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Neonatal Administration of IL-12 Enhances the Protective Efficacy of Antiviral Vaccines

Bernard P. Arulanandam, * James N. Mittler, † ‡ William T. Lee, † ‡ Margot O’Toule, § and Dennis W. Metzger* 

Neonates are highly susceptible to infectious agents and are known to display polarized expression of Th2-like cytokines and Abs. This neonatal immune bias has important implications for the development of vaccine strategies, particularly against viral infections. We now report that coadministration of IL-12 and an influenza subunit vaccine at birth enhances the protective efficacy of antiviral vaccination. Immunization and treatment with IL-12 within 24 h of birth resulted in elevated expression of IFN-γ, IL-10, and IL-15 mRNA in the spleens of newborn mice compared with animals exposed to vaccine only. In addition, these animals showed dramatic increases in IFN-γ, IL-2, and IL-4-secreting cells, and in IgG2a Ab levels upon adult challenge compared with mice primed with vaccine alone. Most importantly, animals vaccinated and simultaneously treated with IL-12 at birth displayed enhanced survival after lethal challenge with infectious influenza virus as adults compared with infected animals that had been primed with vaccine alone. This augmented protection required B cells and could be transferred to naive mice by immune serum. Collectively, these results provide evidence that administration of IL-12 to neonates induces a Th1-like response in newborns and elicits protective antiviral immune memory. The Journal of Immunology, 2000, 164: 3698–3704.

The limited ability of newborns to respond to vaccine Ags is due to the immaturity of the immune system and sensitivity to tolerogenic signals (1–3). Consequently, there is a high degree of susceptibility to infections during this early period of ontogeny, and protection against pathogenic organisms appears to be primarily mediated by maternal Abs (4). As a result of this window of vulnerability, there is continued interest in effective induction of neonatal vaccine immunity (5).

Recent evidence indicates that the neonatal immune system can be primed to recognize foreign Ags under certain conditions. Matzinger and colleagues (6) showed that the nature of Ag presentation in newborn animals determines the state of immune responsiveness. Specifically, they found that the transfer of adult dendritic cells to newborn mice allowed the animals to be primed rather than tolerized to allogenic spleen cells. Neonatal B cells that are unresponsive to polysaccharide Ags at birth were also recently shown to become responsive in vitro by incubation with recombinant cytokines such as IL-1 and IL-6 (7). Forsthuber et al. (8) demonstrated that injection of neonatal animals with protein Ags in IFA actually induces immune deviation rather than tolerance and stimulates preferential expression of Th cell type 2 (Th2) cytokines and Ab isotypes. Similarly, Siegriest and colleagues (9, 10) found that neonatal vaccine immunization results in biased Th2-type responses that still remain after adult boosting. Collectively, these findings have significant implications for neonatal vaccination strategies and suggest that redirection of the neonatal immune system to a Th1 pathway might increase responsiveness to vaccine Ags.

IL-12 is a 70-kDa heterodimeric protein that is produced by APCs and has pleiotrophic effects on innate and adaptive immune responses (11–14). We (15–17) and others (18–22) have demonstrated the effectiveness of IL-12 as an adjuvant for humoral immunity in adult animals. In addition, we have found that neonatal animals have reduced IL-12 expression in the periphery and that IL-12 delivered at birth can serve as an adjuvant to induce a Th1-like cytokine response and cause priming for enhanced Ab responses to DNP-OVA and hen eggwhite lysozyme (HEL) (23). Similarly, Cairo and colleagues (24) showed that human cord blood cells have reduced expression of IL-12 and IL-15 compared with adult peripheral blood. The direct therapeutic efficacy of IL-12 treatment in young animals has been demonstrated during neonatal cryptosporidial and streptococcal B infections (25, 26). Therefore, immunoregulatory molecules such as IL-12 could contribute significantly in shaping the immune responses of neonates to a variety of infectious organisms.

In the present study, we have examined for the first time whether administration of IL-12 with an influenza subunit vaccine at birth enhances the protective efficacy of antiviral vaccination. The immune response of mice to influenza virus is especially well characterized to a Th1 pathway might increase responsiveness to vaccine Ags.

**Materials and Methods**

**Mice**

BALB/c mice were obtained from the National Cancer Institute (Bethesda, MD). C57BL/6 IgM deficient (μMT) and C57BL/6 wild-type mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were

*Abbreviations used in this paper: HEL, hen eggwhite lysozyme; HPRT, hypoxanthine phosphoribosyl transferase; H1, hemagglutinin subtype 1; N1, neuraminidase subtype 1, NMS, normal mouse serum; ELISPOT, enzyme-linked immunospot.

