

## A Regulatory Role for Interleukin 4 in Differential Inflammatory Responses in the Lung following Infection of Mice Primed with Th1- or Th2-Inducing Pertussis Vaccines

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Protection against infectious pathogens at mucosal surfaces is dependent on local antibody responses, production of inflammatory mediators, and recruitment of immune effector cells to the site of infection. Since Th1 and Th2 cells produce cytokines with pro- and anti-inflammatory activities, immunization with vaccines that induce these T-cell subtypes may regulate the subsequent inflammatory response to infection. We have demonstrated that immunization of mice with pertussis whole-cell or acellular vaccines (Pw or Pa) selectively induces Th1 and Th2 cells, respectively. In this study we have used a murine respiratory-infection model to demonstrate that priming with a Th1- or Th2-inducing pertussis vaccine can influence the local inflammatory response and immune effector cells in the lung following aerosol challenge with *Bordetella pertussis*. Analysis of bronchoalveolar lavage (BAL) fluid taken during the course of *B. pertussis* infection of naïve mice or mice immunized with Pw revealed an early influx of neutrophils and local production of interleukin 1 $\beta$  (IL-1 $\beta$ ) in the lungs. In contrast, neutrophil infiltration and IL-1 $\beta$  production were not observed following challenge of mice immunized with the Th2-inducing Pa. Conversely, during infection local production of IL-6 and IL-1ra was significantly greater in mice immunized with Pa than in those immunized with Pw. Studies of knockout mice revealed neutrophil and lymphocyte infiltration in the lungs following *B. pertussis* infection of IL-4-defective (IL-4<sup>-/-</sup>) mice but not in wild-type mice immunized with Pa. Furthermore, the levels of IL-1 $\beta$ , IL-6, and IL-1ra in Pa-immunized IL-4<sup>-/-</sup> mice were comparable to those in mice immunized with Pw. These results demonstrate distinct influences of Th1- and Th2-inducing vaccines on the protective inflammatory responses in the lungs following challenge with *B. pertussis* and implicate IL-4 as an important regulator of inflammatory-cell recruitment.

Although local humoral immunity is considered to be a key element in protection against infection at mucosal surfaces, cellular immunity also plays a role in controlling pathogenic microorganisms that invade the lungs and gastrointestinal tract. The production of inflammatory mediators and recruitment of immune effector cells to the site of infection is an important feature of protective cellular immune response. Proinflammatory cytokines and chemokines, including interleukin 1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ) and MIP-2, promote the infiltration of neutrophils, macrophages, and lymphocytes to the local site of infection. However, anti-inflammatory cytokines, soluble cytokine receptors, and receptor antagonists normally control these inflammatory responses, which can result in local pathology and systemic and centrally controlled adverse events. CD4<sup>+</sup> T cells also play a role in the regulation of inflammation (1). Gamma interferon (IFN- $\gamma$ ) produced by Th1 cells as well as CD8<sup>+</sup> T cells, NK cells, and  $\gamma\delta$  T cells, has proinflammatory activity, activating phagocytic cells, and synergizes with lipopolysaccharide (LPS) in the production of IL-1 $\beta$  (12). In contrast, cytokines produced by Th2 cells, such as IL-4, IL-6, IL-10, and IL-13, in addition to promoting antibody responses also have anti-inflammatory activity

and may limit immunopathology associated with Th1-mediated responses (1).

Using a murine model of respiratory infection with *Bordetella pertussis*, we have demonstrated that natural infection or immunization with a whole-cell pertussis vaccine (Pw) selectively induces Th1 cells (5, 23, 29). In contrast, acellular pertussis vaccines (Pa), comprising the protective antigens detoxified pertussis toxin (PT), filamentous hemagglutinin (FHA), and pertactin adsorbed to alum, generate Th2 cells (5, 29). A similar dichotomy is observed in infected and vaccinated children; peripheral blood mononuclear cells from children recovering from whooping cough or immunized with Pw secrete IFN- $\gamma$  but undetectable IL-4 or IL-5, whereas immunization with Pa induces T cells with a mixed Th1-Th2 profile (3, 30, 31). This clear dichotomy between Th1 and Th2 induction with two different vaccines against the same pathogen provides an ideal model to examine the effect of Th1 or Th2 priming on the local immune response in the lung following infection.

Although the reported protection rates of both Pw and Pa in children are quite variable, (36 to 96 and 59 to 85% against World Health Organization-defined whooping cough for Pw and Pa, respectively), all three- and five-component Pa had efficacies in clinical trials which approached that of European Pw (2, 9, 10, 28, 36). Furthermore, the reactogenicity of Pa is considerably reduced compared with that of Pw (1, 9, 10, 28, 36). However, the data from the murine model has suggested that immunity induced by Pa is largely mediated by antibody, whereas Pw may activate cellular as well as humoral effector mechanisms (24). It is now well established that *B. pertussis* can be taken up by macrophages and polymorphonuclear leuko-

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cytes (PMN) (34, 38). Therefore, recruitment and activation of macrophages and PMN may be a critical element of protective cellular immunity to *B. pertussis* in the lungs. IFN- $\gamma$  is known to trigger macrophage activation by inducing the production of IL-1 $\beta$  (8, 12). IL-1 $\beta$  is also a potent stimulus for neutrophil accumulation through upregulation of adhesion molecules, which facilitate neutrophil adherence and transendothelial migration (7, 11, 26). Furthermore, it has been shown that IL-1 $\beta$  is capable of inducing mRNA expression of the neutrophil chemoattractant MIP-2 in the rat lung (43).

