Interleukin-12 Is Produced by Macrophages in Response to Live or Killed *Bordetella pertussis* and Enhances the Efficacy of an Acellular Pertussis Vaccine by Promoting Induction of Th1 Cells

BERNARD P. MAHON, MARK S. RYAN, FIONA GRIFFIN, AND KINGSTON H. G. MILLS*

*Infection and Immunity Laboratory, Department of Biology, St. Patrick's College,
Maynooth, County Kildare, Ireland*

Using a murine respiratory infection model, we have demonstrated previously that infection with *Bordetella pertussis* or immunization with a whole-cell pertussis vaccine induced antigen-specific Th1 cells, which conferred a high level of protection against aerosol challenge. In contrast, immunization with an acellular vaccine, consisting of the *B. pertussis* components detoxified pertussis toxin, filamentous hemagglutinin, and pertactin adsorbed to alum, generated Th2 cells and was associated with delayed bacterial clearance following challenge. In this study, we demonstrated that addition of interleukin-12 (IL-12) either in vitro or in vivo enhanced type 1 T-cell cytokine responses induced with an acellular vaccine. Furthermore, the rate of bacterial clearance in mice coinjected with IL-12 and the acellular vaccine was similar to that observed following immunization with a potent whole-cell vaccine. Analysis of IL-12 secretion by murine macrophages suggested that this cytokine is produced in vivo following *B. pertussis* infection or immunization with the whole-cell vaccine. IL-12 was detected in the supernatants of lung, splenic, and peritoneal macrophages infected with live *B. pertussis* or stimulated with heat-killed whole *B. pertussis* or *B. pertussis* lipopolysaccharide. In contrast, IL-12 could not be detected following stimulation of macrophages with the bacterial antigens filamentous hemagglutinin, detoxified pertussis toxin, and pertactin, the components of acellular vaccines. Our findings suggest that induction of endogenous IL-12 may contribute to the high efficacy of pertussis whole-cell vaccines and also demonstrate that it is possible to attain these high levels of protection with a less reactogenic acellular vaccine incorporating IL-12 as an adjuvant.

Colonization of the respiratory tract by the gram-negative coccobacillus *Bordetella pertussis* results in whooping cough, a significant cause of morbidity and mortality in human infants. Immunization with a whole-cell vaccine has proved efficacious in controlling pertussis, but concern has been raised over its reactogenicity (6, 30). Pertussis acellular vaccines are significantly less reactogenic but of varying efficacy (1, 12, 13, 30). The rational design of a high-potency acellular vaccine is dependent on a thorough understanding of the mechanism of immune protection against *B. pertussis*. Until recently, the bacterium was thought to occupy a purely extracellular niche during infection, and consequently, humoral immune mechanisms were assumed to be paramount in protection (30). However, there is increasing evidence from human and murine studies that *B. pertussis* can also occupy an intracellular niche through invasion and survival within lung macrophages and other cell types (7, 9, 33). These observations have led to a reexamination of the mechanisms of protective immunity. While antibody undoubtedly plays a role in bacterial toxin neutralization and in the prevention of bacterial attachment following transudation of circulating immunoglobulin into the lung, evidence is now emerging that cell-mediated immunity also plays a significant role in protection against *B. pertussis* (4, 20, 21, 27–29).

In previous studies (20, 29) using a murine respiratory model, we have demonstrated that protective immunity against *B. pertussis* induced by infection is mediated by a CD4$^+$ T-cell population that secreted interleukin-2 (IL-2) and gamma interferon (IFN-γ), namely, Th1 cells (24). Adoptive transfer experiments demonstrated that protection could be conferred with T cells in the absence of detectable antibody responses (20). In a study of vaccine-induced immunity, we have also reported that immunization with the whole-cell pertussis vaccine selectively induced Th1 cells, whereas an acellular vaccine, consisting of the *B. pertussis* antigens detoxified pertussis toxin (PT), filamentous hemagglutinin (FHA), and pertactin, induced Th2 cells (29). Furthermore, the induction of a Th1 response following infection or immunization with the whole-cell vaccine was associated with earlier bacterial clearance following respiratory challenge.

The polarization of CD4$^+$ T-cell cytokine production towards type 1 or type 2 responses following in vivo priming appears to be controlled by a number of factors, including the nature of the immunogen, the route of immunization, and the antigen-presenting cell and the regulatory cytokine milieu at the site of T-cell stimulation (4, 10, 25). The regulatory cytokine, IL-12, is also a key cytokine in the development of type 1 responses (15, 39). IL-12 can induce the secretion of IFN-γ by NK cells and by CD4$^+$ T cells and can promote the differentiation and development of Th1 cells from Th0 precursor populations (5, 18, 26, 35). Furthermore, IL-12 may also induce the production of opsonizing antibodies by promoting IFN-γ-mediated immunoglobulin (Ig) class switching in favor of IgG2a in the mouse (23). Since Th1 cells play an important role in the resolution of infections with intracellular organisms, IL-12 can influence the course of bacterial, viral, and parasitic infections by altering the balance of Th1 and Th2 cells in favor of IFN-γ production (8, 11, 14, 16, 26, 34, 38, 40–43).

The aim of the present study was to examine the ability of live and killed *B. pertussis* and *B. pertussis* components to stimulate IL-12 production by macrophages and to investigate the role of IL-12 in modulating the type of T-cell response to

* Corresponding author. Phone: 353 1 7083838. Fax: 353 1 7083845. Electronic mail address: kmills@may.ie.

B. pertussis induced by immunization. Our findings suggest that IL-12 is secreted by macrophages following intracellular infection with *B. pertussis* or stimulation with killed *B. pertussis* (whole-cell vaccine) but not with acellular vaccine components. Furthermore, we have demonstrated that a Th2 response normally induced following immunization of mice with an acellular vaccine preparation can be switched to a Th1 or Th0 response by incorporation of IL-12 into the vaccine formulation. The use of IL-12 as an adjuvant in an acellular pertussis vaccine significantly increased its protective efficacy; the rate of *B. pertussis* clearance from the lungs following respiratory challenge was equal to that observed with a potent whole-cell vaccine. Our findings demonstrate a regulatory influence of IL-12 on the induction of *B. pertussis*-specific Th1 cells following infection or immunization and provide further evidence for the role of Th1 cells in protective immunity against *B. pertussis*.

MATERIALS AND METHODS

Mice. Female BALB/c mice were bred and maintained under the guidelines of the Irish Department of Health. All mice were 8 to 12 weeks old at the initiation of experiments.

