice. Groups of four C57BL/6 or IL-1R$^{-/-}$ mice were inoculated with 5×10^5 CFU of *B. pertussis* and sacrificed on days 0, 3, 7, 14, and 21 p.i. TNF-α, IFN-γ, IL-10, and IL-17 concentrations in the lungs (A to D) or cell culture supernatants of splenocytes (E to H) treated with medium alone (unstimulated [U]) or with medium containing heat-killed *B. pertussis* (stimulated [S]) are expressed as means ± the standard errors. *, $P \leq 0.05$; **, $P \leq 0.01$ (for wild-type versus IL-1R$^{-/-}$ mice [A to D] or wild-type versus IL-1R$^{-/-}$ splenocytes stimulated with *B. pertussis* [E to H]). Results are representative of two independent experiments.

high in the IL-1R$^{-/-}$ mice (Fig. 7E). IFN-γ concentration in the culture supernatants of splenocytes from both types of mice increased to high levels by day 7 p.i. and stayed high thereafter (Fig. 7F). Interestingly, production of IL-10 by C57BL/6 splenocytes kept increasing throughout the time course (Fig. 7G, squares), whereas IL-1R$^{-/-}$ splenocytes failed to increase IL-10 production after an early peak on day 3 p.i. and had produced significantly less IL-10 by day 21 p.i. (Fig. 7G, triangles) than wild-type splenocytes. Splenic IL-17 production by C57BL/6 mice cells was high (~2,000 pg/ml) by day 7 p.i., kept increasing thereafter, and reached ~10,000 pg/ml on day 21 p.i., whereas IL-1R$^{-/-}$ mouse splenocytes barely produced any IL-17 (Fig. 7H). Together, these data indicate that IL-1R$^{-/-}$ mice produce substantially increased amounts of TNF-α and IFN-γ but virtually no IL-17 in response to *B. pertussis* infection.

Intranasal administration of rmIL-17 did not reduce *B. pertussis* numbers in IL-1R$^{-/-}$ mice. Since very little IL-17

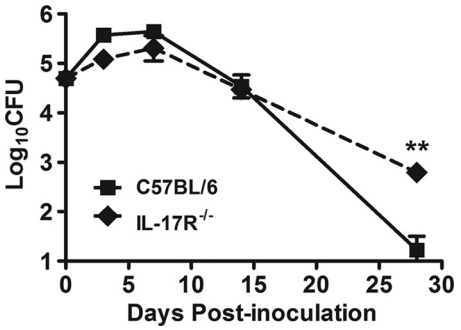

FIG. 8. IL-17R is not essential for controlling *B. pertussis* loads in the lungs during the first weeks p.i. Groups of three to four C57BL/6 or IL-17R$^{-/-}$ mice were inoculated with 10^5 CFU of *B. pertussis.* Bacterial numbers in the lungs on days 0, 3, 7, 14, and 28 p.i. are expressed as mean $\log_{10}$ CFU ± the standard error. **, $P \leq 0.01$, for wild-type versus IL-17R$^{-/-}$ mice. Results are representative of three independent experiments.

response was detected in the lungs of IL-1R$^{-/-}$ mice or was produced by splenocytes from IL-1R$^{-/-}$ mice and since IL-17 has been implicated in the control of *B. pertussis* colonization (3, 20), we hypothesized that the lack of IL-17 responses in IL-1R$^{-/-}$ mice might partially account for the failure of these mice to control *B. pertussis* numbers. To test this, C57BL/6 and IL-1R$^{-/-}$ mice were inoculated with *B. pertussis*, intranasally administrated PBS, or PBS containing rmIL-17 on days 3 and 6 p.i. and sacrificed on day 7 p.i. Although rmIL-17 treatment restored the IL-17 level in the lungs of IL-1R$^{-/-}$ mice to that of wild-type mice, PBS- or rmIL-17-treated IL-1R$^{-/-}$ mice harbored similar numbers of *B. pertussis* bacteria in their lungs, tracheas, and nasal cavities, and the bacterial loads were ~30-fold higher than those in C57BL/6 mice (data not shown), indicating that restoration of IL-17 levels in the lungs on day 7 p.i. is not sufficient to decrease *B. pertussis* burdens in IL-1R$^{-/-}$ mice. To directly test the role of IL-17 signaling during *B. pertussis* infection, we inoculated wild-type and IL-17R$^{-/-}$ mice with 10^5 CFU of *B. pertussis* and determined bacterial numbers. Similar numbers of *B. pertussis* bacteria were recovered from the lungs of C57BL/6 and IL-17R$^{-/-}$ mice on days 3, 7, and 14 p.i. *B. pertussis* was cleared from most wild-type animals by day 28 p.i., but thousands of CFU were recovered from the lungs of IL-17R$^{-/-}$ mice (Fig. 8), suggesting that IL-17R signaling contributes to controlling *B. pertussis* numbers only after 2 weeks p.i. Together, these results suggest that low IL-17 responses in IL-1R$^{-/-}$ mice are not the major cause of their failure to control *B. pertussis* numbers.