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housed and bred at the Albany Medical College and provided food and water ad libitum. Animal care and experimental procedures were performed in compliance with the Institutional Animal Care and Use Committee guidelines.

Neonatal immunization procedures

One-day-old mice were injected i.p with 1 μg of subunit influenza vaccine, which consisted 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, New York, NY). The Ag was mixed with 2 mg/ml of alum (Rehydrolg Low Viscosity Gel; Reheis, Berkeley Heights, NJ). Mice were also injected i.p on day 1 with 1 μg of recombinant murine IL-12 that was diluted in PBS containing 1% BALB/c mouse serum (PBS-NMS) or, in the case of control mice, with PBS-NMS only. The mice were allowed to mature to adulthood, and, at 5–6 wk of age, they were boosted with the same amount of Ag mixed in alum. No toxicity was observed with this treatment regimen. Sera were prepared by bleeding mice from the orbital plexus 7 days after the boost.

RNA isolation and RT-PCR

Total RNA isolation from snap-frozen spleens 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. Following 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 70% ethanol, and solubilized in diethyl pyrocarbonate-treated water. The concentration of total RNA was determined by spectrophotometric analysis at 260 nm. Three micrograms of total RNA were reverse transcribed into cDNA using a reverse transcription kit (Life Technologies, Gaithersburg, MD) using oligo(dT)16 primers. The resulting cDNA was amplified using specific primers for IFN-γ and IL-10 with hypoxanthine phosphoribosyl transferase (HPRT) primers as a control. The sense and antisense primers had the following sequences: IFN-γ: 5′-TGAACCGCTA CACACTGATCTTTG-3′ and 5′-GCAGCTTCCCCGCTTCTTGAG-3′; IL-10: 5′-ATGGCAAGCTTAAAGGATCTGGGT-3′ and 5′-ATTTCGGAAGAGAAAACACGGGT-3′; and HPRT: 5′-GTTG GTAACAGGCAGATTTTGTT-3′ and 5′-GATTACACTGCGCTA TCTTAGGC-3′. PCR amplification was performed by the manufacturer’s instructions. Briefly, 10 μg of total RNA were hybridized to 3P-labeled RNA probes overnight at 50°C. The nucleic acid was treated with RNase for 45 min at 30°C to digest single-stranded RNA, and the mixture was subjected to phenol-chloroform extraction and to a 1:10 dilution of pooled serum and challenged 6 h later with infectious influenza virus intranasally.

Results

H1N1 subunit vaccine plus IL-12 administration to neonates enhances the expression of Th1-type cytokines

IL-12 has strong potentiating effects on the immune system through its ability to preferentially activate Th1 and NK cells and induce IFN-γ production (13, 14). To determine the effects of vaccination and IL-12 administration on newborn mice, we analyzed cytokine gene expression in the spleens of 3-day-old mice that had been immunized 2 days previously with H1N1 subunit vaccine mixed with alum. It was found that neonatal mice that received PBS-NMS or Ag 24 h after birth did not express appreciable levels of splenic IFN-γ mRNA (Fig. 1). However, neonates that were immunized with H1N1 subunit vaccine and simultaneously treated with IL-12 exhibited significant induction of IFN-γ expression. We also examined the expression of IL-10, which is known to be induced in both neonates and adult mice by IL-12 treatment (23, 28, 29). Analysis of neonatal splenic mRNA revealed an absence of IL-10 mRNA expression in animals that received vaccine alone or PBS-NMS alone. In contrast, IL-10 mRNA was significantly elevated in the spleens of IL-12-treated animals. Simultaneous amplification of HPRT mRNA confirmed that equal amounts of nucleic acid were used in all RT-PCR.

To quantify and further examine cytokine profiles after neonatal immunization with influenza vaccine, levels of cytokine transcripts...
were measured by a multiplex RNase protection assay (Fig. 2). It was found that IFN-γ mRNA levels were increased 25-fold in animals treated at birth with H1N1 plus IL-12 compared with mice treated with the vaccine alone (Table I). Furthermore, IL-10 mRNA expression was enhanced 50-fold after IL-12 treatment. Coadministration of the influenza vaccine plus IL-12 also caused an 18-fold increase in expression of IL-15 mRNA levels. There was no detectable expression of other cytokines such as IL-4 or IL-5 after neonatal vaccination.