Cytokines and antigens. Recombinant murine IL-12 was kindly provided by Stanley Wolf, Genetics Institute Inc., Cambridge, Mass. The third British reference preparation for pertussis vaccine (88/522) was used as the whole-cell vaccine. Heat-killed *B. pertussis* for use in proliferation assays was prepared by incubation of cells at 80°C for 30 min. PT, FHA, and pertactin, prepared from *B. pertussis* Tohama, were kindly provided by Carine Capiau at SmithKline Beecham, Rixensart, Belgium. Chemically detoxified PT (PTd) for immunization experiments was prepared by treatment with 0.2 to 0.5% formaldehyde for 7 days followed by dialysis against phosphate-buffered saline (PBS) containing 0.01% formaldehyde. Inactivated PT (iPT) for use in proliferation assays was prepared by heating active PT at 80°C for 30 min (active PT refers to untreated native PT throughout). Lipopolysaccharide (LPS) from *B. pertussis* W28 (89/670) was obtained from The National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, United Kingdom. LPS from *Escherichia coli* (prepared by phenolic extraction and gel filtration chromatography) was purchased from Sigma Chemical Co., Poole, Dorset, United Kingdom.

Immunizations. In studies of the adjuvant effect of IL-12 for a pertussis acellular vaccine, groups of 20 mice received two intraperitoneal immunizations 4 weeks apart with one-fifth of a human dose (0.8 IU) of the whole-cell vaccine (88/522) or an acellular vaccine consisting of 5 μg each of FHA, pertactin, and PTd with or without recombinant murine IL-12 (0.5 μg per mouse). Control mice received PBS alone. Two weeks after the second immunization, the mice were either sacrificed to assess immune responses or challenged with *B. pertussis*.

Aerosol infection. Respiratory infection of mice was initiated by aerosol challenge by the method originally described by Sato et al. (32), with the following modifications. *B. pertussis* W28 phase I was grown under agitation conditions at 36°C in Stainer-Scholte liquid medium. Bacteria from a 48-h culture were resuspended at a concentration of approximately 2×10^{10} CFU/ml in physiological saline containing 1% casein. The challenge inoculum was administered as an aerosol over a period of 12 min with a nebulizer directed into an aerosol chamber containing groups of 20 to 24 mice. Four mice from each experimental group were sacrificed at 2 h and 2, 5, and 9 days after aerosol challenge to assess the number of viable *B. pertussis* bacteria in the lungs.

Enumeration of viable bacteria in the lungs. Lungs were removed aseptically and homogenized in 1 ml of sterile physiological saline with 1% casein on ice. One hundred microliters of undiluted homogenate or of serially diluted homogenate from individual lungs were spotted in triplicate onto each of three Bordet-Gengou agar plates, and the number of CFU was estimated after 5 days of incubation. Results are reported as the mean number of *B. pertussis* CFU for individual lungs from four mice. The limit of detection was approximately 0.5 $\log_{10}$ CFU per lung.

Macrophage production of IL-12. Murine peritoneal macrophages were obtained from naive animals by plastic adherence of cells obtained by peritoneal lavage. Splenic macrophages were prepared by plastic adherence, and alveolar macrophages were isolated by bronchoalveolar lavage as described previously (29). The murine macrophage cell line J774 was also used in studies of IL-12 production. Macrophages were infected with viable phase I *B. pertussis* bacteria at a bacterium-to-macrophage ratio of 5:1 for 2 h before extensive washing. Extracellular bacteria were killed by treatment with polymyxin B sulfate (100 μg/ml) for 40 min followed by further washing. This treatment reduces the number of extracellular bacteria by 5.0 $\log_{10}$ CFU. Infected macrophages or macrophages stimulated with heat-inactivated bacteria or bacterial antigens were cultured at 2×10^5 cells per ml at 37°C in a 5% CO_2 atmosphere. After 24 or 48 h, cell culture supernatants were removed and the production of IL-12 was determined by bioassay or immunoassay.

T-cell proliferation assays. Spleen cells from immunized or control mice were

tested for in vitro proliferation against heat-killed *B. pertussis* (10^6/ml), heat-inactivated PT (1.0 μg/ml), FHA (1.0 μg/ml), and pertactin (1.0 μg/ml) as described previously (20). Results were calculated as mean counts per minute of [^{3}H]thymidine incorporation for triplicate cultures for groups of four to six mice. Stimulation indices were calculated by dividing the proliferative response to the antigens by the response of control cultures, in which cells were stimulated with medium alone.

Analysis of cytokine production. T-cell cytokine production was assessed with spleen cells stimulated with *B. pertussis* antigens in vitro. Spleen cells (2×10^6/ml) from immunized or naive control mice were cultured with antigens or with medium alone (background control), and supernatants were removed after 24 h to determine IL-2 and after 72 h to determine IFN-γ, IL-4, and IL-5. IL-2 release was assessed by the ability of culture supernatants to support the proliferation of the IL-2-dependent CTLL-2 cell line, and the concentrations of murine IL-4, IL-5, and IFN-γ were determined by specific immunoassays using commercially available antibodies (PharMingen, San Diego, Calif.) as described previously (17). IL-12 was determined by immunoassay and bioassays. In the immunoassay, commercially available (Genzyme Diagnostics, Cambridge, Mass.) anti-IL-12 monoclonal antibodies, clones C17.8 (rat IgG2a) and C15.6 (rat IgG1), which recognize the p40 subunit of murine IL-12 either as a monomer, as homodimers, or as part of the p70 heterodimer, were used for capture and detection, respectively. An alkaline phosphatase-conjugated mouse anti-rat IgG1 (PharMingen) was used to detect the second anti-IL-12 antibody. Biologically active IL-12 concentrations were assessed by the ability of test supernatants to stimulate the production of IFN-γ by naive spleen cell preparations. To ensure that the production of IFN-γ was due to the presence of IL-12, test samples were also assayed in the presence and absence of a specific anti-IL-12 neutralizing antibody (2.5 μg of protein G-purified sheep anti-murine IL-12 per ml; provided by Stanley Wolf, Genetics Institute, Inc., which can completely neutralize up to 5 ng of IL-12 per ml. Cytokine concentrations were determined by comparing either the proliferation or the optical density at 492 nm for test samples with a standard curve for recombinant cytokines of known concentration.