IL-1R signaling contributes to overcoming the effects of pertussis toxin. IL-1R is required for the control of *B. pertussis* but not *B. parapertussis* infection (Fig. 2A, B, and C), suggesting that IL-1R may be important in the host response to some *B. pertussis*-specific virulence mechanisms. Since Ptx is not expressed by *B. parapertussis* (4, 46), we hypothesized that IL-1R might contribute to overcoming the effects of Ptx. To test if Ptx is required for *B. pertussis* to cause lethal diseases in IL-1R$^{-/-}$ mice, we challenged C57BL/6 or IL-1R$^{-/-}$ mice with 2×10^6 CFU of *B. pertussis* or the *B. pertussis* Δ*ptx* mutant and monitored survival. C57BL/6 mice showed no signs of disease following challenge with either strain and were euthanized on day 100 p.i. (Fig. 9A). High-dose wild-type *B. pertussis*-challenged IL-1R$^{-/-}$ mice showed signs of severe disease as early as day 5 p.i., and all of them had to be euthanized by day 13 p.i. to avoid suffering. Interestingly, *B. pertussis* Δ*ptx* mutant-challenged IL-1R$^{-/-}$ mice did not show any sign of disease by day 100 p.i. (Fig. 9A), indicating that the LD_{50} of the *B. pertussis* Δ*ptx* strain is at least 2,000-fold higher than that of wild-type *B. pertussis* in IL-1R$^{-/-}$ mice. To determine whether IL-1R$^{-/-}$ mice are able to control the number of *B. pertussis* Δ*ptx* mutant bacteria, we challenged wild-type and IL-1R$^{-/-}$ mice with 5×10^5 CFU of the *B. pertussis* Δ*ptx* strain, and bacterial loads in the lungs were enumerated on days 0, 3, 7, and 14 p.i. On day 3 p.i., the numbers of *B. pertussis* Δ*ptx* mutant bacteria in the lungs of IL-1R$^{-/-}$ mice were ~10-fold higher than in the lungs of C57BL/6 mice (Fig. 9B), suggesting that IL-1R contributes to the early control of the number of *B. pertussis* Δ*ptx* mutant bacteria. Interestingly, IL-1R$^{-/-}$ mice were able to reduce the number of *B. pertussis* Δ*ptx* mutant bacteria at a rate similar to that of wild-type mice during day 3 and 14 p.i. (Fig. 9B), which is contrary to the inefficient bacterial clearance after wild-type *B. pertussis* infection in IL-1R$^{-/-}$ mice (Fig. 2B). Together, these data suggest that IL-1R signaling is required to prevent lethal disease caused by *B. pertussis* only when Ptx is expressed.

To test if Ptx contributes to the induction of IL-1 by *B. pertussis*, BMM from wild-type mice were stimulated with medium alone or with medium containing *B. pertussis* or its isogenic Ptx-deficient derivative, the *B. pertussis* Δ*ptx* strain, at an MOI of 10, and IL-1α or IL-1β concentration in the cell culture supernatant was determined after 48 h of incubation. Compared to the medium-stimulated cells, *B. pertussis*-stimulated macrophages produced both IL-1α and IL-1β (Fig. 9C), which is consistent with previous findings (Fig. 1A). Interestingly, the *B. pertussis* Δ*ptx* strain induced similar levels of IL-1α but significantly lower levels of IL-1β than wild-type *B. pertussis* (Fig. 9C). These data indicate that Ptx contributes to the induction of IL-1β.