**IL-12 administered at birth induces a mixed Th1 and Th2 recall response in adult mice**

Based on the RT-PCR and RNase protection analyses performed soon after birth, we determined whether the Th1 cytokine response was still maintained in adult mice. Isolated spleen cells from mice primed at birth were tested with an ELISPOT assay for cytokine production to the recall Ag. Neonatal mice vaccinated and treated with IL-12 at birth had increased numbers of IL-2- and IFN-γ-secreting cells compared with animals receiving the vaccine alone (Fig. 3). Interestingly, we also observed an enhancement of IL-4-producing spots after neonatal IL-12 exposure. The ratio of IFN-γ- to IL-4-producing cells was 7-fold greater with IL-12 treatment compared with mice receiving the vaccine alone.

**Mice primed at birth with H1N1 subunit vaccine and IL-12 develop a Th1-like serum Ab response**

Our laboratory has previously demonstrated that IL-12 administered to adult mice i.p. can alter isotype-restricted Ab responses to HEL (15, 16). Furthermore, we recently showed that IL-12 given to neonates enhances serum Ab levels to the DNP hapten and HEL (23). We have now found that IL-12 delivered to newborn animals has similar effects on anti-H1N1 Ab expression. Mice that were immunized at birth with H1N1 showed elevated titers of total anti-H1N1 serum Ab after boosting with homologous Ag as adults compared with animals exposed to Ag only as adults (Fig. 4). IgM, IgG1, and IgG2a Ab levels were all significantly enhanced in mice exposed to the vaccine at birth, confirming the results of our previous findings in model protein and hapten-carrier systems (23). Importantly, treatment with H1N1 plus IL-12 at birth resulted in an even greater enhancement of IgG2a Ab expression upon adult Ag challenge. However, there were no significant differences in IgM and IgG1 Ab levels between mice inoculated at birth with H1N1 was still maintained in adult mice. Isolated spleen cells from mice primed at birth were tested with an ELISPOT assay for cytokine production to the recall Ag. Neonatal mice vaccinated and treated with IL-12 at birth had increased numbers of IL-2- and IFN-γ-secreting cells compared with animals receiving the vaccine alone (Fig. 3). Interestingly, we also observed an enhancement of IL-4-producing spots after neonatal IL-12 exposure. The ratio of IFN-γ- to IL-4-producing cells was 7-fold greater with IL-12 treatment compared with mice receiving the vaccine alone.

**FIGURE 1.** Expression of IFN-γ and IL-10 mRNA in the spleens of neonatal mice. Neonatal mice were injected i.p. on day 1 with H1N1 in alum plus IL-12 or PBS vehicle. Control mice received only PBS vehicle without Ag. Mice were sacrificed 2 days after treatment, and total RNA was isolated from three to four pooled neonatal spleens. Expression of IFN-γ (459 bp), IL-10 (455 bp), and HPRT (162 bp) was assayed by RT-PCR. Molecular size standards (100 bp ladder) are shown to the left of each panel.

**FIGURE 2.** IL-12 administration at birth influences cytokine mRNA expression. Neonatal mice were injected i.p. on day 1 with H1N1 in alum plus IL-12 or PBS vehicle. Control mice received only PBS vehicle without Ag. Mice were sacrificed 2 days after treatment, and total RNA was isolated from three to four pooled neonatal spleens and analyzed by multiplex RNase protection assay. The protected mRNAs were separated on a 6% polyacrylamide gel and visualized by autoradiography. Unprotected free probe is shown to the right of the panel.

**Table I. Cytokine mRNA levels in the spleens of neonatal mice immunized with the influenza subunit vaccinea**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>PBS</th>
<th>H1N1 + PBS</th>
<th>H1N1 + IL-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>2.4</td>
<td>4.3</td>
<td>215</td>
</tr>
<tr>
<td>IL-15</td>
<td>7.6</td>
<td>13</td>
<td>233</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>3.9</td>
<td>8.8</td>
<td>218</td>
</tr>
</tbody>
</table>

*a* One-day-old mice were injected i.p. with H1N1 in alum plus IL-12 or PBS vehicle. Control mice received only PBS vehicle without Ag. Mice were sacrificed 2 days after treatment, and total RNA was isolated from three to four pooled neonatal spleens and analyzed by multiplex RNase protection assay. Relative RNA levels were quantitated on a phosphorimager and normalized to GAPDH. The cytokine mRNA levels are expressed as arbitrary units.