In the present study we examined the hypothesis that Th1- and Th2-inducing pertussis vaccines may mediate protection against *B. pertussis* infection by activating distinct immune effector mechanisms in the respiratory tract. Mice were immunized with Pw or Pa, and the kinetics of cell infiltration and local cytokine production was examined in the lungs following *B. pertussis* respiratory challenge. Rapid infiltration of neutrophils was observed in the lungs following bacterial challenge of mice immunized with Pw but not in mice immunized with Pa. Furthermore, the inflammatory response in the lungs of mice immunized with the Th1-inducing vaccine was associated with elevated levels of IL-1 $\beta$  whereas priming with the Th2-inducing vaccine was associated with increased levels of IL-6 and IL-1ra. The anti-inflammatory effect of Pa was abrogated in IL-4-defective (IL-4<sup>-/-</sup>) mice. Our findings suggest that recruitment and activation of inflammatory cells, such as neutrophils, into the lungs following *B. pertussis* challenge may play a pivotal role in facilitating the early resolution of disease following immunization with Pw. In contrast, protection induced by Pa, which appears to be primarily mediated by antibody, is associated with reduced inflammatory responses.

#### MATERIALS AND METHODS

**Mice.** Specific-pathogen-free BALB/c and C57BL/6 mice were purchased from B&K Universal Ltd., Hull, United Kingdom, and were bred and maintained according to the guidelines of the Irish Department of Health. The IL-4<sup>-/-</sup> mice, generated from wild-type C57BL/6 (*H-2<sup>b</sup>*) mice (14), were obtained from B&K Universal and used with the kind permission of Werner Muller (Institute for Genetics, University of Cologne, Cologne, Germany).

**Immunizations.** Mice (6 to 8 weeks old) were immunized intraperitoneally twice at 4-week intervals with 0.2 human dose of either Wellcome Pw (National Institute for Biological Standards and Control reference reagent 88/522) or Pa prepared with 5  $\mu$ g each of detoxified PT, FHA, and pertactin adsorbed to alum (29). The mice were challenged 2 weeks after the second immunization.

***B. pertussis* infection.** Respiratory infection of mice was performed as previously described (24). Briefly, *B. pertussis* Wellcome 28 was grown at 36°C in Stainer-Scholte medium. Bacteria from a 48-h culture were concentrated to  $2 \times 10^{10}$ /ml in phosphate-buffered saline with 1% casein. Aerosol challenge was administered over 15 min using a nebulizer (0.5 ml/min). The course of *B. pertussis* infection was followed by performing CFU counts on lungs from groups of four mice at various times after aerosol challenge. The lungs were aseptically removed and homogenized in 1 ml of sterile physiological saline with 1% casein on ice. One hundred milliliters of undiluted homogenate or of serially diluted homogenate from individual lungs was spotted in triplicate onto Bordet-Gengou agar plates, and the number of CFU was estimated after 5 days of incubation at 37°C. The results are reported as the mean number of *B. pertussis* CFU for individual lungs from four mice per experimental group per time point. The limit of detection was approximately 0.5 log<sub>10</sub> CFU per lung.

**Analysis of BAL cells.** Bronchoalveolar lavage (BAL) fluids were obtained by injection and aspiration in 0.5-ml volumes (total, 4 to 5 ml) of warm RPMI 1640 medium via cannulation of the trachea. Cells from the lavage fluids were recovered by centrifugation at  $300 \times g$  for 5 min and resuspended in RPMI 1640 medium with 8% fetal calf serum. The cell composition in the lungs during the course of infection was followed by performing a total-leukocyte count and microscopic examination of Romanowsky staining of cytospin preparations of BAL cells. Morphological identification of neutrophils was confirmed by immunofluorescence staining and flow cytometry analysis using phycoerythrin-conjugated anti-LY-6G (clone RB6-8C5, purchased from PharMingen). Cells incubated with an isotype-matched directly conjugated antibody with irrelevant specificity acted as a control. After incubation for 30 min at 4°C, the cells were washed and immunofluorescence analysis was performed on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) and analyzed using Lysys version II.1 software. A total of 10,000 cells were analyzed per sample.

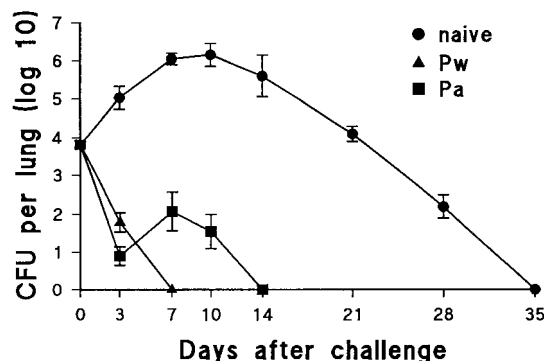


FIG. 1. *B. pertussis* clearance from the lungs of naive and immunized mice. The mice were immunized intraperitoneally at 0 and 4 weeks with Pa or Pw and challenged 2 weeks after the second immunization by exposure to an aerosol of live *B. pertussis*. Naive nonimmunized mice acted as controls. The course of infection was followed by performing CFU counts on individual lung homogenates at intervals after challenge. The results are mean ( $\pm$  standard error) CFU counts for four mice per experimental group at each time point.

**Cytokine levels in BAL fluids.** BAL fluids were concentrated fivefold, and the levels of IL-1 $\beta$ , TNF- $\alpha$ , IL-6, and IL-1ra were determined by specific immunoassays. IL-1 $\alpha$  and TNF- $\beta$  anti-cytokine antibodies were obtained from Genzyme Diagnostics, Cambridge, Mass. IL-6 and IL-1ra anti-cytokine antibodies were kindly provided by Steve Poole, National Institute for Biological Standards and Control, Potters Bar, Hertsfordshire, United Kingdom. Recombinant cytokines of known concentration and potency were used for the generation of standard curves.