RESULTS

Macrophages secrete IL-12 in response to live or killed *B. pertussis*. Our previous studies have demonstrated that respiratory infection with *B. pertussis* or immunization with the pertussis whole-cell vaccine selectively stimulates Th1 cells in vivo, whereas an acellular vaccine consisting of PTd, FHA, and pertactin adsorbed to alum generated predominantly Th2 cells (4, 29). One explanation for this dichotomy may be the induction of endogenous IL-12 secretion by macrophages exposed to live or killed whole bacteria or bacterial components not present in the acellular vaccine. Therefore, we examined the ability of killed whole *B. pertussis* and *B. pertussis* components to stimulate the production of IL-12 by murine macrophages. Adherent cells from the spleens of naive mice stimulated with heat-killed *B. pertussis* produced significant levels of IL-12, as detected by an immunoassay specific for p40 and p70 (Fig. 1A). Moderate levels of IL-12 were also detected in supernatants from macrophages incubated with LPS derived from either *B. pertussis* or another gram-negative bacterium, *E. coli*. These levels were enhanced when IFN-γ was added to the cultures (data not shown). In contrast, little or no IL-12 was produced by macrophages stimulated with FHA, PTd, or pertactin, the components of the acellular vaccine (Fig. 1A). Peritoneal macrophages also produced IL-12 in response to stimulation with heat-killed *B. pertussis* in a dose-dependent manner (Fig. 1A).

To demonstrate that the IL-12 produced was biologically active, we also tested IL-12 production with a bioassay which measured the production of IFN-γ by murine spleen cells in the presence or absence of a neutralizing polyclonal anti-IL-12 antibody. Supernatants from splenic macrophages that had been stimulated with killed bacteria or purified LPS induced naive spleen cells to produce high levels of IFN-γ, which was inhibited by the anti-IL-12 antibody (Fig. 1B). Although supernatants from spleen cells stimulated with active PT did stimulate the production of IFN-γ, this response could not be ablated by the addition of the anti-IL-12 antibody. Furthermore, IL-12 was not detected in supernatants of PT-stimulated macrophages by the immunoassay (Fig. 1A). Therefore, it is
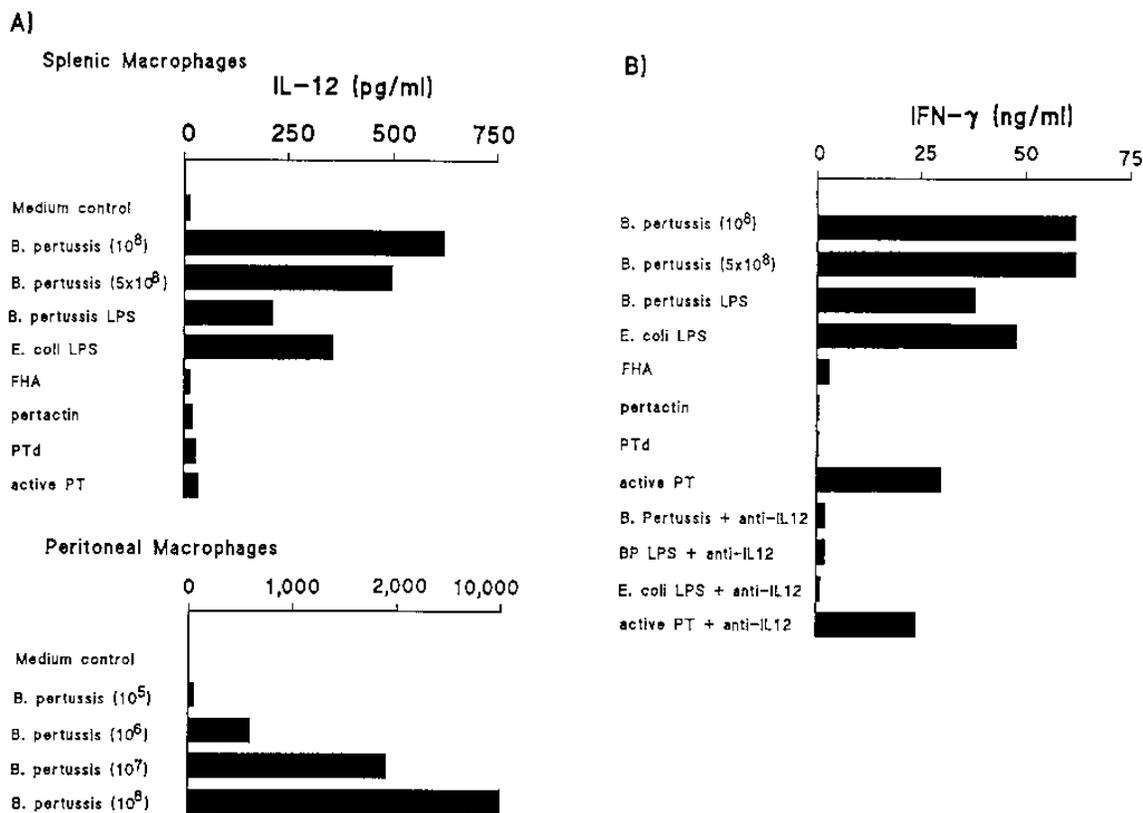
FIG. 1. Macrophage production of IL-12 in response to whole *B. pertussis* cells and bacterial components. IL-12 was tested by immunoassay (A) or by bioassay (B). Results from the immunoassay, which detects p40 and p70, are mean concentrations in supernatants from triplicate cultures of splenic macrophages incubated with heat-killed *B. pertussis* (1×10^8/ml and 5.0×10^8/ml), *B. pertussis* LPS (1 μg/ml), *E. coli* LPS (1 μg/ml), FHA (1 μg/ml), pertactin (1 μg/ml), active PT (1 μg/ml), PTd (1 μg/ml), or peritoneal macrophages incubated with increasing doses (10^5 to 10^8 CFU/ml) of heat-killed *B. pertussis*. The bioassay measured the production of IFN-γ by naive spleen cells incubated for 24 h with supernatants from splenic macrophages (stimulated as described for the immunoassay) in the presence or absence of a polyclonal neutralizing anti-IL-12 at 2.5 μg/ml. Levels of IFN-γ produced in the presence of anti-IL-12 antibody are only shown where positive responses were observed in the absence of the antibody and with one dose (5.0×10^8/ml) of the killed bacteria. Results are means for triplicate assays and are representative of four independent experiments. Standard deviations were less than 20% of the mean values.

unlikely that active PT induces IL-12 from macrophages. Active PT is mitogenic for murine T cells, and we have found that it promotes IFN-γ produced by purified splenic T cells in the presence of irradiated accessory cells (31a). Therefore, the IFN-γ detected in the IL-12 bioassay with supernatants from macrophages stimulated with active PT is likely to result from direct stimulation of T cells in the spleen cell population by active PT carried over in the macrophage supernatants.