**FIGURE 3.** Neonates injected with H1N1 vaccine plus IL-12 at birth exhibit mixed Th1/Th2 memory recall responses as adults. Neonatal mice were injected i.p. on day 1 with H1N1 in alum plus IL-12 or PBS vehicle. Another group of mice received only PBS vehicle without Ag. After 4–5 wk, spleen cells from these mice were challenged in vitro with H1N1, and the numbers of cytokine-secreting cells were then determined. The differences in number of cytokine-secreting cells between each group of mice were significant at \( p < 0.05 \).
and IL-12 and those inoculated with only H1N1. There was no IgA detected in the sera of any of the animals (data not shown).

**IL-12 administration at birth increases the protective effects of influenza subunit vaccination**

The effects of coadministering IL-12 and H1N1 at birth on survival and clinical outcome after challenge with influenza virus were next assessed by treating 1-day-old BALB/c mice with H1N1 vaccine and 1 μg of IL-12 or PBS vehicle. Some mice received only PBS vehicle. Four to 5 wk after immunization, the mice were inoculated intranasally with A/PR8/34 influenza virus and monitored daily for morbidity and mortality. It was found that all mice that were pretreated with only PBS-NMS at birth (no H1N1 subunit vaccine) displayed progressive weight loss and died within 13 days after virus challenge (Fig. 5).

Vaccination with H1N1 alone at birth resulted in 55% survival. However, inclusion of IL-12 during neonatal immunization resulted in 100% survival. Recovery from infection in the surviving mice was evidenced by regaining body weight. Therefore, incorporation of IL-12 in the immunization regimen increased the efficacy of the vaccine and conferred significant protection against subsequent influenza virus challenge.

**Augmented protection against influenza infection after vaccination with H1N1 plus IL-12 at birth is Ab-mediated**

To evaluate the contribution of humoral immunity in protection from influenza virus infection, we examined the host immune responses of B cell deficient C57BL/6 (μMT) mice (30). Mice were vaccinated at birth as described and challenged as adults with lethal influenza virus infection intranasally. It was found that μMT mice pretreated at birth with PBS-NMS alone succumbed to infection by day 9 compared with wild-type mice, which survived significantly longer (Fig. 6). All the μMT mice that received the H1N1 vaccine alone also died by day 9 compared with 67% survival in wild-type littermates. Among μMT mice that were vaccinated and treated with IL-12, all but one succumbed to infection. In contrast, every wild-type mouse that was similarly treated survived.

Thus, B cells rather than cytotoxic T cells play a crucial role in the enhanced protection conferred by IL-12 treatment at birth.

To further examine the role of serum Abs in protection against influenza virus infection, we performed passive transfer studies. Pooled serum from mice vaccinated at birth was transferred into naive animals, which were subsequently challenged with infectious A/PR8/34 influenza virus. It was found that animals that received serum from mice immunized at birth with vaccine alone exhibited 63% survival, while all but one animal that received serum from IL-12-treated mice survived viral challenge (Fig. 7).

**Discussion**

Newborns and young infants are at heightened risk of acquiring major infections, and there is a lack of suitable vaccine adjuvants that can be administered safely to neonates and that elicit strong Th1-type immune responses for defense against viral infections. We have now shown that coadministration of IL-12 at birth with...
an influenza subunit vaccine induces a Th1-type cytokine profile, primes animals for heightened IgG2a Ab responses, and increases protection against subsequent viral infection as adults. We previously demonstrated that neonates have a reduction in IL-12 expression that could be responsible for poor responsiveness and a bias toward Th2 immune responses (23). IL-12 is a pivotal cytokine that regulates the biological functions of T cells and NK cells and enhances the production of IFN-γ and other cytokines (13, 14). In addition, IFN-γ stimulates production of opsonizing Abs such as IgG2a (15, 17, 31). In the present study, we found that coadministration of IL-12 and influenza vaccine at birth resulted in significant increases in neonatal IFN-γ mRNA levels. Expression of IL-15, which has similar biological activities as IL-2 and induces proliferative responses in T cells, B cells, and NK cells (32–34), was also increased by neonatal IL-12 treatment, as was IL-10. IL-10 is an important regulator of T cells and is suggested to be involved in a feedback mechanism to modulate the Th1 pathway and down-regulate the effects of IFN-γ. While enhancing Th1-type cytokines, IL-12 had no effects on Th2-type cytokines such as IL-4 or IL-5 in neonates. The impaired cytokine responses of newborns and young infants is an important consideration in any neonatal vaccine strategy. Cairo and colleagues (24) have shown that IL-12 and IL-15 are expressed at lower levels in human cord blood, and this reduction in cytokine expression was speculated to contribute to the impairment of neonatal cell-mediated immunity. In addition, Barrios et al. (9, 10) demonstrated that neonatally immunized animals secrete higher levels of IL-5 and lower levels of IFN-γ compared with adults. These findings have crucial implications for neonatal vaccine strategies that require robust Th1-type immune responses, particularly against viral diseases such as influenza, respiratory syncytial virus, and HIV. Our results establish the ability of IL-12 administered at birth to direct the generation of Th1 immunity and serve as a potent adjuvant for viral vaccines.