**Statistical analysis.** All statistical analysis of data was performed with the statistical software package StatWorks. Levels of statistical significance were assessed using analysis of variance. Data for each treatment group were compared at each time point, and levels of significance between Pw and Pa or C57BL/6 and wild-type mice are indicated on the figures.

#### RESULTS

##### Immune response and protection induced with Pw and Pa.

We have previously demonstrated that spleen cells from mice immunized with Pw produced high levels of IFN- $\gamma$  and undetectable IL-4 and IL-5 following stimulation with *B. pertussis* antigens in vitro, whereas immunization with Pa induced T cells that secreted IL-4 and IL-5 but not IFN- $\gamma$  (23, 29). The selective priming of Th2 cells by Pa was confirmed through the generation of antigen-specific CD4<sup>+</sup>-T-cell lines and clones from immune mice (5). The association between T-cell subtype induction and protection against infection was assessed by performing CFU counts on lung homogenates at intervals after *B. pertussis* challenge of immunized and control mice. Respiratory challenge of BALB/c mice by aerosol exposure to live *B. pertussis* results in a reproducible infection in the lungs (24). In naive mice, the bacteria multiply in the lungs, reach a peak 7 to 14 days after infection, and are eventually cleared after 5 weeks (Fig. 1). Live bacteria were undetectable in the lungs of mice immunized with Pw 7 days after challenge. However, in mice immunized with Pa, low numbers of bacteria were still detectable on days 7 and 10 and there was a delay in complete bacterial clearance to day 14 (Fig. 1).

**Kinetics of cell infiltration in the lungs of naive or immunized mice following infection with *B. pertussis*.** Cellular influx into the lungs during the course of *B. pertussis* infection was examined in naive and immune mice by performing morphological analysis and total-leukocyte counts on cells recovered from BAL samples at intervals after challenge. Following *B. pertussis* infection of naive mice, the numbers of neutrophils recovered from the lungs increased dramatically from almost undetectable levels on day 0 to almost  $10^5$  cells per lung on day

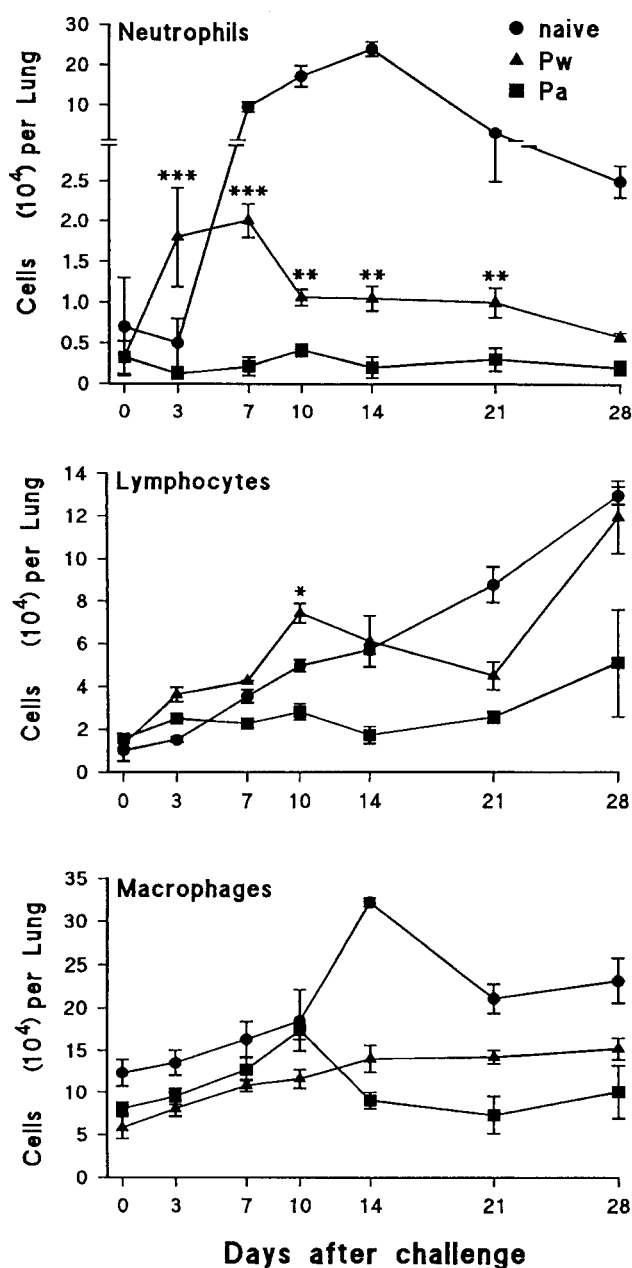


FIG. 2. Kinetics of cell infiltration in the lungs of naive and Pw- or Pa-immunized BALB/c mice infected with *B. pertussis*. Naive mice or mice immunized twice with Pw or Pa were challenged with *B. pertussis* 2 weeks after the second immunization. The cell composition in the lungs during the course of infection was studied by microscopic examination of Romanowsky staining of cytospin preparations of cells recovered from BAL samples. The results are expressed as mean ( $\pm$  standard error) values for four mice per experimental group tested individually in triplicate at each time point. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (Pw- versus Pa-immunized mice).

7 and then started to decline after day 14 (Fig. 2). BAL fluids recovered from the lungs of mice immunized with Pw revealed an early influx of neutrophils, which was found to persist for up to 1 week after challenge (Fig. 2). In contrast, neutrophil infiltration was not observed following challenge of mice immunized with Pa (Fig. 2). The absolute numbers of neutrophils recovered from the lungs of mice immunized with Pw was significantly lower than those obtained from infected nonim-

mized mice at days 7, 10, and 14 after *B. pertussis* challenge ( $P < 0.001$ ), and this may simply reflect the lower bacterial load in the immune animals.

