The Journal of Immunology

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J Immunol 2006; 177:7980-7989; ;
doi: 10.4049/jimmunol.177.11.7980
http://www.jimmunol.org/content/177/11/7980

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

TLR4 Mediates Vaccine-Induced Protective Cellular Immunity to *Bordetella pertussis*: Role of IL-17-Producing T Cells[1]

Sarah C. Higgins,* Andrew G. Jarnicki,* Ed C. Lavelle,† and Kingston H. G. Mills[2]*

Whole cell pertussis vaccines (Pw) induce Th1 responses and protect against *Bordetella pertussis* infection, whereas pertussis acellular vaccines (Pa) induce Ab and Th2-biased responses and also protect against severe disease. In this study, we show that Pw failed to generate protective immunity in TLR4-defective C3H/HeJ mice. In contrast, protection induced with Pa was compromised, but not completely abrogated, in C3H/HeJ mice. Immunization with Pw, but not Pa, induced a population of IL-17-producing T cells (Th-17), as well as Th1 cells. Ag-specific IL-17 and IFN-γ production was significantly lower in Pw-immunized TLR4-defective mice. Furthermore, treatment with neutralizing anti-IL-17 Ab immediately before and after *B. pertussis* challenge significantly reduced the protective efficacy of Pw. Stimulation of dendritic cells (DC) with Pw promoted IL-23, IL-12, IL-1β, and TNF-α production, which was impaired in DC from TLR4-defective mice. *B. pertussis* LPS, which is present in high concentrations in Pw, induced IL-23 production by DC, which enhanced IL-17 secretion by T cells, but the induction of Th-17 cells was also dependent on IL-1. In addition, we identified a new effector function for IL-17, activating macrophage killing of *B. pertussis*, and this bactericidal activity was less efficient in macrophages from TLR4-defective mice. These data provide the first definitive evidence of a role for TLRs in protective immunity induced by a human vaccine. Our findings also demonstrate that activation of innate immune cells through TLR4 helps to direct the induction of Th1 and Th-17 cells, which mediate protective cellular immunity to *B. pertussis*. *The Journal of Immunology,* 2006, 177: 7980–7989.

Immunization with whole cell pertussis vaccines (Pw)[3] is effective at preventing whooping cough in children. However, Pw have been associated with a number of local and systemic reactions, and although still used in developing countries, have been replaced in developed countries by acellular pertussis vaccines (Pa), prepared with highly purified Ags from *Bordetella pertussis* administered with alum as the adjuvant (1, 2). Although the safety profile of Pa is considerably higher, the protective efficacy and the persistence of immunity can be lower than that seen with most Pw (3).

The mechanism of vaccine-induced protective immunity against *B. pertussis* is not fully understood. Studies on immunized children have suggested that high levels of circulating Abs against the *B. pertussis* virulence factors, pertussis toxin (PT), and pertactin may be important for protection (4, 5). However, there is increasing evidence of a role for cell-mediated immunity in protection, especially with Pw (6). Cellular immune responses are more persistent than Abs, and it appears that T cells play a critical role in long-term protection (6, 7). An examination of T cell responses in immunized children and in a mouse model, in which protection correlates with vaccine efficacy in children (8), has shown that Pw induce Th1 cells, whereas Pa generate T cells with a Th2-biased or mixed Th1/Th2 cytokine profile (7, 9, 10). IFN-γ has been shown to play an important role in innate and adaptive immunity to *B. pertussis*; IFN-γ or IFN-γ receptor-defective mice and mice depleted of NK cells, which infiltrate the lung and secrete IFN-γ early in infection, develop disseminating lethal infections (11–13). Furthermore, Pw-immunized IFN-γ receptor-defective mice clear the bacteria more slowly than wild-type mice. In contrast, immunity induced with Pa does not appear to involve IFN-γ and is largely mediated by IgG1 Abs in mice (8).

The induction of protective Th1 responses by previous infection with *B. pertussis* or by immunization with Pw has been associated with IL-12 production by macrophages or dendritic cells (DC), and this has been linked with LPS and active PT present in the live bacteria and residual endotoxin in Pw preparations (14). The addition of exogenous IL-12 augments the protective efficacy of Pa to that of a potent Pw by enhancing the induction of IFN-γ-producing T cells (14). However, despite a reduction in IFN-γ production, the rate of bacterial clearance is not significantly lower following *B. pertussis* challenge of naive or Pw-immunized IL-12p35-defective mice (M. T. Brady and K. H. G. Mills, unpublished observation), suggesting a redundant role for IL-12 in protective adaptive immunity to *B. pertussis*.

It has been reported recently that IL-17 can act as important effector T cell cytokine in inflammatory responses and IL-17-producing T cells (Th-17) have been shown to play a pathogenic role in autoimmune diseases (15–17). IL-17 production from $CD4^+$ and $CD8^+$ T cells stimulates inflammatory cytokine and chemokine production and neutrophil recruitment (18). There is more limited evidence of a role for IL-17 in protection against bacterial infections, including *Klebsiella pneumoniae* (19) and *Mycobacteria tuberculosis* (20), and its role in vaccine-induced immunity has not been addressed. We have reported previously that TLR4 is required for clearance of primary infections with *B. pertussis* (21). In this study, we examined the role of TLR4 in adaptive immunity to *B. pertussis* induced by vaccination. Our findings demonstrate

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Received for publication February 27, 2006. Accepted for publication September 13, 2006.

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[1] This work was supported by a Science Foundation Ireland Principal Investigator Award (00/PI.I/B045; to K.H.G.M.).

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[3] Abbreviations used in this paper: Pw, whole cell pertussis vaccine; DC, dendritic cell; FHA, filamentous hemagglutinin; IL-1ra, IL-1R antagonist; LAL, *Limulus* amebocyte lysate; Pa, acellular pertussis vaccine; PT, pertussis toxin; Th-17, IL-17-producing T cell.

0022-1767/06/$02.00

that TLR4 plays a critical role in protective cellular immunity elicited by Pw, and this involves IL-23 and IL-1-driven IL-17, which enhances the bactericidal activity of macrophages.

Materials and Methods

Mouse immunizations

Specific pathogen-free C3H/HeN, C3H/HeJ, and BALB/c mice were obtained from Harlan. C57BL/6 and IL-1 type I receptor-defective (IL-1R1$^{-/-}$) mice were bred in house from established colonies and housed under specific pathogen-free conditions. Mice were maintained according to the regulations and guidelines of the Irish Department of Health. The Pa used in this study was a two-component vaccine (JNIH-3 from National Institute for Biological Standards and Control (NIBSC)) comprised of detoxified PT and filamentous hemagglutinin (FHA), which was assigned a potency of 40 IU by the manufacturer (using a Japanese-modified Kendrick test). The Pw used in this study (the third international standard preparation, 66/303 from NIBSC) was a thiomersal killed *B. pertussis* vaccine and was assigned a potency of 46 IU per ampoule. Mice were immunized i.p. twice (wk 0 and 4) with 0.2 human dose of Pa or Pw, and were challenged with *B. pertussis* by aerosol inoculation or sacrificed 2 wk after second immunization. In other experiments, mice were immunized once with Pa or Pw into the footpad, and lymph node and spleen cells were recovered 7 days later.

B. pertussis *respiratory challenge*

Respiratory infection of mice was performed by aerosol challenge, as previously described (8). The course of *B. pertussis* infection was followed by performing CFU counts on lungs from groups of four to five mice at intervals after challenge. The lungs were aseptically removed and homogenized in 1 ml of sterile physiological saline with 1% casein on ice. Undiluted and serially diluted homogenate (100 μl) from individual lungs was spotted in triplicate onto Bordet-Gengou agar plates, and the number of CFU was calculated after 5 days incubation at 37°C. The limit of detection was ~0.6 $\log_{10}$ CFU per lung.

