Intranasal Interleukin-12 is a Powerful Adjuvant for Protective Mucosal Immunity

Bernard P. Arulanandam,[1,a] Margot O'Toole,[2] and Dennis W. Metzger[1,a]

[1]*Department of Microbiology and Immunology, Medical College of Ohio, Toledo;* [2]*Genetics Institute, Cambridge, Massachusetts*

The use of interleukin (IL)–12 as a new vaccine adjuvant for stimulating protective antiviral mucosal immunity has been examined. Mice were immunized intranasally (in) with an influenza vaccine consisting of soluble hemagglutinin (H1) and neuraminidase (N1) plus IL-12. This treatment resulted in elevated levels of lung and splenic interferon-γ and IL-10 mRNA. Total and IgG2a anti-H1N1 antibody levels in serum were significantly elevated, as were total, IgG1, IgG2a, and secretory IgA antibody levels in bronchoalveolar lavage (BAL) fluids compared with animals receiving vaccine alone. Mice immunized in with vaccine and IL-12 also exhibited decreased weight loss and dramatically enhanced survival after lethal challenge with infectious influenza virus. Protection was dependent upon the presence of B cells and could be transferred to naive mice by inoculation of either serum or BAL fluid from IL-12–treated mice. These findings show for the first time that soluble IL-12 delivered in serves as a powerful respiratory adjuvant for protective antiviral immunity.

Mucosal surfaces represent both the first line of defense against pathogenic organisms and the primary interface between the host and the environment [1]. As a result, there is continued interest in the effective induction of mucosal immunity for prevention of infections. In fact, there is an urgency to develop more potent vaccines and targeted methods for delivery that elicit both mucosal and systemic immunity [2]. Unfortunately, current vaccine strategies involving mucosal routes of immunization are limited by a lack of suitable mucosal adjuvants that can be safely given to humans [3, 4]. Mucosal adjuvants, such as cholera toxin (CT) and *Escherichia coli* enterotoxin (LT) are potent adjuvants but cause severe diarrhea in humans and induce Th2 responses that can exacerbate lung pathology rather than enhance protection [5, 6].

Interleukin (IL)–12 is a well-characterized heterodimeric cytokine that is produced by antigen-presenting cells such as macrophages, dendritic cells, and Langerhans cells [7–9]. IL-12 has profound regulatory effects on the immune system through its ability to preferentially activate Th1 and NK cells and to induce interferon (IFN)–γ production [9, 10]. A number of studies have shown that IL-12 also strongly stimulates serum IgG2a and IgG3 antibody responses to protein and hapten-carrier antigens [11–18]. Recent evidence indicates the importance of IL-12 in mucosal-associated immune defenses. Therapeutic IL-12 treatment of mice has been shown to decrease infection by various respiratory pathogens, including *Klebsiella pneumonia* and *Mycobacterium avium* [19, 20]. In addition, individuals with deficiencies in IL-12 receptor expression have difficulty in developing protective immunity to respiratory mycobacterial and enteric salmonella infections [21, 22].

Because of its immune stimulating properties, there is continual interest in utilizing IL-12 as a vaccine adjuvant. However, there is very little information about the application of soluble recombinant IL-12 as a vaccine adjuvant for mucosal immunization. Marinaro et al. [23] reported that oral administration of liposome-encapsulated IL-12 redirected systemic immune responses toward Th1-type profiles with enhancement of serum IgG2a and IgG3 antibody production. However, oral IL-12 inoculation did not affect secretory IgA levels. In addition, Okada et al. [24] showed that intranasal (in) immunization with a human immunodeficiency virus (HIV) DNA vaccine in an IL-12– and granulocyte-macrophage colony-stimulating factor–expressing plasmid augmented mucosal and cell-mediated immune responses against HIV-1 antigens. Intratracheal inoculation of IL-12 has been reported to inhibit anti-adenovirus IgA antibody expression in bronchoalveolar lavage (BAL) fluid without affecting IgG levels, but the effects of IL-12 were not thoroughly characterized in terms of IgG isotypes [25]. In addition, the intratracheal route of IL-12 delivery was invasive and thus not pertinent to vaccination strategies. There are no reports as yet examining the ability of IL-12 given in to enhance the protective capacity of mucosal vaccines against pathogenic organisms.

We previously showed the ability of IL-12 delivered in together with a model hapten-carrier antigen to enhance both

Received 11 January 1999; revised 18 May 1999; electronically published 8 September 1999.

Presented in part: 3d National Symposium on the Basic Aspects of Vaccines, Washington, DC, April 1998 (abstract C1).

Grant support: NIH (AI38380, AI41715 and HL621200).

[a] Present affiliation: Center for Immunology and Microbial Disease, Albany Medical College, Albany, NY.

Reprints or correspondence: Dr. Dennis W. Metzger, Center for Immunology and Microbial Disease, Albany Medical College, 47 New Scotland Ave., Albany, NY 12208 (metzge.d@mail.amc.edu).

The Journal of Infectious Diseases 1999; 180:940–9

0022-1899/1999/18004-0002$02.00

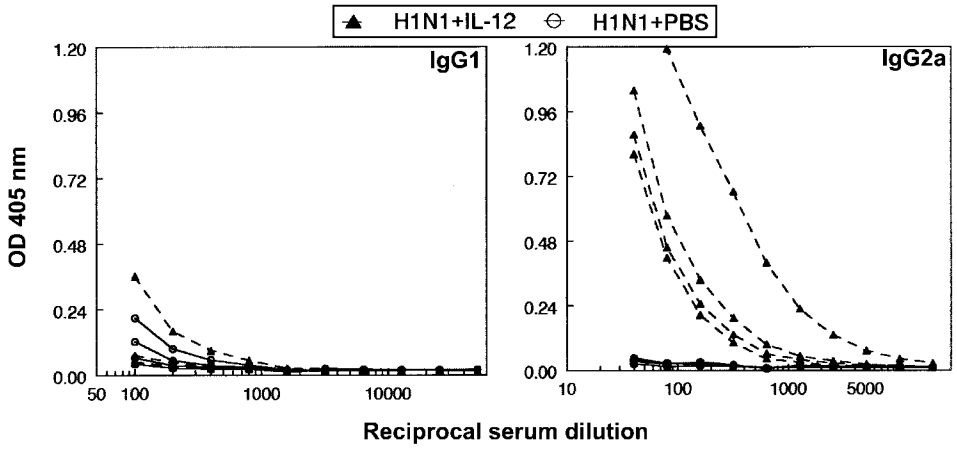

Figure 1. Effects of interleukin (IL)–12 administered in on early systemic antibody responses to subunit influenza vaccine. Mice were immunized in on day 0 with H1N1 subunit influenza vaccine and treated in with either IL-12 (▲) or PBS vehicle (○) on days 0, 1, 2, and 3. Serum anti-H1N1 antibody levels on day 14 were determined by isotype-specific ELISA by use of H1N1-coated microtiter plates. Each line represents binding of antibody from an individual mouse (4 mice per group). The difference in binding between mice immunized with vaccine and IL-12 and those immunized with vaccine and PBS vehicle was significant at $P < .05$ for IgG2a.

mucosal and systemic anti-hapten antibody responses [26]. In the present study, we have examined whether in administration of IL-12 with an influenza subunit vaccine is able to enhance the protective efficacy of vaccination to a respiratory pathogen. The immune response of mice to influenza virus is especially well characterized and provides an attractive model to analyze the adjuvant properties of IL-12 at mucosal surfaces. Furthermore, Monteiro et al. recently reported that endogenous IL-12 is important in the immune response of mice to influenza virus [27]. Our results show that IL-12 coadministered with an subunit influenza vaccine in markedly enhances protection against lethal challenge with influenza virus. These findings show that IL-12 delivered in is a potent vaccine adjuvant for augmenting protective mucosal immunity.