Assessment of BAL fluids also revealed a lymphocyte infiltration in the lungs of naive and Pw-immunized mice but not Pa-immunized mice (Fig. 2). However, an influx of macrophages was detected in the lungs during infection of mice immunized with Pa. Although the peak response in Pa-immunized mice was higher than that in Pw-immunized mice, it was not as persistent and the numbers were significantly lower ( $P < 0.001$ ) than in naive mice at 14, 21, and 28 days after challenge (Fig. 2).

**Local cytokines in the lungs.** BAL fluids recovered 4 h and 3, 7, 10, 14, 21, and 28 days after *B. pertussis* challenge were assessed for local production of cytokines during infection. A marked increase in IL-1 $\beta$  levels was found in BAL fluids 4 h after infection of mice immunized with Pw and 3 to 7 days after infection of naive mice (Fig. 3). In both cases the increase in IL-1 $\beta$  preceded the infiltration of neutrophils into the lungs (Fig. 2). In contrast, no significant increase in the levels of IL-1 $\beta$  was observed in the BAL fluids throughout the course of infection in mice immunized with Pa (Fig. 3). However, a 12-fold increase in the level of IL-6 was observed in the lungs 4 h after *B. pertussis* infection of mice immunized with Pa (Fig. 3). By comparison, IL-6 levels from the lungs of naive and Pw-immunized mice displayed only a five- and twofold increase, respectively. A significant increase in the levels of TNF- $\alpha$  was found in the BAL fluids 4 h after infection of both naive and Pa-immunized mice (Fig. 3). In contrast, no marked increase in the levels of TNF- $\alpha$  was observed in the lungs of mice immunized with Pw. Furthermore, BAL fluids from mice immunized with Pa had threefold-higher levels of IL-1ra 4 h after aerosol infection with *B. pertussis* (Fig. 4).

**Neutrophil infiltration in the lungs of Pa-immunized IL-4-defective mice.** Since immunization with Pa induces a Th2 response in mice, and IL-4 has been shown to influence inflammation, we examined the role of IL-4 in controlling the inflammatory response to *B. pertussis* in mice immunized with Pa. IL-4-defective (IL-4 $^{-/-}$ ) and wild-type C57BL/6 mice were immunized with either Pw or Pa and then challenged with live *B. pertussis*.

Similar to the findings for BALB/c mice, an influx of neutrophils was observed in the lungs 3 days after challenge of C57BL/6 mice immunized with Pw (Fig. 5). An increase in the numbers of infiltrating macrophages and lymphocytes was also observed on day 7. In contrast, only a modest infiltration of neutrophils was detected in the lungs following infection of C57BL/6 wild-type mice immunized with Pa, with numbers approximately eightfold lower than those seen in mice immunized with Pw (Fig. 5). Immunofluorescence analysis with a neutrophil-specific antibody, LY-6G, confirmed the morphological data; 3 days after *B. pertussis* challenge neutrophils accounted for 1 and 9% of cells in BAL fluids from wild-type mice immunized with Pa and Pw, respectively (not shown). Only small increases in the numbers of infiltrating macrophages and lymphocytes were found in the BAL fluids after *B. pertussis* challenge of wild-type mice immunized with Pa (Fig. 5). In contrast, infection of IL-4 $^{-/-}$  mice immunized with Pa was associated with a significant infiltration of neutrophils on day 3, with a further increase in these numbers by day 7 ( $P < 0.01$  and  $P < 0.001$ , respectively, versus the wild type). Immunofluorescence analysis with the anti-neutrophil antibody showed 7% positive cells in the BAL fluids of IL-4 $^{-/-}$  mice compared with 1% in those of the wild-type mice. In addition, significantly greater ( $P < 0.05$ ; 4 h and 3 and 7 days) lymphocyte influx was detected in the lungs of knockout mice than in