T cell cytokine production

Spleen mononuclear cells (2×10^6/ml) or lymph node cells (1×10^6/ml) were cultured at 37°C and 5% CO_2 with heat-killed *B. pertussis*, formalin-treated *B. pertussis* sonicate, or purified FHA. Stimulation with PMA (250 ng/ml; Sigma-Aldrich) and anti-mouse CD3 (1 μg/ml; BD Biosciences) or medium only was used as positive and negative controls, respectively. In certain experiments, rIL-23 (10 ng/ml) was added to the cultures with or without Ag. Supernatants were removed after 72 h, and IL-5, IL-10, IL-17, and IFN-γ concentrations were determined by two-site ELISA.

Influence of conditioned medium from B. pertussis *LPS-stimulated DC on IL-17 production*

DC were generated by culturing bone marrow cells for 10 days in medium with 40 ng/ml GM-CSF from a GM-CSF-expressing cell line, as described previously (22). DC were stimulated with 10, 100, or 1000 ng/ml *B. pertussis* LPS (reference reagent from NIBSC; this reagent did not induce cytokine production by DC or macrophages from TLR4-defective mice, suggesting that it is not contaminated with non-TLR4 agonists) or with medium only as a control, and supernatants were recovered after 24 h. $CD4^+$ T cells were purified using mouse $CD4^+$ T cell enrichment columns (R&D Systems). Spleen cells or purified $CD4^+$ T cells (2×10^5/ml) and APC (irradiated naive spleen cells, 2×10^6/ml) were stimulated with or without Ag (*B. pertussis* formalin-treated sonicate; 2 μg/ml) in the presence or absence of supernatants from LPS- or medium-stimulated DC. rIL-23 (10 ng/ml), IL-12 (10 ng/ml), or 10 μg/ml neutralizing Abs to IL-23 (eBioscience), IFN-γ (BD Biosciences) IL-12 (R&D Systems), or IL-1α and IL-1β (R&D Systems), or 1 μg/ml IL-1R antagonist (IL-1ra, rat; a gift from S. Poole, NIBSC, Potters Bar, U.K.) were also added to certain cultures. The anti-IL-12 Ab was a polyclonal Ab, raised against IL-12, and neutralizes IL-12 (R&D Systems; Ab AF-419-NA specification sheet). We found that it neutralizes IL-12, but not IL-23, presumably by virtue of its binding to IL-12p35 (our unpublished observations). Alternatively, T cells were stimulated with medium only or 10 ng/ml IL-23, IL-1α or IL-1β, or IL-23 in the presence of IL-1α or IL-1β. Recombinant cytokines were purchased from R&D Systems and eBioscience. Supernatants were removed after 72 h, and concentrations of IL-17 were determined by two-site ELISA.

B. pertussis-*specific Abs*

Serum Ab responses to *B. pertussis* were quantified by ELISA using plate-bound *B. pertussis* sonicate (5 μg/ml). Bound Abs were detected using biotin-conjugated anti-mouse IgG, IgG1, or IgG2a Abs (Caltag Laboratories), and peroxidase-conjugated streptavidin (BD Pharmingen). Ab levels are expressed as the mean endpoint titer (±SE), determined by extrapolation of the linear part of the titration curve to 2 SE above the background value obtained with nonimmune mouse serum.

Anti-IL-17 treatment in vivo

Two groups of five BALB/c mice were immunized with Pw and injected i.p. with 100 μg/mouse of either anti-IL-17 Ab (MAB421; R&D Systems) or an isotype control Ab (R&D Systems) 2 h before and 3 and 7 days after *B. pertussis* aerosol challenge. Nonimmunized mice challenged with *B. pertussis* acted as a further control. Mice were sacrificed on day 10, and lungs were homogenized in 1 ml of 1% casein solution. CFU counts were performed, as described above. The remaining lung homogenates were frozen at −20°C and used later to determine the concentrations of IL-6 (BD Pharmingen) and MIP-2 (R&D Systems) by two-site ELISA.

Cytokine secretion by DC and macrophages

Bone marrow-derived immature DC (10^6/ml) were cultured at 37°C for 24 h with Pw or Pa (0.008–0.5 IU/ml). In some experiments, DC were also stimulated with *B. pertussis* LPS (10 ng/ml), live *B. pertussis* (10^7/ml), or 5 μg/ml phosphorothioate-stabilized oligodeoxynucleotide-containing CpG motifs (5′-tccatgacgttcctgatgct-3′; synthesized by Sigma-Genosys). Supernatants were removed after 18 h, and the concentrations of IL-1β, TNF-α, IL-10, IL-23, IL-12p70, and IL-12p40 were quantified by two-site ELISA (BD Biosciences, R&D Systems, or eBioscience).

Flow cytometric analysis

Mononuclear cells were purified from the lungs of naive and *B. pertussis*-infected mice by mechanical disruption of lung tissue (22). Lung mononuclear cells were incubated with goat anti-mouse IL-17R (S-18; Santa Cruz Biotechnology), followed by donkey anti-goat IgG FITC (Santa Cruz Biotechnology) and PE-Cy5.5-conjugated anti-F4/80 (Caltag Laboratories). Incubation with the second Ab only or isotype-matched control Abs served as controls. Fc receptor binding of Abs was minimized by preincubation with rat anti-mouse CD16/CD32 (FcRγIII/II) mAb (mouse Fc block; BD Biosciences). GM-CSF-amplified bone marrow-derived DC or adherent peritoneal lavage cells were incubated with Abs specific for CD11c (Caltag Laboratories) or isotype control Abs. Immunofluorescence analyzed was performed on a FACSCalibur (BD Biosciences), with CellQuest software.

Macrophage bactericidal assay

Peritoneal cells were recovered from the lavage fluid of mice, and macrophages were purified by adherence to plastic. This yielded cell populations that were 78–86% $F4/80^+$ and 10–16% $B220^+$. The alveolar macrophage cell line, MHS, was obtained from the American Type Culture Collection. Macrophages (2×10^6 cells/ml; triplicate wells) were stimulated with rIL-17, IFN-γ, or TNF-α (1–50 ng/ml; R&D Systems; LPS content of cytokines was <0.01 EU/ml) for 2 h and then infected with *B. pertussis* (10 bacteria to 1 macrophage). After a further 2-h incubation at 37°C, the supernatants were removed, and ice-cold 1% casein in water was added to lyse the macrophages. Neat and 1/10 serial dilutions of the lysed macrophages (100 μl) from each well were plated onto Bordet-Gengou blood agar plates in duplicate. The number of CFU was calculated after 5 days' incubation at 37°C.

LPS assay

Endotoxin content of vaccine preparations was tested using the chromogenic *Limulus* amebocyte lysate (LAL) assay (Associates of Cape Cod) and the pyrogene recombinant factor C endotoxin assay (Cambrex), according to the manufacturer's instructions. Vaccines were sonicated before testing and tested over a wide range of dilutions.

Statistical analyses

One-way ANOVA was used to test for statistical significance of differences between more than two experimental groups. Student's *t* test was used for analysis when two groups were compared.