Based on our data, we hypothesized that Ptx-induced IL-1β, through IL-1R mediated signaling events, contributes to IL-10 production, which is important for controlling inflammatory responses and preventing overwhelming growth of *B. pertussis*. To test if IL-1R is involved in IL-10 induction by Ptx, we stimulated BMM from C57BL/6 and IL-1R$^{-/-}$ mice with a range of concentrations of active pertussis toxin and measured IL-10 production by these cells after 24 h of incubation. Control cells incubated with medium alone produced little IL-10. Ptx-stimulated wild-type macrophages produced IL-10 in a dose-dependent manner (Fig. 9D). In comparison, IL-10 production by IL-1R-deficient cells was significantly lower when they were stimulated with 0.05 to 5 μg/ml Ptx, suggesting that IL-1R is involved in Ptx-induced IL-10 production.

To determine if IL-1R is essential for the induction of a balanced proinflammatory and anti-inflammatory cytokine response *in vivo*, we performed ELISPOT analyses to quantify IFN-γ- and IL-10-producing cells in the lungs of *B. pertussis*-inoculated wild-type and IL-1R$^{-/-}$ mice on day 3 p.i., a time point when bacterial loads are similar in wild-type and IL-1R$^{-/-}$ mice. Wild-type mice have more IL-10-producing cells than IFN-γ-producing cells (Fig. 9E and F), indicating a dominating regulatory cytokine response in these mice. In comparison, mice lacking IL-1R had significantly fewer IL-10-produc-