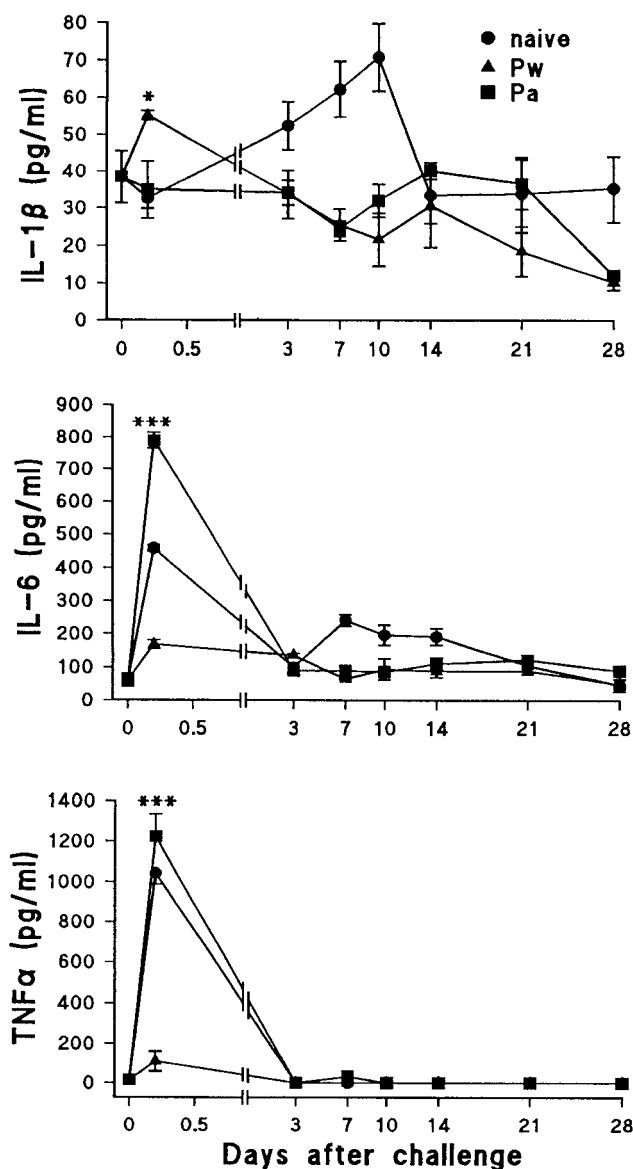


FIG. 3. Local cytokine production in the lungs following *B. pertussis* infection of BALB/c mice immunized with Pw or Pa. Naïve mice or mice immunized twice with Pw or Pa were challenged with *B. pertussis* 2 weeks after the second immunization. BAL fluids were concentrated fivefold, and the levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  were assessed by specific immunoassays. The results are mean ( $\pm$  standard error) values for four mice in each experimental group (assays performed in triplicate). \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$  (Pw- versus Pa-immunized mice).

the wild-type strain (Fig. 5). Surprisingly, when compared with that in the wild-type C57BL/6 mice, the influx of neutrophils was significantly reduced ( $P < 0.05$ ) 3 and 7 days after challenge of IL-4 $^{-/-}$  mice immunized with Pw. However, consistent with our previous findings (20) and a recent report demonstrating that IL-4 is required for an effective antitumor cell-mediated immunity (35), we have observed reduced antigen-specific IFN- $\gamma$  production in IL-4 $^{-/-}$  mice immunized with Pw (data not shown).

**Levels of IL-1 $\beta$ , IL-1ra, and IL-6 in the lungs of immunized IL-4 $^{-/-}$  mice.** In an attempt to establish whether the differences in neutrophil infiltration between wild-type and IL-4 $^{-/-}$

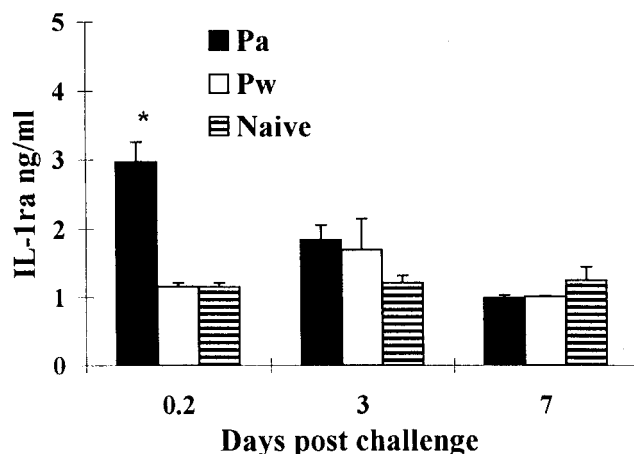


FIG. 4. Local production of IL-1ra in the lungs following respiratory challenge of BALB/c mice immunized with Pw or Pa. Naïve mice or mice immunized twice with Pw or Pa were challenged with *B. pertussis* 2 weeks after the second immunization. BAL fluids recovered 4 h, 3 days, and 7 days after challenge were concentrated fivefold, and the levels of IL-1ra were assessed by specific immunoassay. The results are mean ( $\pm$  standard error) values for four mice from each group. \*,  $P < 0.05$  (Pw- versus Pa-immunized mice).

mice immunized with Pa were related to differences in local cytokine production, IL-1 $\beta$ , IL-1ra, and IL-6 levels were measured in BAL fluids following challenge. When compared to the wild-type C57BL/6 mice, significantly ( $P < 0.01$ ) higher levels of IL-1 $\beta$  were detected in BAL fluids 4 h after infection of IL-4 $^{-/-}$  mice immunized with Pa (Fig. 6). Furthermore, the levels of IL-1 $\beta$  in IL-4-deficient mice were comparable to those measured in mice immunized with Pw. Conversely, 4 h after aerosol infection, the levels of both IL-1ra and IL-6 were significantly ( $P < 0.05$ ) higher in the BAL fluids of Pa-immunized wild-type mice than in those of Pa-immunized IL-4 $^{-/-}$  or Pw-immunized mice (Fig. 6). These results indicate that IL-4 plays a regulatory role in inflammatory responses, suppressing the production of the proinflammatory cytokine IL-1 $\beta$  and enhancing the production of the anti-inflammatory mediators IL-6 and IL-1ra.

**Course of infection in immunized IL-4 $^{-/-}$  mice.** In order to establish whether the altered inflammatory response in immunized IL-4 $^{-/-}$  mice affected the course of infection, naïve and Pw- or Pa-immunized IL-4 $^{-/-}$  and wild-type C57BL/6 mice were challenged by exposure to an aerosol of *B. pertussis*. The course of infection was not significantly different in Pa- or Pw-immunized IL-4 $^{-/-}$  mice than in the wild-type strain (Fig. 7). However, the rate of bacterial clearance was marginally, but not significantly, enhanced in the naïve IL-4-defective mice (Fig. 7).