Results

A Pw fails to protect against B. pertussis *in TLR4-defective mice*

We examined the role of TLR4 in vaccine-induced protective immunity to *B. pertussis* by immunization of C3H/HeN and TLR4-defective C3H/HeJ mice with Pw and Pa. Mice were immunized

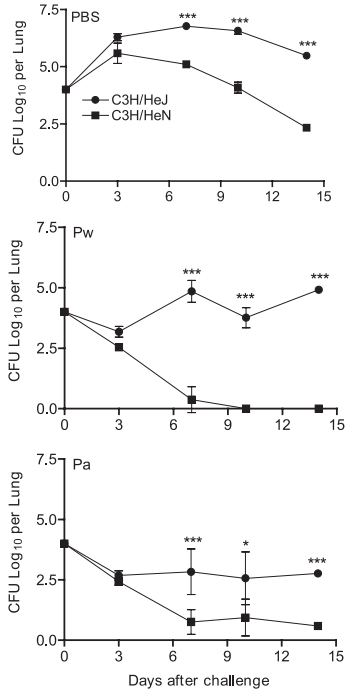

FIGURE 1. Reduced efficacy of Pw and Pa against *B. pertussis* infection in TLR4-defective mice. C3H/HeN and C3H/HeJ mice were immunized i.p. at 0 and 4 wk with PBS, Pw, or Pa, and challenged by aerosol exposure to *B. pertussis* 2 wk later. Mice were sacrificed 0, 3, 7, 10, and 14 days after challenge, and *B. pertussis* CFU counts were determined on individual lung homogenates. Results are expressed as mean (±SD) CFU for five mice per group at each time point and are representative of three experiments. *, $p < 0.05$; ***, $p < 0.001$, C3H/HeN vs C3H/HeJ.

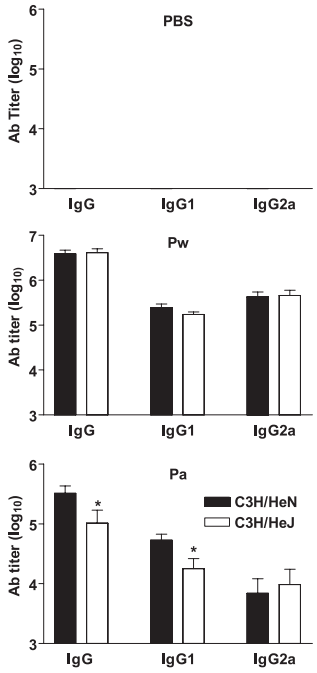

FIGURE 2. *B. pertussis*-specific Ab responses in C3H/HeJ and C3H/HeN mice immunized with Pw or Pa. C3H/HeN and C3H/HeJ mice were immunized, as described in the legend to Fig. 1. Two weeks after the second immunization and before challenge, serum was recovered and *B. pertussis*-specific IgG, IgG1, and IgG2a Ab titers were determined by ELISA. Results are mean endpoint titers for eight mice per group and are representative of two experiments. *, $p < 0.05$; C3H/HeN vs C3H/HeJ.

twice and challenged by exposure to a *B. pertussis* aerosol 2 wk after the second immunization. The bacterial load remained high in nonimmunized mice for at least 10 days after challenge, and the CFU counts were significantly greater in the C3H/HeJ when compared with C3H/HeN mice (Fig. 1). Immunization of C3H/HeN mice with Pw conferred a high level of protection against *B. pertussis* infection. A dramatic decline in bacterial numbers was observed in C3H/HeN mice 7 days after challenge, and the *B. pertussis* infection was completely cleared by day 10 (Fig. 1). In contrast, the Pw elicited very little protection in the TLR4-defective mice with only a $\log_{10}$ reduction in the CFU counts observed in the lungs of Pw-immunized TLR4-defective C3H/HeJ mice when compared with PBS-immunized C3H/HeJ mice 3–14 days after *B. pertussis* challenge. Bacterial counts were significantly ($p < 0.001$) higher in the Pw-immunized C3H/HeJ when compared with C3H/HeN mice 7, 10, and 14 days after *B. pertussis* challenge. Immunization with Pa conferred protection in both C3H/HeN and C3H/HeJ mice, with a decline in CFU counts in the lungs 3 days after challenge (Fig. 1). However, the bacterial burden in lungs of Pa-immunized TLR4-defective C3H/HeJ mice was significantly higher ($p < 0.05$–0.001) than in the C3H/HeN mice 7, 10, and 14 days after challenge with *B. pertussis*. Our findings demonstrate that TLR4 is essential for protective immunity elicited by Pw, but plays a less significant role in protection induced by Pa.

Defective Ag-specific cytokine production in immunized TLR4-defective mice

To assess the influence of TLR4 on adaptive immunity induced with Pw or Pa, *B. pertussis*-specific Ab and T cell responses were examined in immunized mice before challenge with *B. pertussis*. Immunization with Pw or Pa elicited high titers of serum IgG specific for *B. pertussis*, which was higher in mice immunized with Pw (Fig. 2). When responses were tested against PT and FHA, the Ags present in the Pa, responses were significantly higher in the mice immunized with Pa (data not shown). The IgG1:IgG2a ratio was higher in mice immunized with Pa when compared with Pw. There was no significant difference in the titers of *B. pertussis*-specific IgG or IgG subclass between C3H/HeN and C3H/HeJ mice immunized with Pw (Fig. 2). In contrast, the *B. pertussis*-specific IgG and IgG1 titers were significantly ($p < 0.05$) reduced in the Pa-immunized TLR4-defective mice.

T cell responses were assessed by testing Ag-induced cytokine production by spleen cells ex vivo. Immunization of C3H/HeN mice with Pw induced *B. pertussis*-specific T cells that secreted IFN-γ and low concentrations of IL-4 and IL-5, whereas spleen cells from mice immunized with Pa secreted IL-5 and low concentrations of IFN-γ (Fig. 3), which is consistent with previous demonstrations in BALB/c and C57BL/6 mice that Pw and Pa induce Th1- and Th2-biased responses, respectively (9). However, the present study demonstrates that immunization with Pw also induces *B. pertussis*-specific Th-17 cells. IL-17 and IFN-γ production induced with Pw and IL-5 production induced with Pa were significantly impaired in C3H/HeJ mice. Spleen cells from mice immunized with Pw or Pa, but not control nonimmunized mice, proliferated in response to *B. pertussis* Ag, and significantly lower responses were observed in C3H/HeJ when compared with C3H/HeN mice (data not shown).

To confirm our novel finding that Pw induces Th-17 cells, we examined the influence of IL-23, which is known to promote expansion of Th-17 cells, on the Ag-specific responses of T cells from immunized mice. Spleen cells from mice immunized with Pw, Pa, or PBS were restimulated with heat-killed *B. pertussis*, purified FHA, or medium only in the presence or absence of IL-23.