Methods

Mice. Six- to eight-week-old female BALB/c mice were obtained from the National Cancer Institute (Bethesda, MD). C57BL/6 wild-type and IgM-deficient (μMT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in the animal facility at the Medical College of Ohio and provided food and water ad libitum. All animal care and experimental procedures were performed in compliance with the Institutional Animal Care and Use Committee guidelines.

Immunizations. Intranasal treatments were performed on mice that had been anesthetized intraperitoneally (ip) with a combination of Ketamine HCL (Fort Dodge Laboratories, Fort Dodge, IA) and Xylazine (Bayer Corporation, Shawnee Mission, KS). Mice were immunized in on day 0 with 25 μL of sterile PBS containing 1 μg of subunit influenza vaccine consisting of soluble hemagglutinin subtype 1 (H1) and neuraminidase subtype 1 (N1) purified from influenza virus A/PR8/34 (provided by Dr. Doris Bucher, New York Medical College, NY). This was followed on days 0, 1, 2, and 3 with in inoculation of 1 μg of recombinant murine IL-12 in PBS containing 1% normal mouse serum (PBS-NMS) or, in the case of control mice, with PBS-NMS only. Mice were boosted in with the same amount of vaccine on days 14 and 28. On day 28, the mice also received IL-12 in PBS-NMS or PBS-NMS only. No toxicity was observed with this treatment regimen. Sera were prepared by bleeding mice from the orbital plexus.

RNA isolation and ribonuclease protection assay. Total RNA isolation from snap-frozen spleens and lungs was performed with the Ambion Total RNA Isolation Kit (Austin, TX) according to the manufacturer's instructions. Briefly, the frozen tissues were homogenized with a mortar and pestle and immediately transferred into tubes containing 1.0 mL of denaturation solution. After phenol-chloroform extraction, the homogenized samples were centrifuged at 10,000 *g* for 10 min at 4°C. The supernatants were subjected to another round of phenol-chloroform extraction, and the resulting RNA was precipitated with isopropanol, washed twice with 75% ethanol, and dissolved in diethyl pyrocarbonate–treated water. The concentration of total RNA was determined by spectrophotometric analysis at 260 nm. Cytokine mRNA levels were determined by use of the RiboQuant multiprobe ribonuclease protection assay system (Pharmingen, San Diego, CA) according to the manufacturer's instructions. Briefly, 10 μg of total RNA was hybridized to a ^{32}P-labeled RNA probe overnight at 56°C. The single-stranded nucleic acid was digested with ribonuclease for 45 min at 30°C, subjected to phenol-chloroform extraction, and resolved on a 6% denaturing polyacrylamide gel. Transcript levels were quantified on a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Total RNA was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase, and relative cytokine mRNA levels were expressed as arbitrary values.

Collection of bronchoalveolar lavage fluid. For collection of BAL fluid, the mice were killed and their tracheas intubated by using a 0.58 mm OD polyethylene catheter (Becton Dickinson, Sparks, MD). The lungs were then lavaged 2–3 times with PBS

containing 5 m*M* EDTA. The recovered BAL fluid was centrifuged at 12,000 *g* for 5 min at 4°C, and the supernatant was stored at −70°C until use.

Detection of antibody and isotype levels by ELISA. Anti-H1N1 levels in serum and BAL were determined by ELISA with minor modifications to a procedure described elsewhere [11, 28]. Briefly, microtiter plates (Nalge Nunc International, Rochester, NY) were coated overnight with 1 μg/mL of H1N1 in PBS. The plates were washed with PBS containing 0.3% Brij-35 (Sigma, St. Louis) and blocked for 1 h at room temperature with PBS containing 5% fetal calf serum (Hyclone Laboratories, Logan, UT) and 0.1% Brij-35. Serial dilutions of serum were added and the plates incubated for 2 h at room temperature. The plates were washed and incubated with goat anti-mouse total Ig, IgG1, or IgG2a conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). After incubation for 1 h, the plates were washed, and *p*-nitrophenyl phosphatase substrate was added to obtain color development. Plates were read at 405 nm with an ELISA microplate reader (Bio-Tek Instruments, Winooski, VT). To detect IgA, the plates were incubated with goat anti-mouse IgA conjugated to biotin (Sigma), then washed and incubated with alkaline phosphatase-conjugated streptavidin (Bio-Rad, Richmond CA). In all cases, appropriate working dilutions and isotype specificities of the secondary antibody conjugates were determined by use of purified myeloma proteins of known isotypes (Sigma). Statistical significance was determined by using a 2-tailed Student's *t* test. Data were considered statistically significant if *P* values were <.05 by use of 50% end-point titers.

Virus challenge. For protection studies, mice were immunized in on day 0 with 25 mμL of PBS containing 1 μg of H1N1 subunit influenza vaccine. This was followed on days 0, 1, 2, and 3 with in inoculation of 1 μg of IL-12 in PBS-NMS or with PBS-NMS only. Some mice received only IL-12 in PBS-NMS or only PBS-NMS (no H1N1 subunit vaccine). Approximately 4–5 weeks after primary immunization, viral challenge was performed by use of infectious A/PR8/34 influenza virus (provided by Dr. Doris Bucher) administered in to anesthetized mice in 40 μL of sterile PBS. The mice were weighed daily and monitored for morbidity and mortality. Survival data were analyzed by use of a log-rank test and compared by use of χ^2 analysis.

Passive transfer of sera and BAL fluids. For passive transfer experiments, sera were obtained on day 28 after in immunization with the H1N1 subunit vaccine. Mice were injected ip with 100 μL of a 1 : 10 dilution of pooled serum and challenged 5 h later with infectious influenza virus in The BAL fluid collected from mice on day 35 after in immunization with H1N1 subunit influenza vaccine was centrifuged to remove cells, and the supernatant was administered in to anesthetized mice together with virus in a total volume of 40 μL.