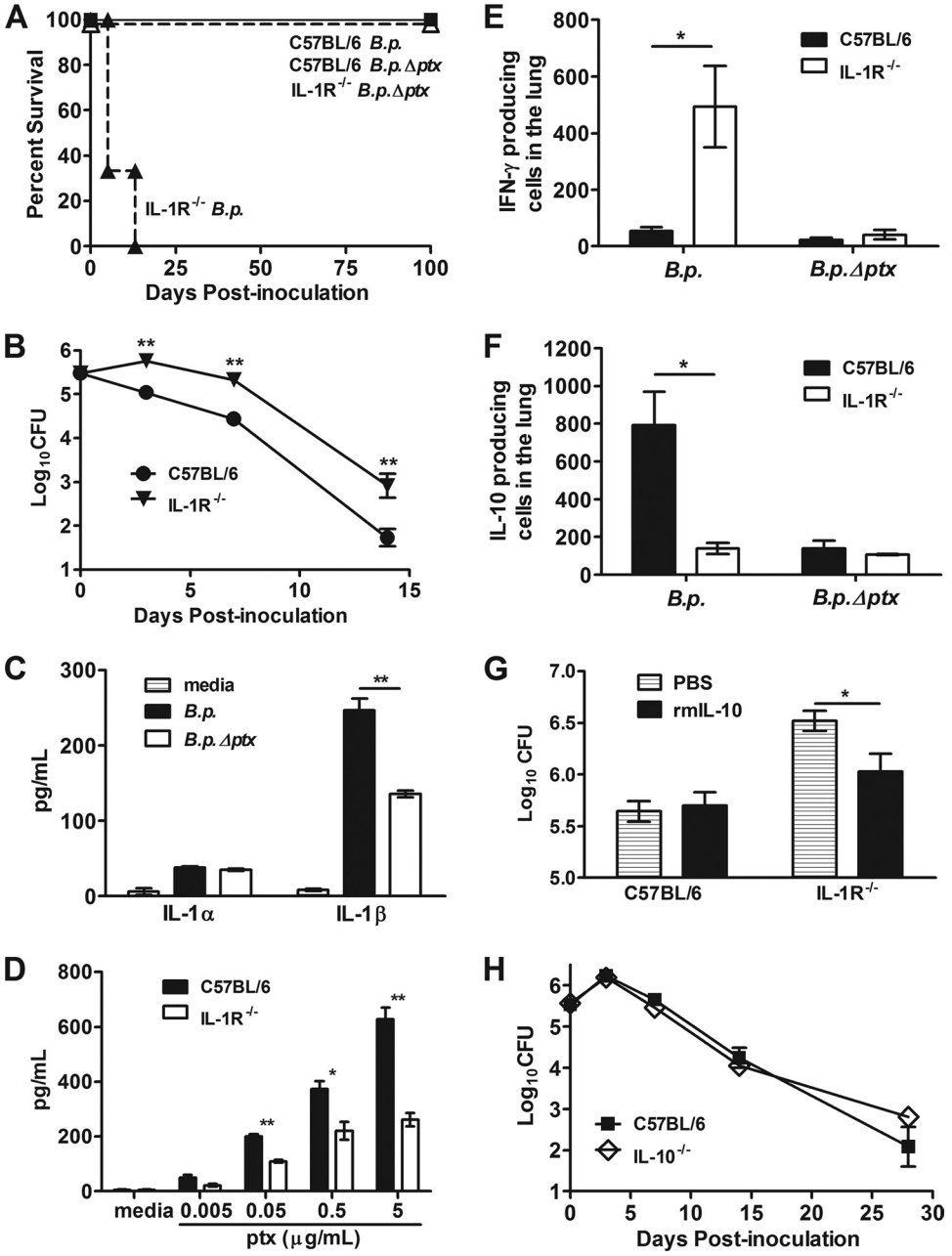

FIG. 9. Pertussis toxin-deficient *B. pertussis* failed to cause lethal infection in IL-1R$^{-/-}$ mice. (A) Groups of 4 C57BL/6 (square) or IL-1R$^{-/-}$ (triangle) mice were inoculated with 2×10^6 CFU of *B. pertussis* (black) or the *B. pertussis* Δ*ptx* mutant (white) and monitored for survival. (B) Groups of four C57BL/6 or IL-1R$^{-/-}$ mice were inoculated with 5×10^5 CFU of the *B. pertussis* Δ*ptx* strain. Bacterial numbers in the lungs on days 0, 3, 7, and 14 p.i. are expressed as mean $\log_{10}$ CFU ± the standard error. (C) C57BL/6 BMM were incubated with medium alone or with medium containing *B. pertussis* (*B.p.*) or its isogenic pertussis toxin-deficient derivative (*B.p.* Δ*ptx*) at an MOI of 10 for 48 h. IL-1α and IL-1β concentrations in the cell culture supernatant are expressed as means ± the standard errors for four similarly treated wells. (D) BMM from groups of three C57BL/6 or IL-1R$^{-/-}$ mice were incubated with medium alone or with medium containing 0.005, 0.05, 0.5, or 5 μg/ml pertussis toxin. IL-10 concentrations by 24 h of incubation are expressed as means ± standard errors for four similarly treated wells. Groups of three to four C57BL/6 or IL-1R$^{-/-}$ mice were inoculated with 5×10^5 CFU of *B. pertussis.* Mice were euthanized on day 3 p.i., and the numbers of IFN-γ-producing (E) or IL-10-producing (F) leukocytes in the lungs were determined by ELISPOT and are expressed as means ± standard errors. (G) Groups of three to four C57BL/6 and IL-1R$^{-/-}$ mice were inoculated with 5×10^5 CFU of *B. pertussis*, intranasally administered PBS or PBS containing 0.1 μg rmIL-10 on days 0 and 3 p.i., and euthanized on day 7 p.i. *B. pertussis* numbers in the lungs are expressed as mean $\log_{10}$ CFU ± the standard error. (H) Groups of four C57BL/6 or IL-10$^{-/-}$ mice were inoculated with 5×10^5 CFU of *B. pertussis.* Bacterial numbers in the lungs on days 0, 3, 7, 14, and 28 p.i. are expressed as mean $\log_{10}$ CFU ± the standard error. Results are representative of two independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$ (between groups indicated by lines or C57BL/6 versus IL-1R$^{-/-}$ mice).

ing cells but more IFN-γ-producing cells. On day 3 after inoculation with the *B. pertussis* Δ*ptx* strain, wild-type and IL-1R$^{-/-}$ mice had similar numbers of IL-10- or IFN-γ-producing cells in their lungs, suggesting that IL-1R is no longer important for the induction of a balanced cytokine response when Ptx is not expressed.