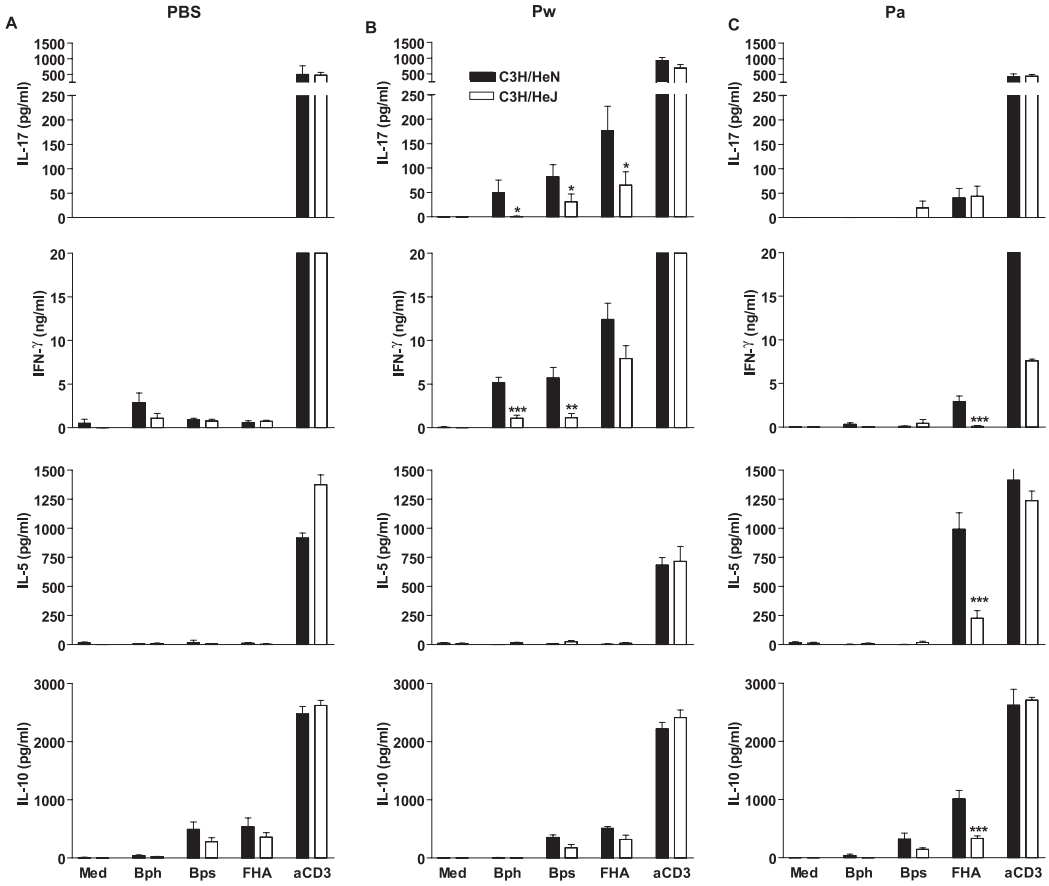

FIGURE 3. Attenuated *B. pertussis*-specific cytokine production in TLR4-defective mice. C3H/HeN and C3H/HeJ mice were immunized i.p. at 0 and 4 wk with PBS (*A*) or 0.2 human doses of Pw (*B*) or Pa (*C*). Two weeks after the second immunization, spleen cells were isolated and restimulated in vitro with heat-inactivated *B. pertussis* (Bph; 1 μg/ml), formalin-treated *B. pertussis* sonicate (Bps; 1 μg/ml), FHA (2 μg/ml), anti-CD3 (0.5 μg/ml) and PMA (25 ng/ml) or medium (Med) only. Supernatants were collected after 72 h, and concentrations of IL-17, IFN-γ, IL-5, and IL-10 were determined by two-site ELISA. *, $p < 0.05$; ***, $p < 0.001$; C3H/HeN vs C3H/HeJ. Results are representative of three experiments.

Spleen cells from mice immunized with Pw secreted IL-17 and IFN-γ production following in vitro restimulation with *B. pertussis* and FHA. IL-17 production was considerably augmented by the addition of exogenous IL-23, whereas IFN-γ production was marginally reduced (Fig. 4). Spleen cells from naive mice or mice immunized with Pa only secreted IL-17 when IL-23 was added to the cultures, whereas spleen cells from Pw-immunized mice secreted IL-17 in the presence or absence of added IL-23. These data demonstrate that Pw induces Ag-specific Th-17 and that in vitro IL-17 production is enhanced by IL-23.

Anti-IL-17 reduces the protective efficacy of Pw

We have shown that the failure of Pw to protect against *B. pertussis* infection in TLR4-defective mice is associated with significantly reduced *B. pertussis*-specific IL-17 production, suggesting that IL-17 may contribute to protection in mice with functional TLR4. Therefore, we examined the role of IL-17 using anti-IL-17-neutralizing Abs in vivo. Two groups of mice were immunized with Pw, and one group was treated with anti-IL-17 2 h before and 3 and 7 days after *B. pertussis* respiratory infection, and a second group was treated with a control Ab. Nonimmunized mice challenged with *B. pertussis* acted as a further control. The nonimmunized mice still had ~4 $\log_{10}$ CFU in the lungs 10 days after challenge, whereas the Pw-immunized control mice had completely cleared the infection from their lungs (Fig. 5). In contrast, bacteria were still detectable in four of five mice treated with anti-IL-17, and the mean CFU were significantly ($p < 0.01$) greater than in mice treated with the control Ab (Fig. 5). An examination of cytokine concentrations in the lung homogenates demonstrated enhancement of IL-6 and MIP-2 following challenge of Pw-immunized mice, and this was partially reversed by treatment with anti-IL-17 (IL-6: naive 30 ± 3 pg/ml, Pw immunized 10 days after challenge 109 ± 26 pg/ml; Pw immunized and anti-IL-17-treated 10 days after challenge 67 ± 9 pg/ml; MIP-2: naive 41 ± 3 pg/ml, Pw immunized 10 days after challenge 161 ± 43 pg/ml; Pw immunized and anti-IL-17 treated 10 days after challenge 127 ± 5 pg/ml). Anti-IL-17 treatment did not fully reverse the protective effects of Pw; the CFU counts were significantly higher in the nonimmunized control mice when compared with the anti-IL-17 Ab-treated Pw-immunized mice (Fig. 5), suggesting that other components of the immune response also contributed to protection. This is consistent with our previous demonstration of a role for IFN-γ (8), and with the data in Fig. 3 that IFN-γ is also significantly reduced in the Pw-immunized TLR4-defective mice.

Pw induces IL-23 and other inflammatory cytokines from DC through TLR4

Having demonstrated defective innate and adaptive immune responses induced by Pa and Pw in TLR4-defective mice in vivo, and because DC play an important role in priming T cell responses, we tested the ability of Pw and Pa to activate innate cytokines from DC in vitro. We used immature myeloid DC, expanded from bone marrow in the presence of GM-CSF using a standard protocol that generated a population that was 70–75% $CD11c^+$. We also used

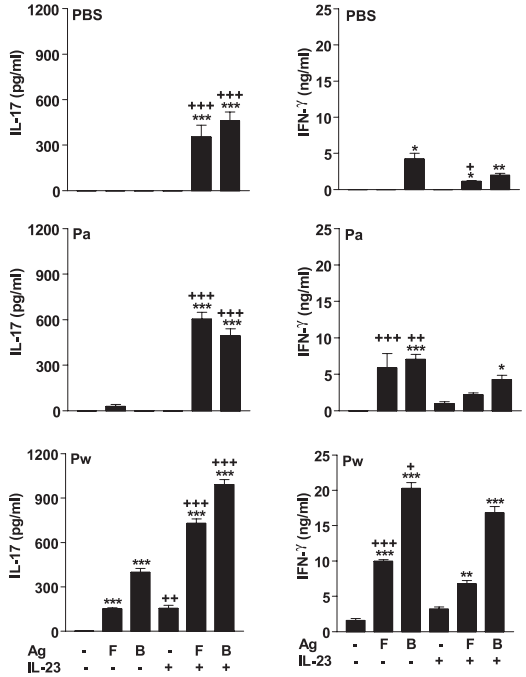

FIGURE 4. Pw induces Th-17, which is enhanced by IL-23. Mice were immunized s.c. in the footpad with Pw or Pa (0.2 human dose) or with PBS only. After 7 days, spleen cells were stimulated with FHA (F; 2 μg/ml), heat-inactivated *B. pertussis* (B; 10^5/ml), or medium with or without IL-23 (10 ng/ml). Supernatants were collected after 72 h, and concentrations of IL-17 and IFN-γ were determined by two-site ELISA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs medium control. +, $p < 0.05$; ++, $p < 0.01$; +++, $p < 0.001$, with vs without IL-23.