## DISCUSSION

The results of this study have demonstrated that priming with Th1- or Th2-inducing pertussis vaccines can have a significant effect on the inflammatory response in the lungs following subsequent infection with *B. pertussis*. A significant neutrophil influx and elevation in local IL-1 $\beta$  production was observed in the lungs after bacterial challenge of mice immunized with the Th1-inducing killed-whole-bacteria vaccine. In contrast, the inflammatory response was suppressed in the lungs of mice immunized with the Th2-inducing subunit vaccine. Significantly higher levels of IL-1ra and IL-6 were found in BAL fluids recovered from the lungs following *B. pertussis*

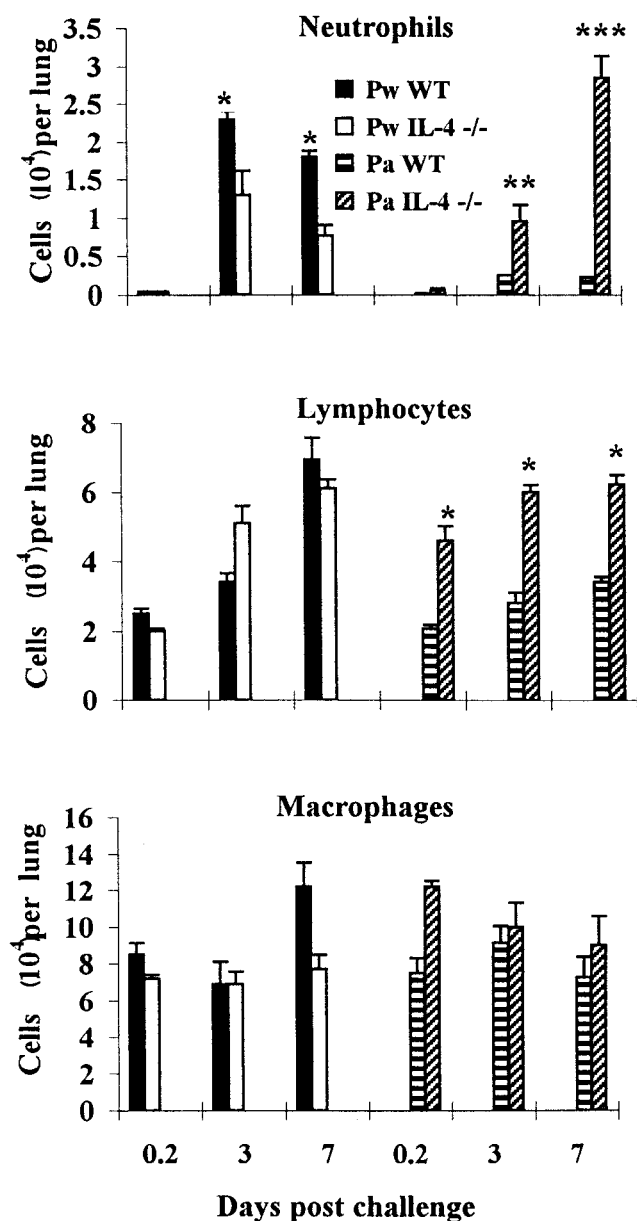


FIG. 5. Cellular infiltration in the lungs following *B. pertussis* challenge of IL-4<sup>-/-</sup> and wild-type mice immunized with Pw or Pa. C57BL/6 wild-type (WT) and IL-4-defective (IL-4<sup>-/-</sup>) mice were immunized twice with Pw or Pa and respiratorily challenged with *B. pertussis* 2 weeks after the second immunization. BAL samples were prepared 4 h, 3 days, and 7 days after challenge and assessed for cell composition as described in the legend to Fig. 2. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (IL-4<sup>-/-</sup> versus wild type). The error bars indicate standard error.

infection of mice immunized with Pa. However, this pattern of pro- and anti-inflammatory activity was reversed in IL-4-defective mice, suggesting that IL-4 may be an important regulator of inflammatory-cell recruitment.

Previous studies have demonstrated a dichotomy in the subtypes of T cells induced with Pw and Pa in mice and in children (3, 5, 23, 29–31) and suggest distinct effector mechanisms induced with the two type of pertussis vaccine. Experiments in a murine model suggested that Th1 cells mediate protective immunity induced by natural infection or by immunization with Pw (4, 5, 20, 23, 24, 29). In contrast, immunization of mice with

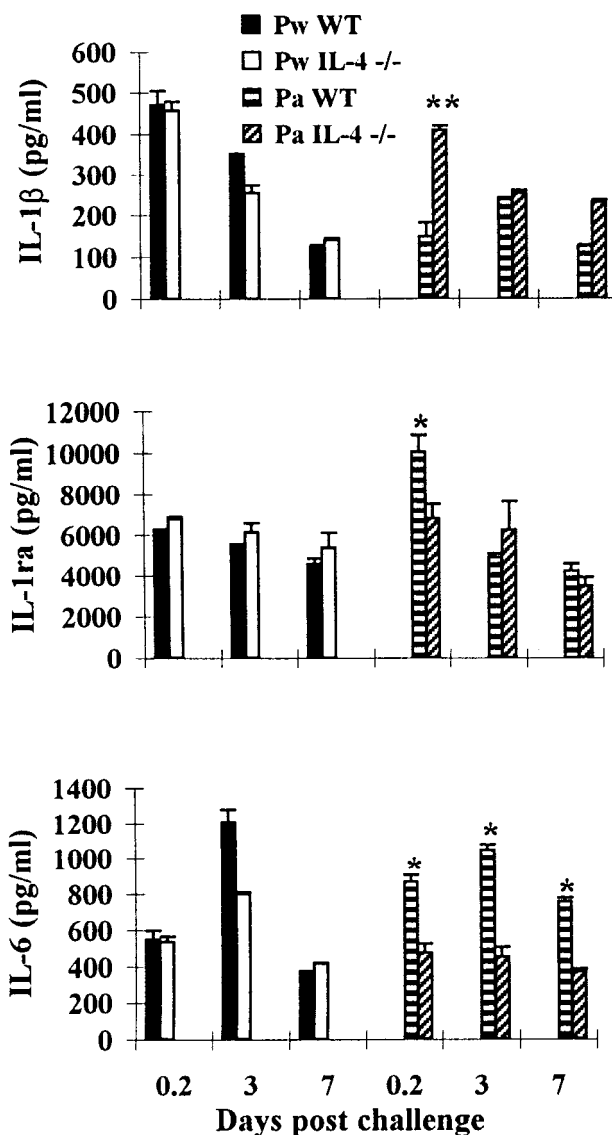


FIG. 6. Cytokine production in the lungs following *B. pertussis* infection of IL-4<sup>-/-</sup> and wild-type mice immunized with Pw or Pa. C57BL/6 wild-type (WT) and IL-4<sup>-/-</sup> mice were immunized and challenged as described in the legend to Fig. 5. BAL fluids were concentrated fivefold, and the levels of IL-1 $\beta$ , IL-1ra, and IL-6 were assessed by specific immunoassays. The results are mean ( $\pm$  standard error) values for four mice in each group. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (IL-4<sup>-/-</sup> versus wild type).