Consistent with previous reports (9, 33), we found that live *B. pertussis* can be taken up and survive within macrophages. We therefore examined the production of IL-12 by macrophages following infection with *B. pertussis*. Murine macrophages from different sources were cultured with live *B. pertussis* for 2 h, washed extensively, and treated with antibiotics to remove extracellular organisms. Culture supernatants were tested for IL-12 after 24 and 48 h. The results in Table 1 demonstrate that murine macrophages derived from the lungs or peritoneal cavity or in the form of an established cell line produce biologically active IL-12 when infected with *B. pertussis*. Although the levels of IL-12 are not as high as that observed following stimulation of peritoneal macrophages with killed bacteria (Fig. 1A), this may reflect the lower concentration of live bacteria used in this experiment. Higher levels of viable *B. pertussis* bacteria were employed in other experiments but resulted in cell death of the macrophage populations used in vitro. In separate experiments, supernatants of alveolar,

peritoneal, J774, and splenic macrophages removed 24 and 48 h after infection with *B. pertussis* were also found to contain IL-12 detected by the immunoassay (data not shown). Furthermore, peritoneal macrophages recovered from mice 24 h after intraperitoneal injection with live *B. pertussis* secreted signifi-

TABLE 1. Murine macrophages secrete IL-12 following infection with *B. pertussis*[a]

Macrophage type	Infected	IFN-γ (pg/ml)[b]	
		−Ab	+Ab
Alveolar	−	<50	<50
	+	700 (±41)	100 (±16)
Peritoneal	−	<50	<50
	+	30,000 (±2,245)	75 (±31)
J774	−	<50	<50
	+	900 (±66)	<50

[a] Macrophages were infected with *B. pertussis* for 2 h and extensively washed and treated with polymyxin B to kill extracellular bacteria prior to culture in the presence (+Ab) or absence (−Ab) of a neutralizing anti-IL-12 antibody. IL-12 production was assessed with a bioassay which measured the production of IFN-γ by naive spleen cells incubated for 24 h with supernatants from infected (+) or control uninfected (−) macrophages.

[b] Results are expressed as the mean (± standard deviation) IFN-γ concentrations in the supernatants of triplicate cultures measured by immunoassay.
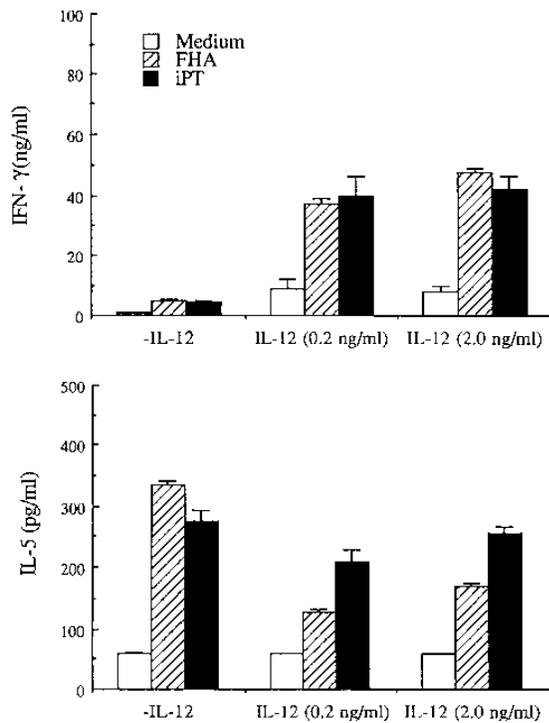
FIG. 2. Addition of IL-12 in vitro augments IFN-γ production by spleen cells from mice primed for a Th2 response. Mice were immunized with FHA and PTd in alum, and spleen cells were stimulated in vitro with FHA or iPT (1.0 μg/ml) or medium alone in the presence of 0, 0.2, and 2.0 ng of recombinant murine IL-12 per ml. The levels of IFN-γ and IL-5 were tested in spleen cell supernatants after 72 h. Results are expressed as the mean (± standard error) cytokine concentration for stimulated spleen cells from four mice per group tested in triplicate.

cant levels of IL-12 (569 pg/ml of culture supernatant in one experiment) without further stimulation in vitro.

Antigen activation of T cells in vitro in the presence of IL-12 modulates an established Th2 response. Immunization of mice with FHA and PTd adsorbed to alum generated a potent Th2 response; ex vivo spleen cells produced high levels of IL-5 and low levels of IFN-γ following specific antigen stimulation in vitro (Fig. 2). However, the addition of 0.2 or 2.0 ng of recombinant murine IL-12 per ml to the spleen cells during antigen stimulation in culture resulted in significantly increased concentrations of IFN-γ and marginally reduced levels of IL-5 (Fig. 2), demonstrating that IL-12 can modulate the pattern of in vitro cytokine secretion by in vivo-primed T cells.

Enhanced T-cell proliferative responses and type 1 cytokine secretion following coinjection of *B. pertussis* antigens with IL-12 in the presence or absence of alum. We tested the ability of IL-12 to modulate immune responses to *B. pertussis* antigens in vivo by immunization of mice with FHA and PTd in the presence or absence of alum. Two weeks after immunization with FHA and PTd in solution, the in vitro proliferative responses of spleen cells against the specific antigens were similar to that observed against medium alone (Table 2). In contrast, immunization with FHA and PTd in the presence of IL-12 resulted in enhanced proliferative responses to FHA, iPT (Table 2), and killed whole bacteria (data not shown). The addition of IL-12 to the alum-adsorbed antigens also augmented the *B. pertussis*-specific proliferative responses, although this did not reach a level of statistical significance (Table 2).

Coinjection of soluble antigens and IL-12 enhanced the level of IFN-γ secreted in vitro by antigen-stimulated spleen cells; IL-5, a Th2 type cytokine, was not detected from spleen cells from these animals (Table 2). In contrast, spleen cells from mice immunized with FHA and PTd in the presence of alum secreted high levels of IL-5 and moderate levels of IFN-γ, confirming the known effect of alum to favor the induction of Th2 type responses in mice. However, coinjection of IL-12 with FHA and PTd adsorbed to alum resulted in a reduction in IL-5 production but not a significant increase in the level of IFN-γ secreted, when compared with spleen cells from animals which had received antigens formulated with alum in the absence of IL-12 (Table 2).