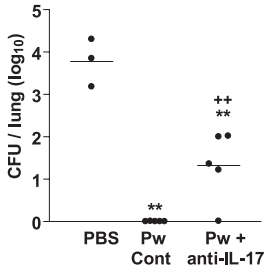

FIGURE 5. Neutralization of IL-17 in vivo reduces the protective efficacy of Pw. BALB/c mice were immunized with PBS or Pw and treated with anti-IL-17 or a control Ab 2 h before and 3 and 7 days after aerosol challenge with *B. pertussis*. Mice were sacrificed 10 days after challenge, and *B. pertussis* CFU counts were determined on individual lung homogenates. Results are expressed as CFU counts for individual mice with mean values indicated by horizontal bars. **, $p < 0.01$, vs PBS; ++, $p < 0.01$, Pw and anti-IL-17 vs Pw and control Ab.

highly purified DCs (>95% $CD11c^+$), enriched from day 10 bone marrow cultures on CD11c MACS columns, with essentially identical results (data not shown). Pw at concentrations as low as 0.008 IU/ml induced high concentrations of TNF-α and IL-12p40 and lower concentrations of IL-1β, IL-23, and IL-12p70 (Fig. 6). In contrast, the anti-inflammatory cytokine, IL-10, was only detected at higher concentrations of Pw. We found that IL-10 inhibits LPS-induced IL-12p70 and IL-23 (our unpublished observations), and this may explain the reduction in these proinflammatory cytokines with increasing concentrations of Pw. Pw-induced production of IL-1β, TNF-α, IL-12p40, IL-23, and IL-10 was significantly lower ($p < 0.01$–0.001) in DC from C3H/HeJ when compared with C3H/HeN mice. The higher concentrations of Pa (0.125–0.5 IU/ml) stimulated IL-1β, TNF-α, and IL-12p40 production by DC from C3H/HeN mice (Fig. 6), and this was significantly ($p < 0.001$) lower in DC from TLR4-defective C3H/HeJ mice. Pa failed to induce IL-12p70, IL-23, or IL-10 from DC at all concentrations tested.

The reduction in cytokine production by Pa- and Pw-stimulated DC from TLR4-defective mice suggested that both vaccines contained LPS or other TLR4 ligands. Therefore, we assessed the LPS content of Pa and Pw used in these studies using a chromogenic LAL assay and the Cambrex pyrogene recombinant factor C endotoxin assay; the latter is more sensitive and less susceptible to interference. The LPS concentration in the Pw preparation used was 133,450 EU/ml by the chromogenic LAL assay and 225,680 EU/ml by the pyrogene assay, and the LPS concentration in the Pa preparation was 6.96 EU/ml by the chromogenic LAL assay and 9.29 EU/ml by the pyrogene assay.

It has already been reported that IL-23 production in response to *K. pneumoniae* is TLR4 dependent (23). In this study, we examined the role of TLR4 in *B. pertussis* and *B. pertussis* LPS-induced IL-23 and IL-12p70 production and used the TLR9 agonist, CpG, as a positive control. High concentrations of IL-23 were detected in DC supernatants from C3H/HeN mice following stimulation with *B. pertussis*, *B. pertussis* LPS, or CpG (Fig. 7). Significantly lower concentrations of IL-23 were detected in DC from C3H/HeJ mice in response to live bacteria or *B. pertussis* LPS, whereas CpG induced similar concentrations to those observed in the DC from C3H/HeJ mice. IL-12p70 was also induced by live *B. pertussis* and *B. pertussis* LPS in DC from C3H/HeN, but was almost undetectable in DC from C3H/HeJ mice. *B. pertussis* and CpG induced comparable concentrations of IL-23, but *B. pertussis* and *B. pertussis* LPS induced considerable lower concentrations of IL-12p70 relative to that induced with CpG. These findings demonstrate that *B. pertussis* and Pw induce IL-12 and IL-23 production, largely due to LPS-activated signaling through TLR4. This may explain the induction of Th1 and Th-17 cells with Pw in vivo and the reduction in Ag-specific IFN-γ and IL-17 observed in TLR4-defective mice immunized with Pw.

IL-23 and IL-1 produced by B. pertussis *LPS-stimulated DC enhance IL-17 production*

It is well established that IL-12 promotes the induction of Th1 cells, and we have already demonstrated that IL-12 promotes *B. pertussis*-specific IFN-γ production in vivo and in vitro (14). However, evidence is emerging that IL-17 is produced by a distinct population of T cells under the influence of IL-23 (16, 17) or TGF-β and IL-6 (24, 25). Therefore, we examined the role of *B. pertussis*-stimulated IL-23 and other innate cytokines in the expansion of Th-17 from naive or immunized mice. Spleen cells from naive or Pw-immunized mice were stimulated in vitro with supernatants (10%) from control or LPS-stimulated DC in the presence or absence of rIL-23 or IL-12 or neutralizing Abs to IL-23, IL-12, and IFN-γ. Stimulation of spleen cells from naive mice with supernatants from LPS-stimulated, but not medium-stimulated, DC induced IL-17 production, which was completely abrogated by anti-IL-23, but was not affected by anti-IL-12 or anti-IFN-γ (Fig. 8*A*). In contrast, IFN-γ production was inhibited by anti-IL-12 and enhanced by addition of rIL-12, but not by addition of rIL-23 (data not shown). Stimulation of naive spleen cells with rIL-23 induced low concentrations of IL-17, but costimulation with supernatants from LPS-stimulated DC (which contained 400 pg/ml IL-23 as well as other inflammatory cytokines) induced high concentrations of IL-17 (Fig. 8*A*), suggesting that soluble mediator(s) produced by LPS-stimulated DC cooperated with