IL-10, a regulatory cytokine, was produced in smaller quantities by lung leukocytes from IL-1R$^{-/-}$ mice in response to *B. pertussis* than by wild-type cells (Fig. 9F). If this contributes to high inflammatory responses and bacterial loads in *B. pertussis*-inoculated IL-1R$^{-/-}$ mice, then giving these mice IL-10 should reduce bacterial loads. To test this, C57BL/6 and IL-1R$^{-/-}$ mice were challenged with *B. pertussis* and intranasally administrated PBS or PBS containing rmIL-10, and bacterial numbers in the lungs were determined on day 7 p.i. Since IL-10 was detected at a relatively high level as early as day 3 p.i. (Fig. 7C), 0.1 μg/ml rmIL-10 was administered at the time of inoculation and on day 3 p.i. The rmIL-10 treatment had little effect on *B. pertussis* numbers in the lungs of wild-type mice but was able to significantly reduce bacterial loads in the lungs of IL-1R$^{-/-}$ mice (Fig. 9G). To further explore the impact of IL-10 deficiency alone on *B. pertussi*s infection, we challenged wild-type and IL-10$^{-/-}$ mice with *B. pertussis*, and bacterial numbers in the lungs were enumerated over time. We observed similar *B. pertussis* loads in the lungs of wild-type and IL-10-deficient mice at all time points (Fig. 9H). Together, these data suggest that the induction of an IL-10 response plays a critical role in controlling *B. pertussis* loads in IL-1R-deficient mice but not in wild-type mice.

DISCUSSION

IL-1R, Toll-like receptor (TLR), and TNF receptor (TNFR) engagement of their distinct ligands results in activation of overlapping signal transduction cascades that share signaling intermediates. The Toll-like receptor/IL-1 domain recruits the myeloid differentiation primary response gene 88 (MyD88) adaptor protein upon activation, leading to further activation of signal transduction events that induce cellular responses known to regulate the innate immune responses. Elucidating the specific and/or redundant functions of these receptor-mediated signaling pathways is critical to understanding the innate immune response. TLR4-deficient mice are colonized to higher numbers after the first week of *B. pertussis* infection and have higher pathology scores and cellular infiltration than wild-type mice (33). TNF-α-deficient mice also have increased *B. pertussis* loads and elevated inflammation in their lungs (74). We showed here that mice lacking IL-1R are more susceptible to *B. pertussis* infection, likely due to uncontrolled inflammation and atypical disseminated infection. Thus, IL-1 is not a dispensable cytokine downstream of TLR4, and its signaling pathway is an essential component of the immune response to *B. pertussis* infection. Blocking IL-1 secretion or IL-1R-mediated signaling has been a proposed treatment of chronic inflammatory diseases, such as rheumatoid arthritis and Crohn's disease (6, 25). Some respiratory infections have been identified in patients using drugs blocking IL-1R signaling (22). In line with these findings, our data (Fig. 2A and B) suggest that therapeutic strategies involving IL-1 blockage may lead to increased susceptibility to *B. pertussis* infection.

IL-1R signaling has been shown to play detrimental or beneficial roles during inflammatory responses on mucosal surfaces (17). In the digestive tract, for example, blockage of components of IL-1R signaling during *Y. enterocolitica* or *S. flexneri* infection controls inflammation (19, 61, 62), whereas IL-1 signaling deficiency leads to increased inflammation caused by *C. rodentium* (40). Similar to the digestive tract, the respiratory tract has a large mucosal area where mounting the proper inflammatory response is critical. However, in comparison, the innate immune signaling pathways important for inducing and resolving inflammatory responses during respiratory pathogen invasions are less defined. Here, we showed that during infection by the respiratory pathogen *B. pertussis*, IL-1R$^{-/-}$ mouse lungs had extensive infiltration of neutrophils and lymphocytes (Fig. 4). In contrast, the wild-type mice showed only mild lymphoid peribronchiolar cuffing. The increased inflammation and lung pathology were associated with increased proinflammatory cytokine responses by BMDC from IL-1R$^{-/-}$ mice (Fig. 5), both at the site of infection and systemically during *B. pertussis* infection (Fig. 7). Thus, consistent with the observation during *C. rodentium* infection, we defined a beneficial role of IL-1R signaling in controlling inflammatory responses by *B. pertussis*.