Incorporation of IL-12 in a pertussis acellular vaccine augments protection against *B. pertussis* challenge by promoting the induction of Th1 cells. Since we had previously demonstrated that a highly protective whole-cell vaccine induces a Th1 response, we decided to compare the immune responses and levels of protection induced with a whole-cell vaccine and with an acellular vaccine administered in the presence or absence of IL-12. Groups of mice received two immunizations 4 weeks apart with either a whole-cell vaccine, an acellular vac-

TABLE 2. Coinjection with IL-12 augments cellular immune responses to *B. pertussis* antigens[a]

| Immunization | In vitro stimulation | Proliferation | | IFN-γ (ng/ml) | IL-5 (pg/ml) |
		cpm	SI		
FHA + PTd	Medium	118 ± 40		−	−
	FHA	129 ± 53	1.1 ± 0.4	1.8 ± 0.5	−
	iPT	98 ± 34	0.8 ± 0.4	2.2 ± 0.8	−
FHA + PTd + IL-12	Medium	1,347 ± 191		−	−
	FHA	8,412 ± 1,491	6.2 ± 0.9*	12.3 ± 1.0**	−
	iPT	8,985 ± 3,793	6.7 ± 1.6*	11.0 ± 0.9**	−
FHA + PTd + alum	Medium	2,380 ± 168		−	−
	FHA	13,264 ± 3,010	5.6 ± 2.8	5.7 ± 0.6	410 ± 70
	iPT	6,389 ± 2,123	2.7 ± 0.8	12.1 ± 1.3	430 ± 60
FHA + PTd + alum + IL-12	Medium	1,546 ± 823		−	−
	FHA	17,953 ± 4,436	11.6 ± 3.4	7.4 ± 3.0	140 ± 30*
	iPT	7,706 ± 4,128	5.0 ± 3.0	16.3 ± 2.0	110 ± 20*

[a] Spleen cells from mice immunized with soluble or alum-adsorbed FHA and PTd, with or without IL-12 (0.5 μg), were stimulated in vitro with iPT (1.0 μg/ml), FHA (5.0 μg/ml), or medium alone. Proliferative responses were measured by [³H]thymidine incorporation after 4 days and are expressed as counts per minute and stimulation indices (SI). The levels of IFN-γ and IL-5 were tested in supernatants after 72 h of culture. Results are mean (± standard deviation) responses for triplicate cultures for four mice in each group. −, below the level of detection. * and **, P < 0.01 and P < 0.001, respectively, compared with the corresponding value for mice immunized in the absence of IL-12, determined by Student's t test.
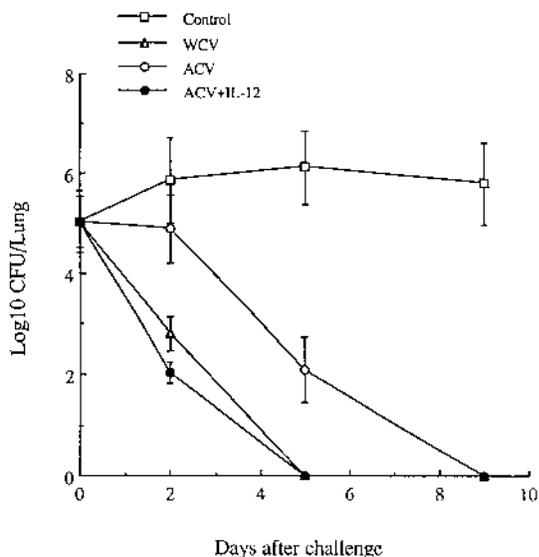
FIG. 3. IL-12 as an adjuvant enhances the protective efficacy of an acellular pertussis vaccine. BALB/c mice were immunized at 0 and 4 weeks with a whole-cell vaccine (WCV), an acellular vaccine (5 μg each of soluble PTd, FHA, and pertactin) with or without 0.5 μg of IL-12 (ACV + IL-12 and ACV, respectively), or PBS alone (control). Mice were challenged by aerosol inoculation with *B. per-tussis* 2 weeks after the second immunization. The course of respiratory infection was monitored by performing viable counts at intervals after challenge. Results are mean (± standard error) CFU counts performed on individual lungs in triplicate for four mice per group at each time point.

cine preparation in solution in the presence or absence of IL-12 (0.5 μg per mouse), or PBS alone as a control. Mice were challenged by exposure to a *B. pertussis* aerosol 2 weeks after the second immunization, and the course of infection was monitored by performing CFU counts on the lungs at intervals after respiratory challenge. The levels of bacteria in the control mice were still high 9 days after challenge (Fig. 3). The time course of respiratory infection in mice immunized with the whole-cell vaccine was very short, with complete clearance by day 5. Bacterial clearance in mice immunized with the acellular vaccine was slower; complete clearance did not occur until day 9 postchallenge. However, the addition of IL-12 to the acellu-lar vaccine formulation significantly enhanced its protective efficacy. Bacterial clearance was complete by day 5, and the bacterial burden on day 2 was lower than that observed in mice immunized with the whole-cell vaccine (Fig. 3).

To confirm our earlier suggestions on the protective role of Th1 cells, and to establish that the superior protective efficacy observed with the acellular vaccine injected with IL-12 was due to enhanced cell-mediated immunity, we tested the immune response of the immunized mice on the day of challenge. Proliferative responses were detected against whole killed *B. pertussis*, FHA, iPT, and pertactin in spleen cells from mice immunized with the acellular vaccine, but these were signifi-cantly enhanced in the presence of IL-12 and approached the levels observed with the whole-cell vaccine (reference 29 and data not shown). An examination of the cytokine profiles pro-duced by spleen cells stimulated with specific antigen in vitro revealed that spleen cells derived from mice immunized with the whole-cell vaccine secreted IL-2 and IFN-γ but no detect-able IL-5 (Fig. 4). In contrast, spleen cells from mice which received the acellular vaccine in the absence of IL-12 secreted low levels of IL-2 and IFN-γ and low but detectable levels of IL-5. However, spleen cells from mice immunized with the acellular vaccine in the presence of IL-12 secreted significant

levels of IL-2 and IFN-γ. Interestingly, IL-12 appeared to have differential effects on T cells of different antigen specificities, potentiating IL-2 production by T cells specific for FHA and pertactin and IFN-γ production by PT-specific T cells. Overall, the immune response induced with the acellular vaccine incor-porating IL-12 as an adjuvant is best described as a mixed Th1-Th2 or Th0 profile.