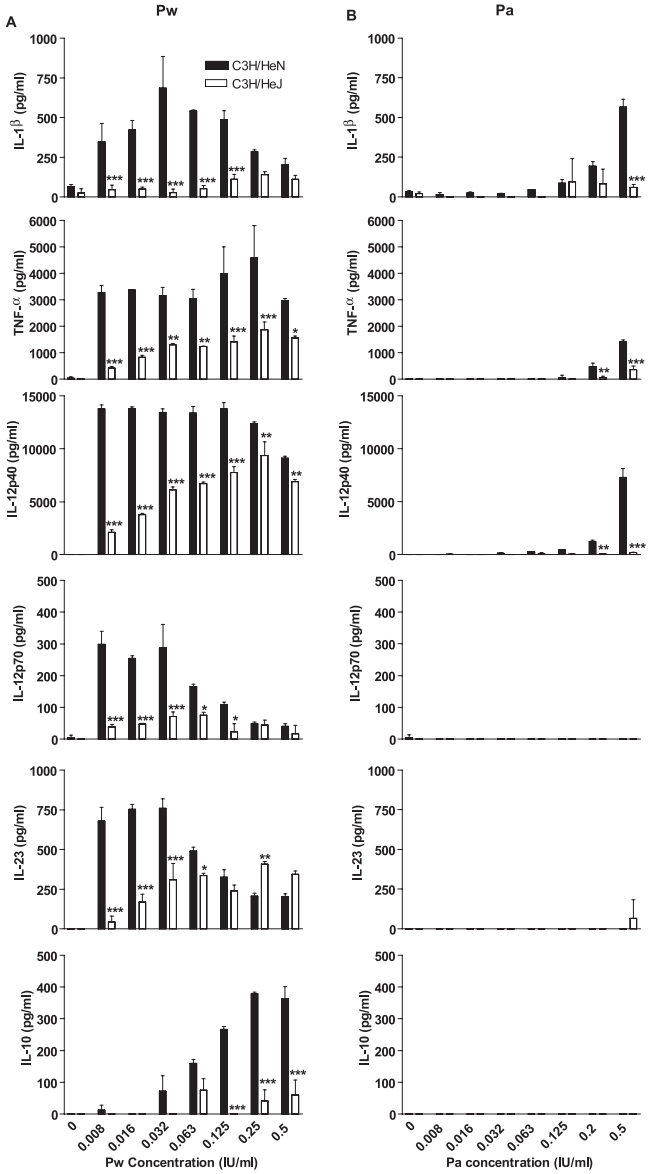

FIGURE 6. Pw induces pro- and anti-inflammatory cytokine production by DC thorough TLR4. Bone marrow-derived DC from C3H/HeN or C3H/HeJ mice were stimulated with a range of concentrations (0.008–0.5 IU/ml) of Pw (*A*) or Pa (*B*) or with medium only. Supernatants were removed after 18 h, and concentrations of IL-1-β, TNF-α, IL-12p40, IL-12p70, IL-23, and IL-10 were determined by two-site ELISA. Values represent means ± SD values for triplicate cultures. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, C3H/HeN vs C3H/HeJ.

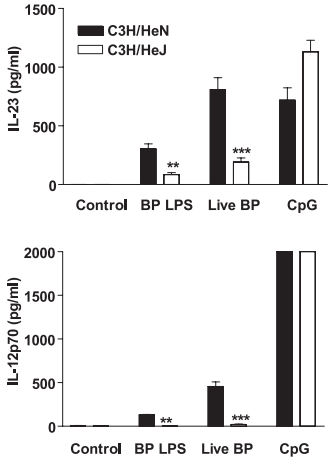

FIGURE 7. *B. pertussis* and *B. pertussis* LPS induce IL-23 and IL-12 production from DC through TLR4. DC from C3H/HeN and C3H/HeJ mice were stimulated with live *B. pertussis* (10 bacteria to 1 cell), *B. pertussis* LPS (100 ng/ml), CpG (1 μg/ml), or medium only. Supernatants were removed after 18 h, and concentrations of IL-12p70 and IL-23 were determined by two-site ELISA. **, $p < 0.01$; ***, $p < 0.001$; C3H/HeN vs C3H/HeJ.

IL-23 to induce IL-17 production. Other studies in the laboratory demonstrated an absolute requirement for IL-1 in IL-23-driven IL-17 production in experimental autoimmune encephalomyelitis (26), and because we had observed that *B. pertussis*-induced IL-1β was significantly impaired in TLR4-defective mice, we examined the role of IL-1 in promoting IL-17 production. Purified T cells from IL-1RI$^{-/-}$ and wild-type C57BL/6 mice were stimulated with supernatants from *B. pertussis* LPS-stimulated DC from C57BL/6 mice. Although the concentrations of IL-17 were lower than that observed for spleen cells from C3H/HeN mice, supernatants from LPS-stimulated DC, but not control DC, promoted IL-17 production by T cells from C57BL/6 mice, and this IL-17 was inhibited by anti-IL-23 and marginally augmented by addition of rIL-23 (Fig. 8*B*). In contrast, T cells from IL-1RI$^{-/-}$ mice failed to secrete IL-17 following stimulation with supernatants from LPS-stimulated DC, whereas T cells from wild-type C57BL/6 mice secreted significant concentration of IL-17 (Fig. 8*B*). This is consistent with our previous report that Th-17 are induced in wild-type, but not IL-1RI$^{-/-}$ mice following immunization with Ags in the presence of TLR agonists (26). T cells from IL-1RI$^{-/-}$ mice did secrete IL-17 in response to direct stimulation with PMA and anti-CD3, but the concentrations were significantly lower than that produced by T cells from wild-type mice. We have already reported defective IL-17, but normal IFN-γ, production by T cells from IL-1RI$^{-/-}$ mice (26). A similar pattern was observed for T cells from Pw or naive mice, with or without costimulation with specific Ag in vitro (data not shown). We confirmed the role of IL-1 in IL-17 induction using IL-1ra and neutralizing anti-IL-1 Abs. Addition of IL-1ra or anti-IL-1α and anti-IL-1β significantly inhibited IL-17 production by CD4$^+$ T cells in responses to supernatants from *B. pertussis* LPS-stimulated DCs (Fig. 8*C*). Finally, we examined the influence of rIL-1α and IL-1β on IL-23-induced IL-17 production. IL-17 could not be detected in supernatants from spleen cells stimulated with IL-23, IL-1α, or IL-1β alone, but was detected following stimulation with IL-23 in combination with IL-1α or IL-1β (Fig. 8*D*). These findings demonstrate that IL-1 and IL-23 synergize to promote the expansion of Th-17 cells and provide further evidence of a role for innate immunity in directing adaptive immune responses.

IL-17 activates macrophage killing of B. pertussis

There is evidence that cell-mediated immunity to *B. pertussis* may involve macrophage killing of *B. pertussis*, through NO-dependent and independent mechanisms (27, 28). We have also established that Pw are less protective in macrophage-depleted mice (P. Byrne and K. H. G. Mills, unpublished observations). Therefore, we examined the role of macrophages in the effector phase of cellular immunity to *B. pertussis* and the role of IL-17 and TLR4 in this response. We found that activation with increasing concentrations of IL-17 significantly enhanced macrophage killing of *B. pertussis* (Fig. 9*A*). Bacteria were undetectable in macrophages 2 h after stimulation with high concentrations (50 ng/ml) of IL-17. IFN-γ and TNF-α also enhanced the bactericidal activity of macrophages