Pa, comprising purified antigens of *B. pertussis* adsorbed to alum, induces Th2 cells in mice (5, 29) and a mixed Th1-Th2 profile in infants (3, 31). It appears that immunity induced with Pa, especially soon after immunization, is largely mediated by antibody, whereas both cellular and humoral immunities play a role in protection induced by previous infection or immunization with Pw (4, 20, 24). Although the exact role of T-cell subtypes in immunity to *B. pertussis* is not fully resolved, it is considered that Th2 cells provide help for immunoglobulin (Ig) production, especially IgG1, IgE, and IgA. In contrast, Th1 cells activate the antimicrobial activity of phagocytic cells and in addition stimulate B cells to produce opsonizing and complement-fixing antibodies of the IgG2a subclass. The results of the present investigation provide direct evidence of

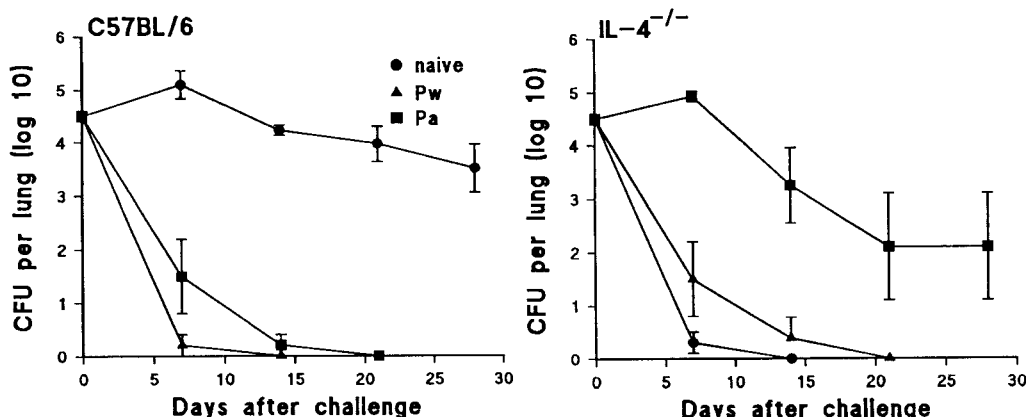


FIG. 7. *B. pertussis* clearance from the lungs of naïve and immunized mice and IL-4<sup>-/-</sup> and wild-type mice. C57BL/6 and IL-4<sup>-/-</sup> mice were immunized intraperitoneally at 0 and 4 weeks with Pa or Pw and challenged 2 weeks after the second immunization by exposure to an aerosol of live *B. pertussis*. Naïve nonimmunized mice acted as controls. The course of infection was followed by performing CFU counts on individual lung homogenates at intervals after challenge. The results are mean ( $\pm$  standard error) CFU counts for four mice per experimental group at each time point.

distinct effector mechanisms at the site of infection in mice immunized with Pa and Pw.

Consistent with a previous report from our laboratory (21), a dramatic influx of neutrophils was observed in the lungs following *B. pertussis* infection of nonimmunized mice. Neutrophil infiltration was also observed in the lungs of mice immunized with the Th1-inducing Pw, and this is consistent with the demonstration that Th1 cells are potent activators of antimicrobial effector cells, such as PMN (8), as well as the murine IgG2a antibody subclass involved in opsonization of bacteria (5, 20, 24) and neutralization of viruses (18). In contrast, the majority of infiltrating cells isolated by BAL from mice immunized with Pa were macrophages, with a small proportion of lymphocytes, but no neutrophil infiltration was observed. Furthermore, cytokine analysis of BAL fluids after challenge revealed that mice immunized with Pa had lower concentrations of IL-1 $\beta$  and increased levels of IL-6 and IL-1ra compared to naïve or Pw-immunized mice. In addition, IL-1 $\beta$  in the lungs of both naïve infected and Pw-immunized mice was detected prior to neutrophil infiltration, indicating that production of this cytokine, possibly by alveolar macrophages, represents a crucial step in the induction of an inflammatory response. Proinflammatory cytokines, such as IL-1 $\beta$ , IFN- $\gamma$ , and TNF- $\alpha$ , upregulate the expression of the adhesion molecule ICAM-1, which has been shown to mediate the efficient extravasation of PMN from the vasculature to the tissues by the ICAM-1-Mac-1-LFA-1 adhesion pathways (11, 43). Although IFN- $\gamma$  levels are higher in mice immunized with Pw, we observed increased levels of TNF- $\alpha$  in mice immunized with Pa, suggesting that in our model TNF- $\alpha$  does not play a major role in neutrophil recruitment.

Although initially thought to be a proinflammatory cytokine, recent evidence suggests that IL-6 has anti-inflammatory and immunosuppressive activities (39). Administration of anti-IL-6 has been shown to enhance the acute neutrophil exudation caused by intranasal administration of *Faeni rectivirgula* (6). Furthermore, antibody-mediated neutralization of IL-6 was found to suppress the expression of mRNA and synthesis of IL-1ra in both Peyer's patches and circulating neutrophils in a model of oral infection with *Yersinia enterocolitica* (13). These findings are in agreement with our own observations of decreased neutrophil infiltration and increased levels of IL-6 and

IL-1ra in the BAL fluids after *B. pertussis* challenge of mice immunized with Pa.