DISCUSSION

The significant new findings of this study are that tissue macrophages, including those recovered from the lungs, spleen, or peritoneal cavity of naive mice, produce IL-12 fol-lowing exposure to live or killed *B. pertussis* and that the ad-dition of IL-12 as an adjuvant to a pertussis acellular vaccine enhances its protective efficacy by promoting type 1 T-cell cytokine production. We have demonstrated that immuniza-
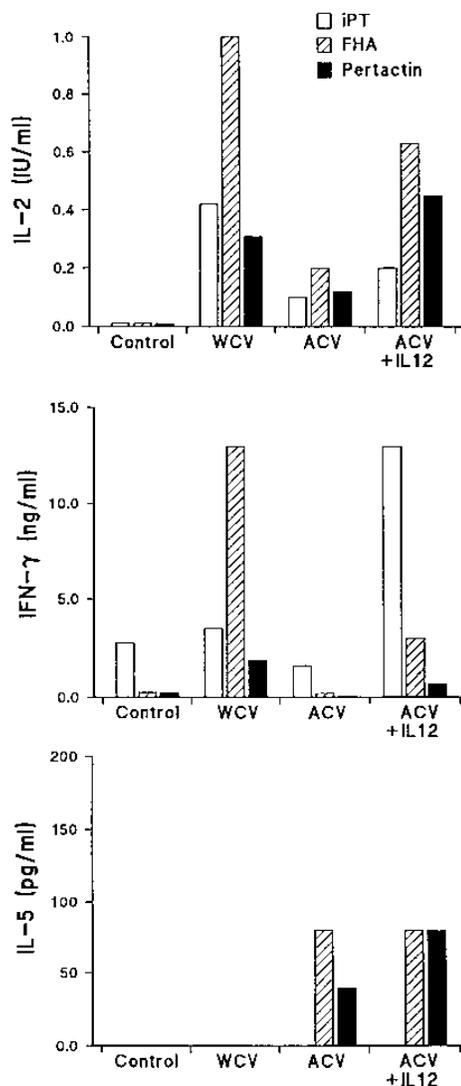

FIG. 4. IL-12 switches a Th2 to a Th1-Th0 response with an acellular per-tussis vaccine. Mice were immunized as described in the legend to Fig. 3, and the secretion of IL-2, IFN-γ, and IL-5 was tested following stimulation of spleen cells with iPT (0.2 to 1.0 μg/ml), FHA (0.2 to 5.0 μg/ml), and pertactin (0.2 to 5.0 μg/ml). Results are expressed as the mean cytokine concentration to the opti-mum concentration of antigen for spleen cells from four mice per group tested in triplicate.

tion of mice with a pertussis acellular vaccine consisting of PTd, FHA, and pertactin adsorbed to alum generated a Th2 response in mice and was associated with delayed bacterial clearance following respiratory challenge (4, 29). In contrast, previous infection or immunization with a whole-cell vaccine induced Th1 cells and conferred a high level of protection (20, 28, 29). Here we provide one explanation for the Th1-Th2 cell dichotomy in acquired immunity to *B. pertussis*. We demonstrate that macrophages secrete IL-12 following infection with *B. pertussis* or stimulation with the whole-cell vaccine or *B. pertussis* LPS but not with PT, FHA, or pertactin, the components of the acellular vaccine. Furthermore, the addition of exogenous IL-12 to the acellular vaccine results in a switch in the response from Th2 to Th1 or Th0 and significantly augmented bacterial clearance following respiratory challenge, such that the protective efficacy exceeded that observed with a potent whole-cell vaccine.

Since colonization of the respiratory tract by *B. pertussis* was considered to be exclusively extracellular, it has long been assumed that vaccine-induced immunity is mediated through the generation of humoral immune responses (6, 30). Therefore, it might be predicted that protection would be associated with a type 2 T-cell response. While it is likely that local or serum antibodies that have transudated into the lung do play a role in preventing bacterial attachment and in the neutralization of bacterial toxins, there is now convincing evidence that cell-mediated immunity is also required for clearance of bacteria from the lungs (4, 20, 21, 27–29). We have previously demonstrated the involvement of Th1 cells (4, 29), and the present study provides evidence of a role for IL-12 in protective immunity against *B. pertussis*. It has been reported that endogenously produced IL-12 plays a role in resistance to a number of intracellular pathogens, such as *Leishmania major*, *Schistosoma mansoni*, *Toxoplasma gondii*, *Listeria monocytogenes*, *Cryptosporidium parvum*, *Brucella abortus*, and *Mycobacterium tuberculosis*, through the generation of a robust Th1 response (8, 11, 14, 16, 38, 40–43). The present study demonstrates that murine macrophages, including those obtained from the lungs, peritoneal cavity, and spleen, produce IL-12 in response to infection with *B. pertussis* or stimulation with heat-killed bacteria. This provides indirect evidence of a role for endogenous IL-12 in immunity to *B. pertussis* following respiratory infection or immunization with the whole-cell pertussis vaccine.

The whole-cell pertussis vaccine, which is composed of heat-killed bacteria, is effective at preventing whooping cough in vaccinated infants, and it has been suggested that the efficacy of this vaccine may be related to levels of LPS as well as residual active PT (3, 6, 30). The results of the present study demonstrate that LPS appears to be a major component of *B. pertussis* responsible for stimulating IL-12 production by macrophages. However, since IFN-γ augments IL-12 production by macrophage in response to LPS (37), PT may also be involved in initiating the cytokine cascade through the stimulation of IFN-γ secretion by T cells (31a). This suggests that the high protective efficacy of whole-cell vaccines may reside in part in their ability to induce IL-12 through the presence of low but significant levels of LPS and active PT. Paradoxically, certain of the unwanted side effects of the whole-cell vaccine have also been attributed to LPS and active PT (6, 36). Indeed, concerns over the reactogenicity of the whole-cell vaccine have led to considerable interest in the development of efficacious subunit or acellular vaccines against *B. pertussis*. Recent clinical trials have demonstrated that certain acellular vaccines are effective at preventing severe disease in infants when administered with alum as the adjuvant (12, 13). Although alum is the adjuvant of choice for human use, we and others have shown that immunization with purified native or recombinant proteins adsorbed to alum favors the induction of Th2 responses in mice (4, 22). Furthermore, analysis of cytokine production by peripheral T cells from infants immunized with a pertussis acellular vaccine revealed a mixed Th1-Th2 or Th0 profile (31). Our studies with the murine respiratory model clearly indicated that early bacterial clearance following respiratory challenge of immune mice is associated with the induction of T cells that secrete IL-2 and IFN-γ (4, 29).

IL-12 has been shown to possess adjuvant properties in a murine model of vaccine-mediated protection against *Leishmania major*, *Listeria monocytogenes*, *T. gondii*, and a neurotropic herpesvirus through the preferential induction of Th1 type responses (2, 11, 19, 34). In the present study, we demonstrate that coinjection of IL-12 with a pertussis acellular vaccine enhances cell-mediated responses to *B. pertussis* antigens and significantly improves its protective efficacy against respiratory challenge. Incorporation of IL-12 into the vaccine formulation enhanced the levels of type 1 cytokines, in particular, IFN-γ. The pattern of antigen-specific T-cell cytokine secretion was also altered when administered with alum-adsorbed antigens, an adjuvant which strongly favors a polarized Th2 response in mice. The addition of IL-12 in this situation results in a reduction in the levels of IL-5 observed and an increase in IFN-γ, demonstrating the ability of IL-12 to modulate T-cell responses even under conditions favorable to the induction of a Th2 response. Furthermore, the addition of IL-12 to cultures of spleen cells from mice immunized with an acellular vaccine promoted antigen-induced IFN-γ production. This suggests that IL-12 is also capable of switching on type 1 cytokine production by T-cell populations which appeared to be primed for a type 2 response.