Results

Intranasal IL-12 administration induces expression of Th1-type cytokine responses in the lungs and spleens of immunized mice. IL-12 given parenterally has profound regulatory effects on the immune system through its ability to preferentially activate Th1 and NK cells and induce IFN-γ production [9, 10]. By use of ribonuclease protection assay, it was found that IFN-γ mRNA levels were increased 2-fold in the lungs of animals 24 and 48 h after in treatment with H1N1 plus IL-12, compared with mice that received vaccine alone (table 1). In the spleens of these animals, IFN-γ mRNA was elevated 5-fold at 24 h and 2-fold at 48 h after IL-12 treatment. It has been shown previously that treatment with IL-12 enhances expression of IL-10 mRNA [26, 29–31]. IL-10 mRNA expression was enhanced 5-fold in the lungs after IL-12 treatment. Splenic IL-10 mRNA levels were increased 8-fold at 24 h and 5-fold at 48 h after IL-12 treatment. Similar results were also obtained by use of reverse transcription–polymerase chain reaction (data not shown).

Coadministration of an in vaccine plus IL-12 has potent effects on systemic antibody responses. We previously showed the ability of parenteral administration of IL-12 to alter isotype-restricted antibody responses to hen egg white lysozyme [11]. In addition, we recently showed that IL-12 delivered in modulates both mucosal and systemic immunity to the DNP hapten [26]. The present study shows that IL-12 delivered in has similar effects on antibody responses to H1N1 influenza vaccine. Fourteen days after immunization with vaccine by itself or together with IL-12, there was little detectable serum IgG1 anti-H1N1 antibody (figure 1). In contrast, IgG2a anti-H1N1 antibody levels were markedly enhanced after IL-12 treatment, compared with mice that received vaccine alone. Therefore, in IL-12 treatment resulted in early activation of serum IgG2a antibody responses.

Similar analyses were performed on day 35 sera to determine the long-term effects of in IL-12 treatment. At this time point, IL-12–treated mice had 6-fold higher levels of total anti-H1N1 serum antibody than mice immunized with the vaccine alone (figure 2). Total (nonspecific) Ig was increased 3-fold after in IL-12 treatment (data not shown). IgG2a antibody levels were still dramatically enhanced in mice that received IL-12. Furthermore, IgG1 anti-H1N1 antibodies, evident in both exper-

Table 1. Interferon (IFN)–γ and interleukin (IL)–10 mRNA levels in the lungs and spleens of mice immunized with influenza subunit vaccine.

Organ/time	Cytokine	H1N1 + PBS	H1N1 + IL-12	Fold increase
Lungs				
24 h	IFN-γ	380 ± 6.7	830 ± 61	2.2
	IL-10	1.7 ± .8	8.7 ± 1.3	5.1
48 h	IFN-γ	340 ± 110	630 ± 152	1.9
	IL-10	1.6 ± .5	8.6 ± 3.0	5.4
Spleens				
24 h	IFN-γ	410 ± 115	1900 ± 400	4.6
	IL-10	2.9 ± 1.9	22 ± 4.3	7.6
48 h	IFN-γ	570 ± 109	1000 ± 170	1.8
	IL-10	2.7 ± 1.6	13 ± 5.0	4.8

NOTE. Mice were killed 24 and 48 h after in treatment with H1N1 subunit influenza vaccine ± IL-12. Total RNA was isolated, and IFN-γ and IL-10 transcript levels were analyzed by using multiplex ribonuclease protection assay. Relative RNA levels were quantitated on a phosphorimager and normalized to glyceraldehyde 3-phosphate dehydrogenase. The cytokine mRNA levels are expressed as arbitrary units ± SE.

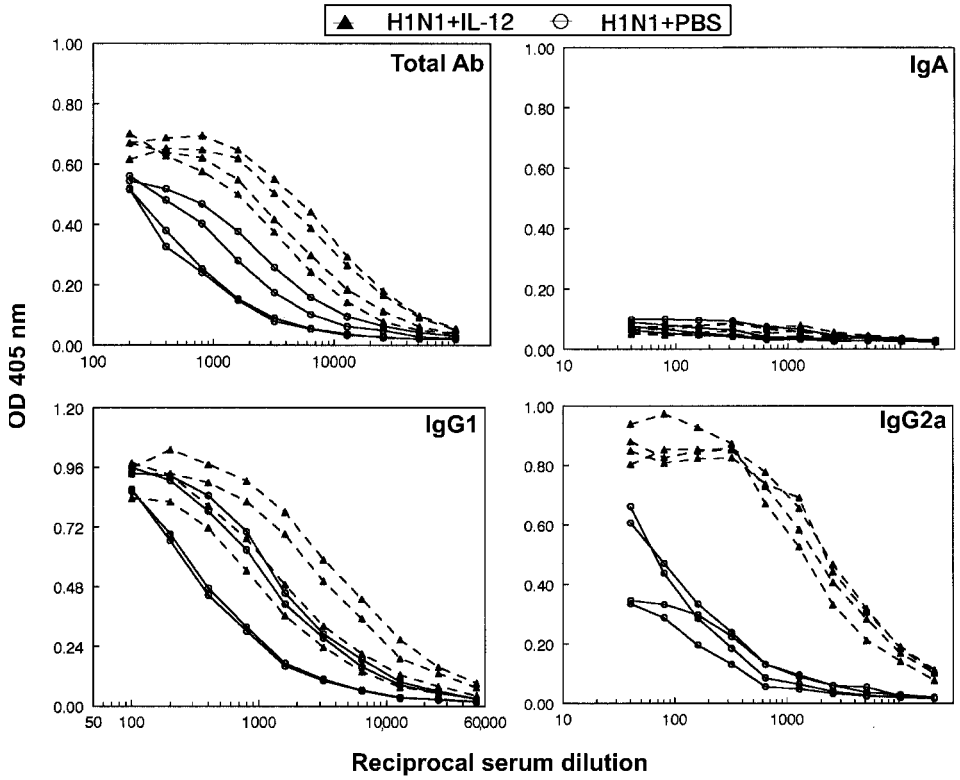

Figure 2. Effects of interleukin (IL)–12 administered intranasally (in) on late systemic antibody responses to subunit influenza vaccine. Mice were immunized in on day 0 with H1N1 subunit influenza vaccine, treated in with either IL-12 (▲) or PBS vehicle (○) on days 0, 1, 2, and 3, and boosted with vaccine on days 14 and 28. On day 28, the mice received a second treatment with IL-12 or vehicle. Serum anti-H1N1 antibody levels on day 35 were determined by isotype-specific ELISA by using H1N1-coated microtiter plates. Each line represents binding of antibody from an individual mouse (4 mice per group). Differences in binding between mice immunized with vaccine and IL-12 and those immunized with vaccine and PBS vehicle were significant at $P < .05$ for total antibody (Ab) and IgG2a.

imental and control groups, were moderately elevated in IL-12–treated mice, compared with mice receiving only vaccine, an observation that is consistent with earlier findings [11]. There was no IgA detected in the sera of any of the mice. The results clearly show the ability of IL-12 coadministered as an adjuvant and delivered in a noninvasive form to enhance serum antibody levels.