IL-17-producing Th17 cells are distinct from Th1 or Th2 cells and have been shown to play pathogenic roles in autoimmune diseases (27, 39, 41, 56). Additionally, this cytokine has been shown to contribute to host defenses against bacterial infections, including *Klebsiella pneumoniae* (26), *Bacteroides fragilis* (11), *Streptococcus pneumoniae* (79), and *M. tuberculosis* (36). IL-1β is essential for the differentiation of IL-17-producing human Th cells (1). Mouse models of arthritis and encephalomyelitis have also revealed the involvement of IL-1 in the generation of IL-17-producing cells (54, 69). The role of IL-1R signaling in induction of local and systemic IL-17 production during bacterial infection has not been previously described. Consistent with the work by Dunne et al. (20), we showed here a severe lack of IL-17 induction in *B. pertussis*-challenged IL-1R$^{-/-}$ mice, which might be attributable to the role of IL-1 in the generation of IL-17-producing cells. Alternatively, since Th17 cells are negatively regulated by IFN-γ (13, 27, 56), the high IFN-γ responses observed in *B. pertussis*-challenged IL-1R$^{-/-}$ mice (Fig. 7B and F) might have an inhibitory effect on IL-17 production in these mice. Although we have established that IL-1R signaling is required for the generation of IL-17 responses during microbial infection, restoring the pulmonary IL-17 peak did not decrease *B. pertussis* burdens in the lung. This could be due to the limitations of our delivery method or could indicate that the defect of IL-1R$^{-/-}$ mice in controlling *B. pertussis* infection is not simply their failure to induce IL-17. IL-17 has been shown to promote macrophage killing of *B. pertussis in vitro* (32). Depletion of IL-17 reduces the efficacy of a *B. pertussis* whole-cell vaccine (32) and affects *B. pertussis* numbers in murine lungs (3). These data suggest a role for IL-17 in immune responses to *B. pertussis*. To directly test whether IL-17 is essential to controlling *B. pertussis* infection, we challenged IL-17R$^{-/-}$ mice with *B. pertussis.* These mice efficiently controlled bacterial numbers during the first 2 weeks p.i. and showed elevated bacterial burdens, compared to wild-type mice, only later during infection (Fig. 8). These observations suggest that although IL-17 plays a role in immunity

against *B. pertussis*, the lack of IL-17 responses does not account for the uncontrolled bacterial growth in IL-1R$^{-/-}$ mice during the first 2 weeks p.i. It is intriguing that Dunne et al. showed higher *B. pertussis* numbers in IL-17-deficient mice than in C57BL/6 mice (20). An independent group observed similar *B. pertussis* loads in wild-type and IL-17A-deficient mice (N. H. Carbonetti, personal communication). The underlying reasons for these discrepancies are not fully understood.