It is not known which of the pleiotropic effects of IL-12 enhance vaccine-mediated bacterial clearance. We have previously suggested that *B. pertussis*-specific cell-mediated immunity may be required for the destruction of an intracellular reservoir of bacteria in the lungs (20, 21). The present findings are consistent with this model in which the presence of IL-12 in an acellular vaccine formulation enhances IFN-γ production by *B. pertussis*-specific Th1 cells, which augments bacterial clearance by activating the intracellular killing of bacteria by macrophages. Furthermore, the induction of a Th1 response promoted by IL-12 may enhance bacterial uptake by macrophages through the induction of *B. pertussis*-specific opsonizing antibody.

In conclusion, the present study demonstrates that murine macrophages secrete IL-12 when stimulated with live or killed *B. pertussis* but not with the putative protective antigens FHA, PT, or pertactin. One mechanism whereby the whole-cell vaccine generates an efficacious Th1 response might be through macrophage production of IL-12 in vivo in response to LPS or other components of the killed bacterial preparation. Conversely, the poor production of IL-12 from macrophages stimulated with components of the acellular vaccine may explain the predominant Th2 response in mice immunized with this vaccine. The adjuvant properties of IL-12 and its ability to modulate the profile of cytokines secreted by CD4+ T-cell populations suggest that IL-12 might prove a useful addition to more efficacious acellular vaccines against pertussis.

Capiau, SmithKline Beecham, for providing purified *B. pertussis* antigens.