Intranasal IL-12 delivery enhances respiratory antibody levels. We next assessed antibody responses in BAL fluid from in immunized mice. Analyses of BAL fluid collected on day 35 of the immune response revealed that IL-12–treated mice had enhanced mucosal antibody responses to H1N1 subunit influenza vaccine. As a group, in IL-12 treatment resulted in 15-fold increases in total anti-H1N1 respiratory antibody production compared with mice immunized with vaccine alone (figure 3). In addition, there was a 13-fold increase in total nonspecific Ig in the BAL fluid of mice that received H1N1 plus IL-12 in (data not shown). Importantly, animals that were immunized and treated with IL-12 displayed elevated BAL fluid IgA anti-H1N1 antibody levels compared with animals not exposed to IL-12. This result is in stark contrast to the absence of detectable IgA in the circulation of these mice. It was also found that levels of both IgG1 and IgG2a anti-H1N1 antibodies were enhanced in BAL fluid after IL-12 administration compared with mice that received vaccine alone. There were only trace amounts of albumin detected in respiratory secretions, showing that the enhancement of IgG was not the result of leakage from blood (data not shown). These results firmly establish the influence of IL-12 delivered in in augmenting respiratory antibody expression.

IL-12 administration increases the protective effects of influenza subunit vaccination. We next assessed the effects of coadministrating IL-12 and H1N1 in on survival and clinical outcome after challenge with influenza virus. Mice were immunized in with H1N1 vaccine on day 0 and treated with 1 μg of IL-12 or PBS vehicle on days 0, 1, 2, and 3. Some mice received only IL-12 or PBS vehicle. Four to 5 weeks after immunization, the mice were inoculated in with infectious A/PR8/34 influenza virus and monitored daily for morbidity and mortality. In the first experiment, a dose of virus was used that allowed 50% survival of mice after exposure to just vaccine (figure 4*A*). It was found that inclusion of IL-12 during vaccination resulted

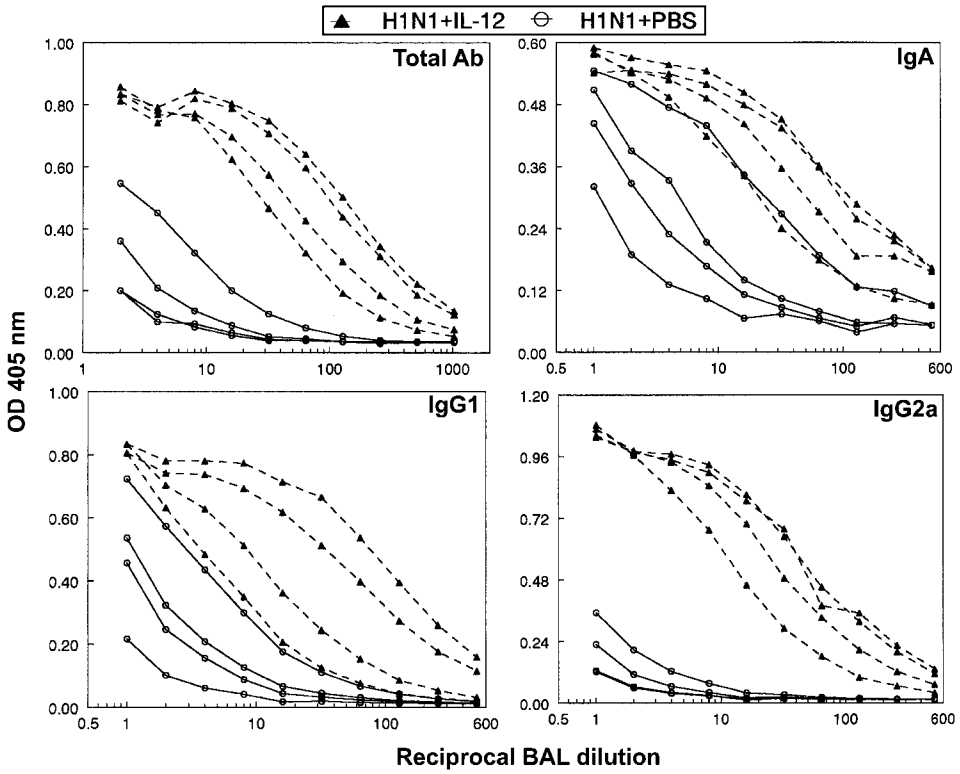

Figure 3. Effects of interleukin (IL)–12 administered intranasally (in) on respiratory mucosal responses. Mice were immunized in on day 0 with H1N1 subunit influenza vaccine, treated in with either IL-12 or PBS vehicle on days 0, 1, 2, and 3, and boosted with vaccine on days 14 and 28. On day 28, mice received a second treatment with IL-12 or vehicle. Mice were killed on day 35, and bronchoalveolar lavage fluid was assayed for anti-H1N1 antibody levels by ELISA by use of H1N1-coated microtiter plates. Each line represents binding of antibody from an individual mouse (4 mice per group). Differences in binding between mice immunized with vaccine and IL-12 and those immunized with vaccine and PBS vehicle were significant at $P < .05$ for total antibody (Ab) , IgG1, IgG2a, and IgA.

in 100% survival and significant reduction in illness, as evidenced by reduced weight loss, compared with mice that received vaccine alone. Mice that were pretreated with IL-12 or PBS-NMS only (no H1N1 subunit vaccine) displayed progressive weight loss, and all died within 11 days after virus challenge.

In a second experiment, a larger dose of virus was used for challenge such that vaccination with H1N1 alone afforded little, if any, significant protection (figure 4*B*). In this case, it was found that vaccination with H1N1 and IL-12 resulted in 50% survival after challenge. Recovery from infection in the surviving mice was evidenced by regaining body weight. As expected, animals that received IL-12 or PBS-NMS alone did not survive virus challenge. Hence, coadministration of IL-12 and the H1N1 subunit influenza vaccine in increased the efficacy of the vaccine and conferred significant protection against lethal doses of live influenza virus.

Enhanced protection against influenza infection after vaccination with H1N1 plus IL-12 is antibody-mediated. To ascertain the role of humoral immunity in protection from influenza virus infection, we examined the responses of B cell–deficient C57BL/6 μMT mice [32]. It was found that all μMT mice pretreated with PBS alone, vaccine alone, or vaccine plus IL-12 succumbed to infection by day 10 (figure 5). Wild-type C57BL/6 mice pretreated with PBS alone died 12 days after infection. In addition, all mice displayed a steady, progressive loss of body weight. Thus, the enhanced protection conferred by IL-12 treatment is a result of augmented B cell function.

To further determine whether protection against influenza virus observed in mice inoculated in with vaccine and IL-12 was mediated by antibody, we transferred pooled serum from wild-type BALB/c mice into naive syngeneic animals, which were then challenged with A/PR8/34 influenza virus 5 h later. Of the animals that received serum from mice inoculated with vaccine or PBS-NMS only, all succumbed to infection (figure 6). However, animals that received serum from mice immunized with the vaccine plus IL-12 exhibited 50% survival after viral challenge.