Our results suggest that recruitment of inflammatory cells into the lung is inhibited by the induction of a Th2-type response following immunization with Pa. No significant neutrophil infiltration following infection with *B. pertussis* was observed in the lungs of BALB/c or C57BL/6 mice immunized with Pa. In contrast, a significant neutrophil infiltration was observed in the lungs of infected IL-4<sup>-/-</sup> mice previously immunized with Pa. Furthermore, BAL fluids recovered from the lungs of IL-4<sup>-/-</sup> mice displayed significantly higher levels of IL-1 $\beta$  and lower levels of IL-6 and IL-1ra than the parent strain. In addition, the influx of pulmonary lymphocytes was increased in IL-4<sup>-/-</sup> mice immunized with Pa. Although there was no significant difference in the course of infection between IL-4<sup>-/-</sup> and wild-type mice immunized with either Pa or Pw, interpretation of these findings is complicated by the observation that while IgG2a antibody levels are enhanced, IFN- $\gamma$  and Th2 cytokines are reduced in immunized IL-4<sup>-/-</sup> mice (24). This is consistent with a report that IL-4 is required for the priming phase of Th1-associated tumor immunity (35). However, we did observe some enhancement of bacterial clearance in naïve (present study) and rechallenged convalescent IL-4<sup>-/-</sup> mice (24). Furthermore, we observed disseminating atypical disease following *B. pertussis* infection of naïve IFN- $\gamma$  receptor-defective (IFN- $\gamma$ R<sup>-/-</sup>) mice and a delay in bacterial clearance following challenge of IFN- $\gamma$ R<sup>-/-</sup> mice convalescing from pertussis or immunized with Pw (20, 24). These findings suggest that IFN- $\gamma$  plays a critical role in activating effector mechanisms, whereas IL-4 may play an important role in regulating the immune response and in reducing inflammation.

Our demonstration that IL-4 plays a key role in suppressing neutrophil recruitment is consistent with reports that have implicated IL-4 in controlling inflammation (33, 37, 41). In a study of antibody-induced glomerulonephritis, IL-4<sup>-/-</sup> mice were found to have increased glomerular pathology as a result of enhanced neutrophil infiltration into the glomeruli (32). Furthermore, in a murine model of immune-mediated lung injury, intratracheal administration of recombinant IL-4 inhibited neutrophil accumulation (25). In addition, recent studies both in vitro and in vivo have shown that IL-4 inhibits IL-1 $\beta$ -induced ICAM-1 upregulation on endothelial cells (11, 41).

Thus, modulation of ICAM-1 expression may be an important mechanism by which IL-4 inhibits neutrophil influx. It is possible, however, that IL-4 suppresses neutrophil migration by additional mechanisms. For example, IL-4 has been demonstrated to inhibit the production of potent neutrophil chemoattractants, such as leukotriene B<sub>4</sub> and IL-8, from monocytes (27, 37). In addition, IL-4 stimulates IL-1ra synthesis while inhibiting IL-1 production (40, 42). These findings are consistent with our observations of increased levels of IL-1 and decreased levels of IL-1ra in IL-4<sup>-/-</sup> mice immunized with Pa.

The reduced inflammatory responses following immunization with Pa compared to those with Pw, while not adversely affecting the protective efficacy, due to the compensating effect of stronger antibody responses, has the considerable benefit of reducing the reactogenicity of the vaccine. Immunization with Pw has been associated with mild-to-severe side effects, including fevers and seizures (2, 9, 10). We have recently provided evidence that the neurological complications of *B. pertussis* infection or immunization with Pw may be associated with increased proinflammatory cytokine production in the brain; IL-1β protein and mRNA expression were detected in the hippocampi and hypothalami of immunized or infected mice (15–17). In contrast, the results of clinical trials have demonstrated that Pa are considerably safer, with significantly fewer adverse systemic and neurological effects (2, 9, 10), and these vaccines did not induce proinflammatory cytokines in the brains of immunized mice (17; C. E. Loscher, S. Donnelly, K. H. G. Mills, and M. A. Lynch, unpublished data). It appears that residual active toxins, in particular PT and LPS, are largely responsible for the proinflammatory activity of Pw (17). LPS and PT stimulate IL-1β and TNF-α production (17), and this is augmented by type 1 responses, in particular IFN-γ, stimulated by LPS-driven IL-12 production (19). In contrast, Pa are devoid of active toxins and induce Th2 cells, which secrete anti-inflammatory cytokines, such as IL-4 and IL-10 (3, 5); in addition, most Pa include FHA as one of the antigens, which we have recently shown to have direct anti-inflammatory activity, capable of inhibiting IL-12 production by an IL-10-dependent mechanism (22). Thus, as a result of their anti-inflammatory activities, Pa have reduced reactogenicity following immunization and may also be associated with reduced inflammatory infiltrate and less tissue damage in the lungs following infection.

In conclusion, we have demonstrated that IL-4 produced by antigen-primed Th2 cells has a major regulatory influence on the inflammatory response following infection with the antigen-bearing pathogen. Furthermore, the data suggest that the anti-inflammatory activity of IL-4 may be mediated through IL-6 and IL-1ra. Our findings on the local inflammatory responses to infection in the lungs in immunized mice also provide direct evidence that the new-generation subunit vaccines confer protection against *B. pertussis* by immune effector mechanisms distinct from that generated with the traditional whole-cell vaccine.

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