REFERENCES

1. **Ad Hoc Group for the Study of Pertussis Vaccine.** 1988. Placebo-controlled trial of two acellular pertussis vaccines in Sweden—protective efficacy and adverse events. Lancet **i:**955–960.
2. **Afonso, L. C. C., T. M. Scharton, L. Q. Vieira, M. Wyssocka, G. Trinchieri, and P. Scott.** 1994. The adjuvant effect of interleukin-12 in a vaccine against *Leishmania major*. Science **263:**235–237.
3. **Bannatyne, R. M., and J. Jackowski.** 1987. Protective efficacy of an endotoxin-depleted pertussis vaccine. Vaccine **5:**268–269.
4. **Barnard, A., B. P. Mahon, J. Watkins, K. Redhead, and K. H. G. Mills.** 1996. Th1/Th2 cell dichotomy in acquired immunity to *Bordetella pertussis*: variables in the in vivo priming and in vitro cytokine detection techniques affect the classification of T cell subsets as Th1, Th2 or Th0. Immunology **87:**372–380.
5. **Bliss, J., V. V. Cleave, K. Murray, A. Wiencis, M. Ketchum, R. Maylor, T. Haire, C. Resmini, A. K. Abbas, and S. F. Wolf.** 1996. IL-12, as an adjuvant, promotes a T helper 1 cell, but does not suppress a T helper 2 cell recall response. J. Immunol. **156:**887–894.
6. **Cherry, J. D., P. A. Brunnell, G. S. Golden, and D. T. Karzon.** 1988. Report of the task force on pertussis and pertussis immunization—1988. Pediatrics **81**(Suppl.)**:**939–984.
7. **Ewanowich, C. A., A. R. Melton, A. A. Weiss, R. K. Sherburne, and M. S. Peppler.** 1989. Invasion of HeLa 229 cells by virulent *Bordetella pertussis*. Infect. Immun. **57:**2698–2704.
8. **Flynn, J. L., M. M. Goldstein, K. J. Triebold, J. Sypek, S. F. Wolf, and B. R. Bloom.** 1995. IL-12 increases resistance of BALB/c mice to *Mycobacterium tuberculosis* infection. J. Immunol. **155:**2515–2524.
9. **Friedman, R. L., K. Nordensson, L. Wilson, E. T. Akporiaye, and D. E. Yocum.** 1992. Uptake and intracellular survival of *Bordetella pertussis* in human macrophages. Infect. Immun. **60:**4578–4585.
10. **Gajewski, T. F., M. Pinnas, T. Wong, and F. W. Fitch.** 1991. Murine Th1 and Th2 clones proliferate optimally in response to distinct antigen presenting cell populations. J. Immunol. **146:**1750–1758.
11. **Gazzinelli, R. T., S. Hieny, T. A. Wynn, S. Wolf, and A. Sher.** 1993. Interleukin 12 is required for T-lymphocyte-independent induction of interferon-γ by an intracellular parasite and induces resistance in T-cell-deficient hosts. Proc. Natl. Acad. Sci. USA **90:**6115–6119.
12. **Greco, D., S. Salmaso, P. Mastrantonio, M. Guiliano, A. G. Tozzi, A. Anemona, M. L. Giori Delgi Atti, A. Giammanco, P. Panel, W. C. Black-welder, D. L. Klein, S. G. F. Wassilak, and the Progetto Pertosase Working Group.** 1996. A controlled trial of two acellular vaccines and one whole-cell vaccine against pertussis. N. Engl. J. Med. **334:**341–348.
13. **Gustafsson, L., H. O. Hallander, P. Olin, E. Reizenstein, and J. Storsaeter.** 1996. A controlled trial of a two-component acellular, a five component acellular, and a whole cell pertussis vaccine. N. Engl. J. Med. **334:**349–355.
14. **Heinzel, F. P., D. S. Schoenhaut, R. M. Rerko, L. E. Rosser, and M. K. Gately.** 1993. Recombinant interleukin 12 cures mice infected with *Leishmania major*. J. Exp. Med. **177:**1505–1509.
15. **Hsieh, C.-S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy.** 1993. Development of Th1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science **260:**547–549.
16. **Hunter, C. A., C. S. Subauste, V. H. van Cleave, and J. S. Remington.** 1994. Production of gamma interferon by natural killer cells from *Toxoplasma gondii*-infected SCID mice: regulation by interleukin-10, interleukin-12, and tumor necrosis factor alpha. Infect. Immun. **62:**2818–2824.
17. **Mahon, B. P., K. Katrak, A. Nomoto, A. J. Macadam, P. D. Minor, and K. H. G. Mills.** 1995. Poliovirus-specific CD4+ Th1 clones with both cytotoxic and helper activity mediate protective humoral immunity against a lethal poliovirus infection in transgenic mice expressing the human poliovirus receptor. J. Exp. Med. **181:**1285–1292.
18. **McKnight, A. J., G. J. Zimmer, I. Fogelman, S. F. Wolf, and A. A. Abbas.** 1994. Effects of IL-12 on helper T cell-dependent immune responses in vivo. J. Immunol. **152:**2172–2179.
19. **Miller, M. A., M. J. Skeen, and H. K. Ziegler.** 1995. Nonviable bacterial antigens administered with IL-12 generate protective immunity against *Listeria monocytogenes*. J. Immunol. **155:**4817–4828.
20. **Mills, K. H. G., A. Barnard, J. Watkins, and K. Redhead.** 1993. Cell-mediated immunity to *Bordetella pertussis*: role of Th1 cells in bacterial clearance in a murine respiratory infection model. Infect. Immun. **61:**399–410.
21. **Mills, K. H. G., and K. Redhead.** Cellular immunity in pertussis. 1993. J. Med. Microbiol. **39:**163–164.
22. **Moore, A., P. McGuirk, S. Adams, W. C. Jones, J. P. McGee, D. T. O'Hagan, and K. H. G. Mills.** 1995. Induction of HIV-specific CD8+ cytotoxic T lymphocytes and CD4+ Th1 cells by immunization with recombinant gp120 entrapped in biodegradable microparticles. Vaccine **13:**1741–1749.
23. **Morris, S. C., K. B. Madden, J. J. Adamovicz, W. C. Gause, B. R. Hubbard, M. C. Gately, and F. D. Finkleman.** 1994. Effects of Il-12 on in vivo cytokine gene expression and Ig isotype selection. J. Immunol. **152:**1047–1056.
24. **Mosmann, T. R., and R. L. Coffman.** 1989. Heterogeneity of cytokine secretion patterns and functions of helper T cells. Adv. Immunol **46:**111–147.
25. **O'Gara, A., and K. Murphy.** 1994. Role of cytokines in determining T-lymphocyte function. Curr. Opin. Immunol. **6:**458–466.
26. **Orange, J. S., and C. A. Biron.** 1996. An absolute and restricted requirement for IL-12 in natural killer cell IFN-γ production and antiviral defense. Studies of natural killer and T cell responses in contrasting viral infections. J. Immunol. **156:**1138–1142.
27. **Peppoloni, S., L. Nencioni, A. Di Tommaso, A. Tagliabue, P. Parronchi, S. Romagnani, R. Rappuoli, and M. T. De Magistris.** 1991. Lymphokine secretion and cytotoxic activity of human CD4+ T-cell clones against *Bordetella pertussis*. Infect. Immun. **59:**3768–3773.
28. **Peterson, J. P., P. H. Ibsen, K. Haslǿv, and I. Heron.** 1992. Proliferative responses and gamma interferon and tumor necrosis factor production by lymphocytes isolated from tracheobroncheal lymph nodes and spleens of mice aerosol infected with *Bordetella pertussis*. Infect. Immun. **60:**4563–4570.
29. **Redhead, K., A. Barnard, J. Watkins, and K. H. G. Mills.** 1993. Effective immunization against *Bordetella pertussis* respiratory infection in mice is dependent on the induction of cell-mediated immunity. Infect. Immun. **61:**3190–3198.
30. **Robinson, A., L. I. Irons, and L. A. E. Ashworth.** 1985. Pertussis vaccine: present status and future prospects. Vaccine **3:**11–22.
31. **Ryan, M., L. Storsaeter, and K. H. G. Mills.** *B. pertussis*-specific Th1/Th2 cells generated following respiratory infection or immunization with an acellular vaccine: comparison of the T cell cytokine profiles in infants and mice. Dev. Biol. Stand., in press.
31a.**Ryan, M. S., and K. H. G. Mills.** Unpublished observations.
32. **Sato, Y., K. Izumiya, H. Sato, J. L. Cowell, and C. R. Manclark.** 1980. Aerosol infection of mice with *Bordetella pertussis*. Infect. Immun. **29:**261–266.
33. **Saukkonen, K., C. Cabellos, M. Burroughs, S. Prasad, and E. Tuomanen.** 1991. The role of the interaction between *Bordetella pertussis* and macrophages in pulmonary colonization. J. Exp. Med. **173:**1143–1149.
34. **Schijns, V. E. C. J., B. L. Haagmans, and M. C. Horzinek.** 1995. IL-12 stimulates an antiviral type 1 cytokine response but lacks adjuvant activity in IFN-γ-receptor deficient mice. J. Immunol. **155:**2525–2532.
35. **Seder, R. A., R. Gazzinelli, A. Sher, and W. E. Paul.** 1993. Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon-γ production and diminishes interleukin 4 inhibition of such priming. Proc. Natl. Acad. Sci. USA **90:**10188–10192.
36. **Sidley, F. M., B. L. Furman, and A. C. Wardlaw.** 1989. Effect of hyperreactivity to endotoxin on the toxicity of pertussis vaccine and pertussis toxin in mice. Vaccine **7:**237–241.
37. **Skeen, M. J., M. A. Miller, T. M. Shinnick, and H. K. Ziegler.** 1996. Regulation of murine macrophage IL-12 production. Activation of macrophages in vivo, restimulation in vitro, and modulation by other cytokines. J. Immunol. **156:**1196–1206.
38. **Sypek, J. P., C. L. Chung, S. E. H. Mayor, J. M. Subramanyam, S. J. Goldman, D. S. Sieburth, S. F. Wolf, and R. G. Schaub.** 1993. Resolution of cutaneous leishmaniasis: interleukin 12 initiates a protective T helper type 1 immune response. J. Exp. Med. **177:**1797–1802.
39. **Trinchieri, G.** 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu. Rev. Immunol. **13:**251–276.
40. **Tripp, C. S., M. K. Gately, J. Hakimi, P. Ling, and E. R. Unanue.** 1994. Neutralization of IL-12 decreases resistance to Listeria in SCID and CB17 mice: reversal by IFN-γ. J. Immunol. **152:**1883–1887.
41. **Urban, J. F., R. Fayer, S.-J. Chen, W. C. Gause, M. K. Gately, and F. D. Finkelman.** 1996. IL-12 protects immunocompetent and immunodeficient neonatal mice against infection with *Cryptosporidium parvum*. J. Immunol. **156:**263–268.
42. **Wynn, T. A., I. Eltoum, I. P. Oswald, A. W. Cheever, and A. Sher.** 1994. Endogenous interleukin-12 (IL-12) regulates granuloma formation induced by eggs of *Schistosoma mansoni* and exogenous IL-12 both inhibits and prophylactically immunizes against egg pathology. J. Exp. Med. **179:**1551–1561.
43. **Zhan, Y., and C. Cheers.** 1995. Endogenous interleukin-12 is involved in resistance to *Brucella abortus* infection. Infect. Immun. **63:**1387–1390.