We also determined whether antibodies generated in the respiratory secretions of immunized mice played a crucial role in protection against influenza virus infection. BAL fluid recovered from unvaccinated BALB/c animals or animals immunized

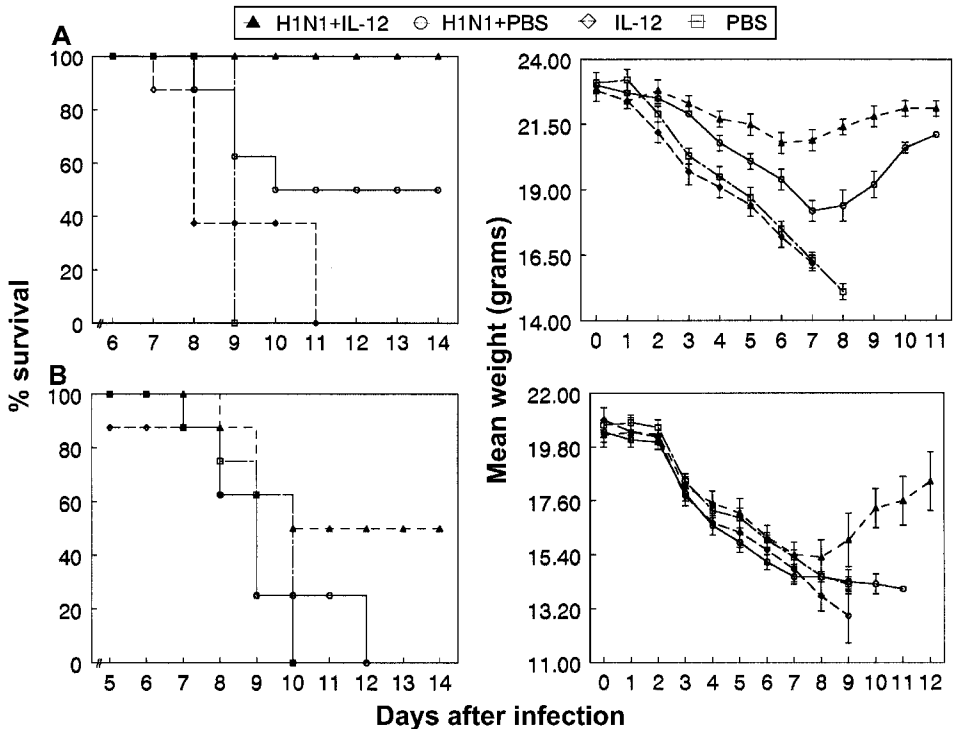

Figure 4. Coadministration of influenza subunit vaccine plus interleukin (IL)–12 protects mice from an subsequent influenza virus infection. Mice were immunized in with H1N1 subunit vaccine on day 0 and treated with 1 μg of IL-12 (▲) or PBS vehicle (○) on days 0, 1, 2, and 3. Some mice received only IL-12 (◇) or PBS vehicle (□). All mice (8 per group) were then challenged in 4–5 weeks later with 10^3 pfu (*A*) or 2×10^3 pfu (*B*) of A/PR/8/34 influenza virus. Mice were monitored daily for mortality and weight loss. Differences in survival between mice immunized with vaccine and IL-12 and those immunized with vaccine and PBS were significant at $P < .05$ at 10^3 pfu and $P < .01$ at 2×10^3 pfu.

with H1N1 ± IL-12 was administered in to naive mice together with live virus. The results showed that virus challenge together with passive transfer of BAL fluid from mice that were treated with PBS-NMS alone resulted in 100% death by day 7 (figure 7). Virus challenge in the presence of BAL fluid from mice immunized with H1N1 alone resulted in survival of only 1 of 8 infected mice. However, 100% of the animals that received BAL fluid from mice treated with H1N1 plus IL-12 were protected against virus infection. These mice exhibited no transient weight loss over the course of the infection, whereas both of the other treatment groups displayed progressive weight loss leading to death. Furthermore, mice that received BAL fluid from animals immunized with vaccine alone had viral lung titers of 10^3 pfu on day 4 after infection, whereas mice that received BAL fluid from animals treated with vaccine plus IL-12 had viral lung titers <100 pfu. Thus, passive transfer of BAL fluid in from mice immunized with H1N1 subunit vaccine plus IL-12 provided dramatic protection against influenza virus challenge.

Discussion

There is substantial interest in developing effective vaccines that can be delivered in a noninvasive fashion and that induce both mucosal and systemic protective immunity. The major reason for stimulating mucosal immunity is to prevent infection at the site of initial contact between the host and the pathogen [1]. A potential limitation of this approach is the lack of effective adjuvants that augment mucosal immune responses and are clinically relevant. We have now shown for the first time that IL-12 delivered in with an influenza subunit vaccine serves as a potent adjuvant and confers increased protection against subsequent viral infection. Use of B cell–deficient mice and passive transfer of serum or BAL fluid showed that the protection induced by IL-12 is mediated by antibody.

Analysis of cytokine mRNA production after in treatment of mice with IL-12 revealed an enhancement of IFN-γ mRNA expression in both lungs and spleen within 24 h. IFN-γ has a variety of immunoregulatory functions, which include induction of Th1 cell differentiation and activation of NK cells [33]. In addition, IFN-γ enhances the production of opsonizing murine antibodies such as IgG2a [11, 13, 14, 17]. IL-10 mRNA expression was also induced in lungs and spleens by in treatment with IL-12. IL-10 is mainly produced by T cells and monocytes and has been shown to inhibit Th1-cell differentiation [34, 35]. Others [29–31] have shown induction of IL-10 after treatment with IL-12, an observation that suggests a feedback mech-

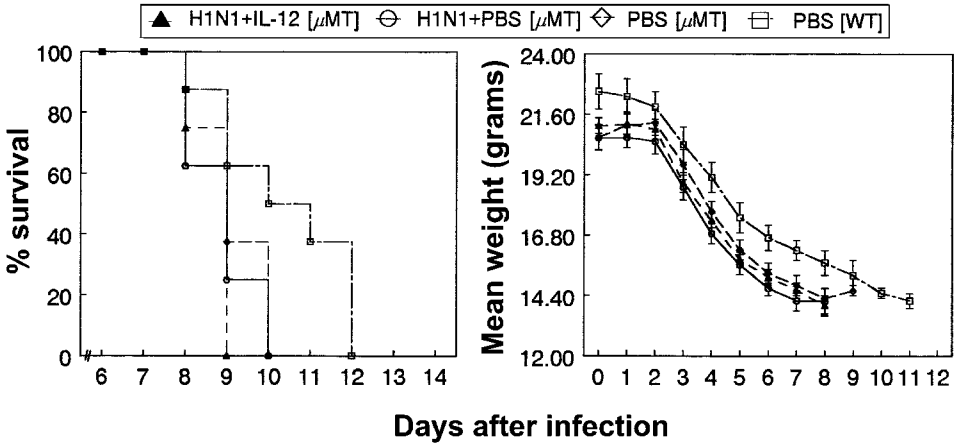

Figure 5. Interleukin (IL)–12 induced protection against influenza virus infection is mediated by B cells. C57BL/6 IgM-deficient (μMT) mice were immunized in with H1N1 subunit vaccine on day 0 and treated with 1μg of IL-12 (▲) or PBS vehicle (○) on days 0, 1, 2, and 3. Some μMT mice received PBS vehicle only (◇). Wild-type C57BL/6 (WT) mice were pretreated with PBS vehicle (□). All mice (8 per group) were then challenged in 6–7 weeks later with 10^3 pfu of A/PR/8/34 influenza virus. Mice were monitored daily for mortality and weight loss. Differences in survival between mice immunized with vaccine and IL-12 and those immunized with vaccine and PBS were $P > .05$.

anism designed to down-regulate the inflammatory effects of IL-12 and IFN-γ.