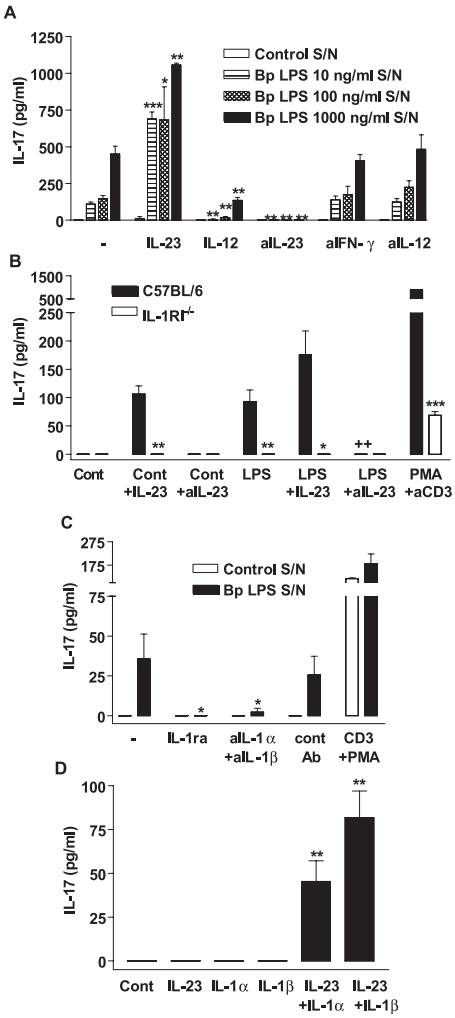

FIGURE 8. *B. pertussis* LPS-induced IL-23 and IL-1 synergize to promote T cell IL-17 production. *A*, DC were stimulated with *B. pertussis* LPS (10, 100, or 1000 ng/ml) or with medium only, and after 24 h supernatants were recovered. Spleen cells from C3H/HeN mice were stimulated with supernatants from LPS or medium-stimulated DC, with rIL-23 (10 ng/ml), rIL-12 (10 ng/ml), neutralizing Abs to IL-23 (anti(a)IL-23; 10 μg/ml), IFN-γ (aIFN-γ; 10 μg/ml), or IL-12 (aIL-12; 10 μg/ml). Supernatants were removed after 72 h, and concentrations of IL-17 were determined by ELISA. *B*, $CD4^+$ T cells were purified from C57BL/6 or IL-$1RI^{-/-}$ mice and stimulated with supernatants from control DC (Cont) or *B. pertussis* LPS-stimulated DC (LPS) in the presence or absence of rIL-23 or anti-IL-23. T cells were also directly stimulated with PMA (250 ng/ml) and anti-CD3 (aCD3; 1 μg/ml). *C*, CD4 T cells were purified from C57BL/6 mice and stimulated with supernatants from control or *B. pertussis* LPS-stimulated DC in the presence or absence of IL-1ra (1 μg/ml) or 10 μg/ml anti-IL-1α and anti-IL-1β or a control Ab or with PMA and anti-CD3. *D*, $CD4^+$ T cells from naive C57BL/6 mice were stimulated with medium only (Cont); 10 ng/ml IL-23, IL-1α, or IL-1β alone; or IL-23 in the presence of IL-1α or IL-1β. Supernatants were removed after 72 h, and concentrations of IL-17 were determined by ELISA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, vs control (*A*), vs wild type (*B*), or vs IL-23 or IL-1 alone (*C*); ++, $p < 0.001$, with vs without anti-IL-23 (*B*).

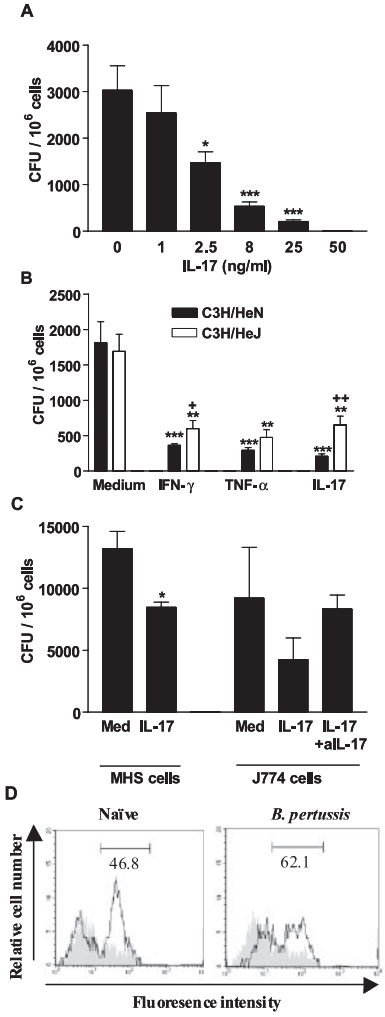

FIGURE 9. IL-17 promotes macrophage killing of *B. pertussis*, which is reduced in TLR4-defective mice. *A*, Peritoneal macrophages from C3H/HeN mice were incubated with 1–50 ng/ml IL-17 or medium only. *B*, Macrophages from C3H/HeN and C3H/HeJ mice were incubated for 2 h with 25 ng/ml IL-17, IFN-γ, or TNF-α, or with medium only. *C*, MHS or J774 macrophages were incubated with medium or IL-17 (10 ng/ml) or IL-17 in the presence of anti-IL-17 (10 μg/ml). After 2 h and incubation, live *B. pertussis* (10 bacteria per macrophage) were added and incubated for an additional 3 h. Supernatants were removed, and CFU counts were performed on cell lysates. *D*, Lung mononuclear cells from naive or *B. pertussis*-infected (day 7) mice were incubated with goat anti-mouse IL-17R (black line) or an isotype control Ab (gray histogram), followed by donkey anti-goat IgG FITC and PE-Cy5.5-conjugated anti-mouse F4/80. Expression of IL-17R was analyzed by flow cytometry following gating on $F4/80^+$ cells. Numbers represent geometric mean fluorescence intensities. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs medium control. +, $p < 0.05$; ++, $p < 0.01$, C3H/HeN vs C3H/HeJ.

(Fig. 9*B*). Furthermore, cytokine-mediated bacterial killing was diminished in macrophages from TLR4-defective mice. However, baseline killing in the absence of cytokines was similar in macrophages from C3H/HeN and C3H/HeJ mice. This suggests that in addition to IL-17, IFN-γ, and TNF-α, other mediators produced through interaction of *B. pertussis* with TLR4 also enhance macrophage killing of *B. pertussis.* IL-17 also promoted killing of *B. pertussis* by an alveolar macrophage cell line, MHS, and by J774 cells, and the effect was reversed by anti-IL-17 (Fig. 9*C*). LPS was undetectable in the recombinant cytokine preparation. We also examined the expression of IL-17R on alveolar macrophages from naive and *B. pertussis*-infected mice. IL-17R expression was detected at low levels on $F4/80^+$ cells in lungs of naive mice, and this was enhanced 7 days after *B. pertussis* challenge (IL-17R on $F4/80^+$ cells, mean fluorescence intensity 48.9 $\pm$ 0.2 for naive mice, mean fluorescence intensity 57.1 $\pm$ 2.6 for day 7 infected mice; $p < 0.05$, $n = 4$; representative profiles shown in Fig. 9*D*).

Discussion

The significant new findings of this study are that signaling though TLR4 is required for the induction and effector phases of adaptive immunity to *B. pertussis*, and that IL-23- and IL-1-driven IL-17 plays a role in protection induced with Pw. It is well established that TLRs play a critical role in the induction of innate immunity to infection (29, 30). However, innate immune responses also direct adaptive immunity, in part through the production of cytokines, such as IL-12, which promote Th1 cell differentiation (31, 32). This study demonstrates that protective immunity induced by Pw involves TLR4-mediated activation of IL-12, IL-23, and IL-1 production from DC, which drives the induction of Th1 and Th-17 cells, and that cytokines from these T cells together with additional TLR4-mediated signals activate the bactericidal activity of macrophages.

To date, there have been few attempts to study the role of TLRs in vaccine-induced adaptive immunity, and the role of Th-17 cells in vaccine-induced immunity has not been addressed. The present study demonstrates an absolute requirement for TLR4 in the protective immune responses generated with Pw in mice and a less significant role in protection induced with Pa. It has been demonstrated that protection induced by previous infection with *B. pertussis* or by immunization with Pw involves the induction of IFN-γ-secreting Th1 cells, IgG2a Abs, and activation of inflammatory responses in the lungs after challenge (8, 11, 33). In contrast, protection induced with Pa is more dependent on IgG1 Ab and Th2 cells (8). In this study, we found that the Ag-specific Th1 responses and the inflammatory cytokine responses in the lungs were reduced after challenge of Pw-immunized TLR4-defective mice. Interestingly, we also observed a reduction in Ag-specific IL-5 and IgG1 Ab responses in TLR4-defective mice immunized with Pa. Activation of innate immune cells through TLR4 has been considered to selectively direct Th1 responses (30, 32). However, we have found that TLR agonists can promote the induction of IL-10-secreting T cells as well as Th1 cells (21, 22) (our unpublished observations). In addition, it has been reported that the T cell responses to inhaled OVA were polarized to Th1 following coadministration of high dose LPS, but to Th2 with low dose LPS, and that these responses were reduced in TLR4-defective mice (34). This is consistent with our demonstration that Pw, which has a high LPS content, induced Th1 and Th-17 cells, whereas the Pa, which had a low, but significant concentration of LPS, induced a more Th2-polarized response. Thus, in addition to alum, the presence of low concentrations of LPS may contribute to the induction of Th2 responses with Pa and many other licensed vaccines that may have residual endotoxin in the Ag preparations.