The contributions of the IL-1R signaling pathway to the control of *B. pertussis* infection can be multifold, including direct and/or indirect effects. Peritoneal macrophages or BMM lacking IL-1R are as effective as wild-type cells in killing *B. pertussis in vitro* (data not shown). The role of IL-1R signaling might be indirect. IL-1R$^{-/-}$ mice showed uncontrolled bacterial growth and extensive lung pathology with elevated cellular infiltration (Fig. 4), as well as increased proinflammatory cytokine production by dendritic cells (Fig. 5), in the lungs and systemically during *B. pertussis* infection (Fig. 7). In addition, antibody (Fig. 6A and B) and *B. pertussis*-specific IFN-γ responses (Fig. 7B) were increased in these mice, which may reflect their higher bacterial burden. However, on day 3 p.i., when bacterial loads were similar between wild-type and IL-1R-deficient mice, we observed more IFN-γ-producing cells in the lungs of IL-1R-deficient mice (Fig. 9E), suggesting that the high bacterial load might not be the only reason for the elevated responses. Moreover, when equivalent amounts of *B. pertussis* antigens (Ags) were given to IL-1R$^{-/-}$ and wild-type mice following acellular vaccination, vaccinated IL-1R$^{-/-}$ mice still showed increased bacterial numbers compared to vaccinated wild-type mice (Fig. 3), further supporting a role for IL-1R in mounting efficient immune responses to *B. pertussis*. In fact, the enhanced adaptive immune responses in IL-1R$^{-/-}$ mice failed to control *B. pertussis* infection and may reflect the relative lack of regulatory cytokine production. Early in infection IL-1R$^{-/-}$ mice had significantly fewer cells in their lungs producing IL-10, a prototypic regulatory cytokine (Fig. 9F), than wild-type mice. Moreover, IL-1R$^{-/-}$ mice failed to increase Ag-specific systemic IL-10 responses in the later stages of infection (Fig. 7G). The involvement of IL-1R in inducing a *B. pertussis*-specific IL-10 response is in line with the finding that the TLR4-mediated innate IL-10 response inhibits Th1 responses and inflammatory pathology in the lungs during *B. pertussis* infection (33) since IL-1R and TLR4 share downstream signaling events. IL-10 has been shown to dampen inflammatory cytokine responses to *B. pertussis* (49). Dirix et al. recently reported that peripheral blood mononuclear cell-derived IL-10 depresses *B. pertussis*-specific IFN-γ production in vaccinated infants, further supporting the role of IL-10 in controlling *B. pertussis*-specific inflammatory cytokine responses (18). These data suggest that during *B. pertussis* infection, IL-1R contributes to the induction of IL-10, which is important for controlling inflammatory responses.

Since adenylate cyclase toxin, produced by both *B. pertussis* and *B. parapertussis*, is involved in inflammasome activation (20), it is not surprising that both *B. pertussis* and *B. parapertussis* induce IL-1 production *in vitro* and *in vivo* (Fig. 1). However, IL-1R is required for controlling only *B. pertussis* and not *B. parapertussis* numbers (Fig. 2B and C), suggesting that it plays a specific role in the context of virulence mechanisms specific to *B. pertussis* and, therefore, other than that of adenylate cyclase toxin. IL-1R signaling deficiency does not lead to lethal infection by *B. parapertussis* or the *B. pertussis* Δ*ptx* mutant (Fig. 2A and B and 9A), both lacking Ptx expression. This highlights an interesting interaction between IL-1R signaling and a specific bacterial virulence factor, Ptx. M. Nasso, et al. showed that human Ptx-treated dendritic cells promote Th1 cytokine secretion by T cells, which are downregulated by IL-10 (55). Mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K), important signaling components downstream of or interacting with IL-1R (15), are required for this effect (55). Our data suggest a role for IL-1R in the induction of IL-10 by Ptx-treated macrophages (Fig. 9D). Moreover, *B. pertussis*-challenged IL-1R$^{-/-}$ mice had fewer IL-10-producing cells in their lungs early during infection (Fig. 9F). It is likely that Ptx induces IL-1β (Fig. 9C), which activates IL-1R-mediated signaling via MAPK and PI3K, leading to the production of IL-10 and thus the control of inflammatory responses. In the absence of IL-1R, lower levels of IL-10 are produced, resulting in overwhelming inflammation, tissue damage, uncontrolled bacterial growth, and ultimately the death of the animal. This is consistent with our data showing that intranasal administration of IL-10 brought down *B. pertussis* numbers in IL-1R$^{-/-}$ mice (Fig. 9G). The *B. pertussis* strain lacking Ptx induces lower inflammatory responses (3, 55) (Fig. 9E), thus eliminating the requirement for IL-1R-mediated regulation.

ACKNOWLEDGMENTS

We thank the staff of the Pennsylvania State University Flow Cytometry facility (Elaine Kunze, Susan Magargee, and Nicole Bern) for technical assistance.

This work was supported by National Institutes of Health grant GM083113 (to E.T.H.).

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Editor: J. B. Bliska