The role of endogenous IL-12 during influenza virus infection has been recently characterized by Monteiro et al. [27]. Specifically, increased expression of IL-12 was detected in the lungs of BALB/c mice during the early onset of influenza virus infection. The endogenous IL-12 was primarily involved in early NK cell–dependent IFN-γ production, and neutralization of IL-12 resulted in significant increases in viral lung titers. Further evidence for the requirement of IL-12 in mucosal-associated defenses was recently shown in individuals who lack IL-12 receptor β1 chain expression and who therefore have an impaired ability to control recurrent mycobacterial and salmonella infections [21, 22]. On the basis of these findings, we examined the ability of exogenous IL-12 to be utilized as a vaccine adjuvant for stimulating specific mucosal immunity.

Our laboratory previously showed that IL-12 given parenterally alters the isotype-restricted antibody response of mice to hen lysozyme [11]. Parenteral injections of IL-12 plus lysozyme greatly elevated antigen-specific serum IgG2a levels and temporarily suppressed IgG1 antibody production. In addition, others [14–18] have shown that ip IL-12 administration enhances serum IgG2a, IgG2b, and IgG3 antibody responses to protein antigens. Parenteral IL-12 can also enhance IgG2a and IgG3 levels against T-independent polysaccharide antigens [28]. Furthermore, we recently showed that IL-12 delivered in together with a model hapten-carrier antigen is capable of influencing anti-hapten antibody responses in a similar manner [26]. In the present study, we examined the effects of in IL-12 on responses to a clinically relevant influenza subunit vaccine. IL-12 treatment was found to have a strong effect on the early onset of the humoral response, as reflected by significant enhancement of IgG2a anti-H1N1 antibody levels. In comparison, animals that received vaccine alone did not develop early IgG2a responses. There was little detectable IgG1 antibody during the early phase of the immune response in animals that received vaccine alone or vaccine and IL-12. After 35 days, IgG2a levels were still enhanced in IL-12–treated mice, and IgG1 levels were also somewhat elevated, an observation that is in agreement with our previous findings in the lysozyme system [11]. These results show the long-lasting effects of IL-12 delivered in and provide further evidence for the use of this route of administration for augmenting systemic humoral immunity. In our in treatment schedule, IL-12 was inoculated for 4 days around the time of vaccine administration. This regimen, although very productive, has limited practicality for human vaccination, and we are currently examining the effectiveness of a single in inoculation of IL-12 and vaccine.

IL-12 in administration also resulted in significant increases in respiratory antibody levels, including IgG and IgA anti-H1N1 antibody levels. IgA is the predominant antibody in mucosal secretions and is thought to play a major role in preventing attachment of pathogens to mucosal epithelial surfaces [36]. Whereas secretory IgA was elevated in the mucosal compartment, only very low amounts of IgA were present in the circulation after in immunization. In a recent study, in application of IL-12 together with tetanus toxoid was also shown to augment secretory IgA antibody levels [37]. The increase in serum and BAL fluid IgG antibody levels after IL-12 treatment provides further support for the use of IL-12 as a potent mucosal adjuvant. Among the murine isotypes, IgG2a is the isotype that mediates optimal complement fixation and opsoni-

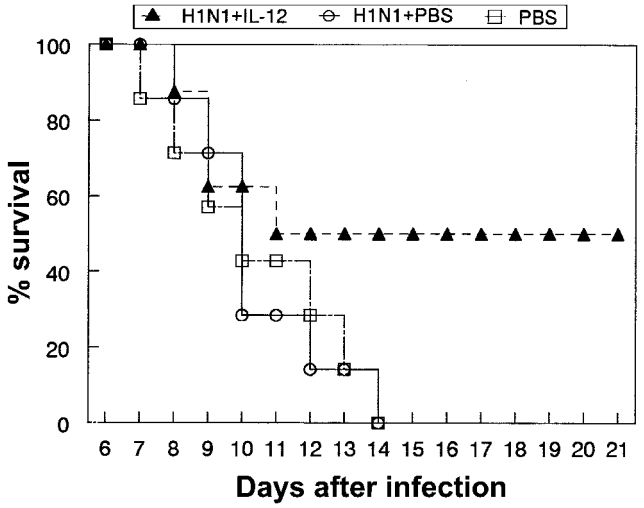

Figure 6. Passive transfer of serum from mice immunized with subunit influenza vaccine plus interleukin (IL)–12 confers protection against influenza virus challenge. Sera were collected from BALB/c mice immunized intranasally with the H1N1 subunit influenza vaccine plus IL-12 (▲), vaccine plus PBS (○), or PBS vehicle only (□). Pooled serum was diluted 1 : 10 in sterile PBS and injected intraperitoneally at a dose of 0.1 mL/mouse. All mice (7–8 per group) were then challenged in 5 h later with 10^3 pfu of A/PR/8/34 influenza virus. Differences in survival between mice immunized with vaccine and IL-12 and those immunized with vaccine and PBS vehicle were significant at $P < .05$.

zation. Tang and Graham [38] have determined that increased IgG2a production in the sera after parenteral IL-12 administration enhances the efficacy of respiratory syncytial viral vaccine; however, mucosal antibody production was not examined. Studies by Nedrud et al. [39] established that protection of the lower respiratory tract from parainfluenza infection is correlated with the presence of IgG antiviral antibodies in BAL fluid. In addition, Palladino et al. [40] have shown that IgG antibody is the principal mediator of recovery from influenza virus pneumonia. Therefore, the ability to enhance the expression of IgG in mucosal-associated compartments is likely to be an important feature of IL-12–mediated protection.

We have also shown that passive transfer of serum or BAL fluid collected from mice immunized with subunit influenza vaccine and IL-12 resulted in significant protection from morbidity and mortality. It has been suggested that cell-mediated immune responses are crucial for recovery from viral infections, whereas humoral immunity is vital for protection against virus challenge [40]. There is also recent evidence indicating that CD8 T cells provide protective immunity against influenza virus B infections in mice lacking Ig and mature B cells [41]. However, it is becoming clear that antibodies play a major role in recovery from viral infections [42]. Several groups have shown that B cell–deficient animals are highly susceptible to lethal influenza A virus infection compared with wild-type littermates [43–45]. In fact, the ability of IL-12 to augment antibody levels and enhance protection against influenza virus infection is completely abrogated in μMT mice. The augmented protection conferred by passive transfer of BAL fluid observed here is likely to be the result of significantly enhanced respiratory antibody levels observed after in IL-12 treatment. Secretory IgA has been reported to be the main mediator of mucosal immunity to influenza virus infection [36, 46]. However, IgA was not detectable in serum which also transferred protection, and there is evidence to suggest the importance of IgG2a antibody expression in viral defenses [39, 40, 42]. Further experiments are in progress to examine which antibody isotype in the BAL fluid is responsible for the observed protection. Wynn et al. previously reported