Our findings suggest that the LPS concentration in the vaccine formulation may have a major impact on vaccine efficacy. Consistent with this, it has been demonstrated that the endotoxin content of different diphtheria, tetanus, and Pw ranged from 11,400 to 181,100 EU/ml, compared with 38 to 1,390 EU/ml for DTPa vaccines (35). The adverse events associated with immunization with Pw, including the induction of fever and seizures, which are significantly less frequent with Pa, have also been linked to residual LPS in the vaccine preparations (35, 36). Conversely, the significant local reactions reported in a high proportion of 4- to 5-year-old children after booster doses of Pa have been linked to strong Th2 responses (37). Therefore, future modifications of Pa should consider the use of Th1-promoting adjuvants, such as IL-12, which has been shown to enhance efficacy of an experimental Pa in a mouse model without toxicity (14).

The reduced Th1 response to Pw in TLR4-defective mice was consistent with defective DC production of IL-12p40 and IL-12p70. The IL-12 family also includes IL-23, which is composed of the common IL-12p40 chain and a distinct IL-23p19 chain, which promotes a distinct population of Th-17 cells (18). Th-17 cells have been shown to play a critical role in autoimmune inflammation (15), and have also been implicated in protective immunity to infection (19, 20). In this study, we have demonstrated for the first time that Ag-specific Th-17 cells can be induced by vaccination and that neutralization of IL-17 in vivo reduced the efficacy of Pw. Furthermore, the generation of Th-17 cells was significantly impaired in TLR4-defective mice, which was consistent with significantly reduced IL-23, IL-1, and IL-12p40 production by C3H/HeJ DC stimulated with Pw in vitro.

Consistent with previous studies (15, 16), we found that IL-23 could amplify IL-17 production in vitro. Supernatants from *B. pertussis* LPS-stimulated DC enhanced IL-17 production, which was reduced by neutralizing anti-IL-23 Ab. Addition of rIL-23 enhanced IL-17 production, but further addition of supernatants from *B. pertussis* LPS-stimulated DC, which contained lower concentrations of IL-23, significantly augmented IL-17 production. We have discovered recently an important role for IL-1 in the induction of pathogenic autoantigen-specific Th-17 cells that mediate experimental autoimmune encephalomyelitis (26). In this study, we found that T cells from IL-1RI$^{-/-}$ mice failed to produce IL-17 in response to supernatants from LPS-stimulated DC, whereas wild-type T cells did secrete IL-17. Furthermore, IL-1α and IL-1β significantly augmented IL-23-driven IL-17 production. Because IL-1, as well as IL-23 and IL-12p40, production was significantly impaired in DC from TLR4-defective mice, our findings suggest that TLR4-mediated IL-1 and IL-23 synergize to drive expansion of Th-17 cells. It has been reported recently that TGF-β and IL-6 can promote the differentiation of IL-17, and whereas IL-23 may expand Th-17 from in vivo activated memory T cells, it did not act as a differentiation factor for naive T cells (24, 25). However, IL-23 was essential for a protective Th-17 response against *Citrobacter rodentium* (24). In agreement with this, we found that IL-23 enhanced IL-17 production by T cells from immune mice. However, we also found that IL-23 in combination with IL-1 promoted IL-17 production from naive T cells. We have reported recently essential roles for PI3K, NF-κB, and novel protein kinase C isoforms in IL-1- and IL-23-mediated IL-17 production (26).

Studies with human monocyte-derived DC have shown that *B. pertussis* stimulates IL-23 and IL-12p40, but not IL-12p70, production, and suggested that IL-23 may be responsible for *B. pertussis*-induced Th1 cells (38). It has also been suggested that different bacteria have selective capacities to induce either IL-12 or IL-23 production (18). We found that *B. pertussis* induced IL-12p40 and IL-23, and low, but detectable concentrations of IL-12p70 from murine DC. Furthermore, IL-12, but not IL-23, enhanced IFN-γ production, whereas IL-23, but not IL-12, enhanced IL-17 production (Fig. 4 and data not shown). Our findings are consistent with recent reports demonstrating that IL-12 and IL-23 promote distinct populations of Th1 and Th-17 cells (16, 17). Furthermore, our results demonstrate that both populations of effector T cells may contribute to protection induced with Pw.

Although it is well established that Th1 cells play a protective role in immunity to many pathogens, including *B. pertussis*, there is very limited information on the role of IL-17 in protective immunity to infection. It has been suggested that a major function of IL-23 and IL-17 is to activate inflammatory cytokines and chemokines and to rapidly recruit neutrophils to the site of infection (39). We found MIP-2 was induced in the lungs following challenge of Pw immunized, and this was reduced in TLR4-defective mice (our unpublished observations) and in mice treated with anti-IL-17. We

have reported previously a defect in the immediate influx of neutrophils into the lungs of TLR4-defective mice following primary infection with *B. pertussis* (21). This is consistent with a report demonstrating that TLR4 is required for recruitment of neutrophils to the lungs, where they mediated Ab-mediated clearance of *Bordetella bronchiseptica* (40). IL-17 has already been shown to play a role in lung CXC chemokine production and neutrophil recruitment in host defense against *K. pneumoniae* infection (19, 41). In addition, IL-17 has also been shown to activate production of the antimicrobial peptide, β-defensin-2, by human airways epithelial cells (42).

The present study has demonstrated a role for IL-17 in protective cellular immunity to *B. pertussis*. Although the mechanism is still not clear, it may involve recruitment of neutrophils and enhancement of the antibacterial activity of macrophages. We found that IL-17R was expressed on lung macrophages, and this was enhanced during infection with *B. pertussis*. Depletion studies in vivo showed that macrophages play an important role in protective immunity to *B. pertussis* induced with Pw, and a less significant role in immunity induced with Pa (P. Byrne and K. H. G. Mills, unpublished observations), which is consistent with the stronger IFN-γ and IL-17 responses induced with Pw. IL-17 and IFN-γ, as well as TNF-α, were found to enhance the macrophage killing of *B. pertussis* in vitro, and the cytokine-activated killing was reduced in macrophages from TLR4-defective mice. It has been reported that protection induced with Pw is compromised in inducible NO synthase-deficient mice (27). We found that NO production in response to *B. pertussis* was significantly reduced in DC and macrophages from TLR4-defective mice (our unpublished observations). NO production was enhanced by IFN-γ, but not by IL-17, suggesting that these cytokines may enhance macrophage killing through distinct mechanisms. This is consistent with the demonstration of NO-dependent and independent mechanism of macrophage killing of *B. pertussis* (28). Collectively, our findings suggest that *B. pertussis* LPS signaling through TLR4 in innate immune cells plays a critical role in the generation of inflammatory cytokines, IL-12, IL-23, and IL-1, which direct the induction of Th1 and Th-17 cells in mice immunized with Pw. Furthermore, the cytokines secreted by these T cell subtypes promote bacterial killing by macrophages, a response that was further enhanced at the effector level by TLR4-mediated activation of macrophages. Thus, TLR4 plays a critical role in the induction and effector phase of the protective cellular immune response to *B. pertussis* induced by vaccination.

Disclosure

K. H. G. Mills is Co-founder, Director and shareholder in Opsona Therapeutics Limited, a university campus company involved in the development of anti-inflammatory therapeutics.

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