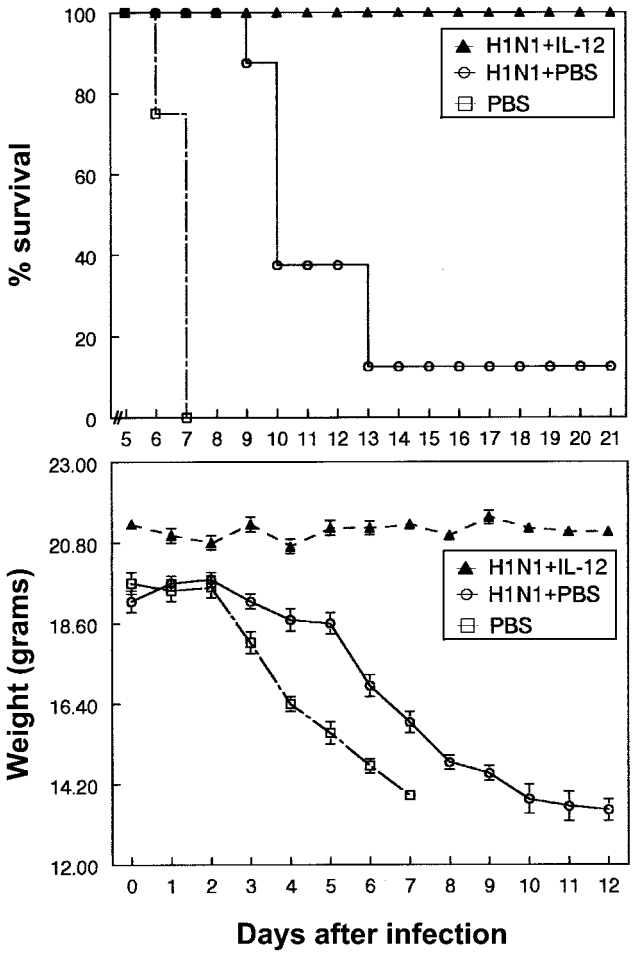

Figure 7. Passive transfer of bronchoalveolar lavage (BAL) fluid in from BALB/c mice immunized with subunit influenza vaccine plus interleukin (IL)–12 confers protection against influenza virus challenge. BAL fluids were collected from BALBc mice immunized with the H1N1 subunit influenza vaccine plus IL-12 (▲), vaccine plus PBS (○), or PBS vehicle only (□). All mice (8 per group) were then inoculated in with pooled BAL fluid and 2×10^3 pfu of A/PR/8/34 influenza virus. Differences in survival between mice immunized with vaccine and IL-12 and those immunized with vaccine and PBS were significant at $P < .001$.

that serum transferred from animals vaccinated and treated ip with IL-12 conferred enhanced protection against subsequent challenge with cercariae of *Schistosoma mansoni,* showing that humoral immunity induced by IL-12 also plays an important role in protection from parasitic diseases [17].

There is continual interest in developing mucosal-based vaccines for a variety of different pathogens, including HIV. Adjuvants that have been used to enhance mucosal immune responses include microbial products such as CT and LT, which have been utilized in a variety of delivery systems [3, 4]. CT is a potent inducer of Th2-type responses, whereas LT elicits a mixed Th1 and Th2 response [23, 47]. However, these adjuvants cause severe diarrhea and are not suitable for use as mucosal adjuvants in humans. There is also a recent report suggesting that CT actually suppresses IL-12 production and IL-12 receptor expression [48]. Furthermore, in respiratory syncytial virus lung infections, Th1 responses are protective, while Th2 responses result in lung pathology [5, 6]. Recently, in IL-12 application was found to inhibit lung eosinophilia after airway challenge in sensitized mice [49]. In the same study, it was reported that low doses of in IL-12 treatment did not cause splenomegaly in these animals. In fact, while parenteral administration of IL-12 can cause significant pathology [50], in delivery of IL-12 may be a preferred route of administration to minimize treatment related toxicity. The ability of IL-12 administered in to enhance the protective efficacy of an influenza vaccine is therefore of direct relevance for mucosal and systemic vaccination protocols.

Acknowledgments

We thank Holly Swiniarski and Lisa C. Merrill for their technical expertise, Cori L Fata for help with plaque assays, and Drs. Alan Sher and Victor H. Van Cleave for helpful comments.

References

1. Staats HF, McGhee JR. Application of basic principles of mucosal immunity to vaccine development. In: Kiyono H, Ogra PL, McGhee JR, eds. Mucosal Vaccines. New York: Academic Press, **1996**:17–39.
2. Van Regenmortel M. Searching for safer, more potent, better-targeted adjuvants. ASM News **1997**;63:136–9.
3. Staats HF, Jackson RJ, Marinaro M, Takahashi, I, Kiyono H, McGhee JR. Mucosal immunity to infection with implications for vaccine development. Curr Opin Immunol **1994**;6:572–83.
4. Elson CO. In defence of mucosal surfaces: regulation and manipulation of the mucosal immune system. In: Paul PS, Francis DH, Benfield, DA, eds. Mechanisms in the Pathogenesis of Enteric Disease. New York: Plenum Press, **1997**:373–85.
5. Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J Clin Invest **1991**;88:1026–33.
6. Graham BS, Henderson GS, Tang YW, Lu X, Neuzil KM, Colley DG. Priming immunization determines T helper cytokine mRNA expression patterns in lungs of mice challenged with respiratory syncytial virus. J Immunol **1993**;151:2032–40.
7. Kobayashi M, Fitz L, Ryan M, et al. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J Exp Med **1989**;170:827–45.
8. Macatonia SE, Hosken NA, Litton M, et al. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive $CD4^+$ T cells. J Immunol **1995**;154:5071–9.
9. Trinchieri G, Scott P. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions. Res Immunol **1995**;146:423–31.
10. Gately MK, Renzetti LM, Magram J, et al. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annu Rev Immunol **1998**;16:495–521.
11. Buchanan JM, Vogel LA, Van Cleave VH, Metzger DW. Interleukin 12 alters the isotype-restricted antibody response of mice to hen egg white lysozyme. Int Immunol **1995**;7:1519–28.
12. Metzger DW, Buchanan JM, Collins JT, et al. Enhancement of humoral immunity by interleukin-12. Ann N Y Acad Sci **1996**;795:100–15.
13. Metzger DW, McNutt RM, Collins JT, Buchanan JM, Van Cleave VH, Dunnick WA. Interleukin-12 acts as an adjuvant for humoral immunity through interferon-gamma-dependent and-independent mechanisms. Eur J Immunol **1997**;27:1958–65.
14. McKnight AJ, Zimmer GJ, Fogelman I, Wolf SF, Abbas AK. Effects of IL-12 on helper T cell-dependent immune responses *in vivo.* J Immunol **1994**;152:2172–9.
15. Morris SC, Madden KB, Adamovicz JJ, et al. Effects of IL-12 on *in vivo* cytokine gene expression and Ig isotype selection. J Immunol **1994**;152:1047–56.
16. Germann T, Bongartz M, Dlugonska H, et al. Interleukin-12 profoundly upregulates the synthesis of antigen-specific complement-fixing IgG2a, IgG2b and IgG3 antibody subclasses *in vivo.* Eur J Immunol **1995**;25:823–9.
17. Wynn TA, Reynolds A, James S, et al. IL-12 enhances vaccine-induced immunity to schistosomes by augmenting both humoral and cell-mediated immune responses against the parasite. J Immunol **1996**;157:4068–78.
18. Bliss J, Van Cleave V, Murray K, et al. IL-12, as an adjuvant, promotes a T helper 1 cell, but does not suppress a T helper 2 cell recall response. J Immunol **1996**;156:887–94.
19. Greenberger MJ, Kunkel SL, Strieter RM, et al. IL-12 gene therapy protects mice in lethal *Klebsiella* pneumonia. J Immunol **1996**;157:3006–12.
20. Doherty TM, Sher A. IL-12 promotes drug-induced clearance of *Mycobacterium avium* infection in mice. J Immunol **1998**;160:5428–35.
21. de Jong R, Altare F, Haagen IA, et al. Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients. Science **1998**;280:1435–8.
22. Altare F, Durandy A, Lammas D, et al. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. Science **1998**;280:1432–5.
23. Marinaro M, Boyaka PN, Finkelman FD, et al. Oral but not parenteral interleukin (IL)–12 redirects T helper 2 (Th2)-type responses to an oral vaccine without altering mucosal IgA responses. J Exp Med **1997**;185:415–27.
24. Okada E, Sasaki S, Ishii N, et al. Intranasal immunization of a DNA vaccine with IL-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF)-expressing plasmids in liposomes induces strong mucosal and cell-mediated immune responses against HIV-1 antigens. J Immunol **1997**;159:3638–47.
25. Yang Y, Trinchieri G, Wilson JM. Recombinant IL-12 prevents formation of blocking IgA antibodies to recombinant adenovirus and allows repeated gene therapy to mouse lung. Nat Med **1995**;1:890–3.
26. Arulanandam BP, Metzger DW. Modulation of mucosal and systemic immunity by intranasal interleukin-12 delivery. Vaccine **1999**;17:252–60.
27. Monteiro JM, Harvey C, Trinchieri G. Role of interleukin-12 in primary influenza virus infection. J Virol **1998**;72:4825–31.
28. Buchanan RM, Arulanandam BP, Metzger DW. IL-12 enhances antibody responses to T-independent polysaccharide vaccines in the absence of T and NK cells. J Immunol **1998**;161:5525–33.

29. Meyaard L, Hovenkamp E, Otto SA, Miedema F. IL-12-induced IL-10 production by human T cells as a negative feedback for IL-12-induced immune responses. J Immunol **1996**; 156:2776–82.
30. Daftarian PM, Kumar A, Kryworuchko M, Diaz-Mitoma F. IL-10 production is enhanced in human T cells by IL-12 and IL-6 and in monocytes by tumor necrosis factor-alpha. J Immunol **1996**; 157:12–20.
31. Gerosa F, Paganin C, Peritt D, et al. Interleukin-12 primes human CD4 and CD8 T cell clones for high production of both interferon-gamma and interleukin-10. J Exp Med **1996**; 183:2559–69.
32. Kitamura D, Roes J, Kuhn R, Rajewsky K. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ chain gene. Nature **1991**; 350:423–6.
33. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. Annu Rev Immunol **1997**; 15:749–95.
34. Fiorentino DF, Zlotnik A, Vieira P, et al. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J Immunol **1991**; 146: 3444–51.
35. Ding L, Shevach EM. IL-10 inhibits mitogen-induced T cell proliferation by selectively inhibiting macrophage costimulatory function. J Immunol **1992**; 148:3133–9.
36. Lamm ME. Interaction of antigens and antibodies at mucosal surfaces. Annu Rev Microbiol **1997**; 51:311–40.
37. Boyaka PN, Marinaro M, Jackson RM, Menon S, Kiyono H, McGhee JR. IL-12 is an effective adjuvant for induction of mucosal immunity. J Immunol **1999**; 162:122–8.
38. Tang YW, Graham BS. Interleukin-12 treatment during immunization elicits a T helper cell type 1–like immune response in mice challenged with respiratory syncytial virus and improves vaccine immunogenicity. J Infect Dis **1995**; 172:734–8.
39. Nedrud JG, Liang XP, Hague N, Lamm ME. Combined oral/nasal immunization protects mice from Sendai virus infection. J Immunol **1987**; 139: 3484–92.
40. Palladino G, Mozdzanowska K, Washko G, Gerhard W. Virus-neutralizing antibodies of immunoglobulin G (IgG) but not of IgM or IgA isotypes can cure influenza virus pneumonia in SCID mice. J Virol **1995**; 69: 2075–81.
41. Epstein SL, Lo CY, Misplon JA, Bennink JR. Mechanism of protective immunity against influenza virus infection in mice without antibodies. J Immunol **1998**; 160:322–7.
42. Gerhard W, Mozdzanowska K, Furchner M, Washko G, Maiese K. Role of the B-cell response in recovery of mice from primary influenza virus infection. Immunol Rev **1997**; 159:95–103.
43. Graham MB, Braciale TJ. Resistance to and recovery from lethal influenza virus infection in B lymphocyte–deficient mice. J Exp Med **1997**; 186: 2063–8.
44. Mozdzanowska K, Furchner M, Maiese K, Gerhard W. $CD4^+$ T cells are ineffective in clearing a pulmonary infection with influenza type A virus in the absence of B cells. Virology **1997**; 239:217–25.
45. Topham DJ, Doherty PC. Clearance of an influenza A virus by $CD4^+$ T cells is inefficient in the absence of B cells. J Virol **1998**; 72:882–5.
46. Renegar KB, Small PA Jr. Passive transfer of local immunity to influenza virus infection by IgA antibody. J Immunol **1991**; 146:1972–8.
47. Takahashi I, Marinaro M, Kiyono H, et al. Mechanisms for mucosal immunogenicity and adjuvancy of *Escherichia coli* labile enterotoxin. J Infect Dis **1996**; 173:627–35.
48. Braun M, He J, Wu C.-Y, Kelsall B. Cholera toxin suppresses interleukin (IL)-12 production and IL-12 receptor β1 and β2 chain expression. J Exp Med **1999**; 189:541–52.
49. Schwarze J, Hamelmann E, Cieslewicz G, et al. Local treatment with IL-12 is an effective inhibitor of airway hyperresponsiveness and lung eosinophilia after airway challenge in sensitized mice. J Allergy Clin Immunol **1998**; 102:86–93.
50. Leonard JP, Sherman ML, Fisher GL, et al. Effects of single-dose interleukin-12 exposure on interleukin-12–associated toxicity and interferon-gamma production. Blood **1997**; 90:2541–8.