Interestingly, animals vaccinated and treated with IL-12 at birth had elevated levels of IL-2, IFN-γ, and IL-4 production upon adult challenge. The augmented IL-4 production observed after IL-12 treatment could be related to the use of alum during vaccination, which primarily induces a Th2 immune response. In addition, IL-12 priming at birth may have a differential effect on the development of neonatal vs adult T cells. In vitro treatment of human neonatal T cells with IL-12 has been shown to induce both IFN-γ and IL-4 secretion, a phenotype associated with maturation of neonatal T cells (35).

It was found that inclusion of IL-12 in the vaccination regimen also resulted in enhancement of Th1-type Ab responses. Specifically, mice primed at birth with the influenza subunit vaccine and IL-12, and boosted as adults with vaccine alone, exhibited a significant increase in serum IgG2a Ab titers compared with animals primed with vaccine alone at birth. These results confirm and extend to a clinically relevant Ag our previous findings showing that IL-12 delivered at birth significantly enhances neonatal B cell memory responses to model T-dependent Ags (23). Animals primed at birth with IL-12 and a protein or hapten carrier, and boosted as adults with Ag only, were found to display elevated IgG1, IgG2a, and IgG2b Ab levels compared with mice injected with Ag alone (23). In addition, we have shown the ability of IL-12 to induce significant amounts of IgG2a Abs in adult animals responding to HEL (15, 16). Murine Abs of the IgG2a isotype are known to be efficient at opsonization and complement fixation and could be beneficial in neonatal vaccination strategies. IgG2a is the dominant Ab isotype elicited by viral infections (36), and, in fact, Graham and colleagues have shown the importance of serum IgG2a in protection against respiratory syncytial virus infections (37). Therefore, the ability of IL-12 to enhance newborn IgG2a Ab levels should have major implications in the development of infant viral vaccines.

The major finding of the present study is that administration of IL-12 at birth together with the influenza subunit vaccine increased the protective effects of the vaccine as shown by enhanced survival after influenza virus challenge. Our laboratory has recently demonstrated that IL-12 administered intranasally to adult mice serves as a potent mucosal adjuvant for anti-viral vaccination (38). In addition, we have now demonstrated in preliminary studies that endogenous IL-12 expression is a crucial component of host defense against influenza virus infection. Specifically, adult IL-12 p35-deficient mice vaccinated intranasally with H1N1 subunit vaccine alone are much more susceptible to subsequent influenza virus challenge compared with wild-type littermates (V. C. Huber and D. W. Metzger, unpublished observations). Inclusion of IL-12 during immunization augmented the protective efficacy of vaccination in IL-12 p35-deficient mice. Similarly, the importance of endogenous IL-12 in the immune response to influenza virus infection has been reported by Trinchieri and colleagues (39).

Use of B cell-deficient mice and passive transfer of serum demonstrated that the protection induced by IL-12 is mediated by Ab. It is believed that cell-mediated immunity is important in recovery from viral infections, whereas humoral responses are pivotal for protection against viral challenge. There is also evidence indicating that CD8 T cells provide protective immunity against influenza B virus infections in adult mice lacking Ig and mature B cells (40). Nevertheless, it is becoming clear that Abs play a major role in recovery from viral infections (41). In fact, several independent groups have shown that B cell-deficient animals are highly susceptible to lethal influenza virus infection compared with wild-type littermates (41–44). The ability of IL-12 to augment protective neonatal B cell memory provides strong evidence for its use as a powerful vaccine adjuvant.

Effective immunization of newborns and young infants against a variety of infectious agents represents a serious challenge to both industrialized and developing countries (45, 46). Due to the preferential Th2 bias of the neonate and increased susceptibility to
infections, there has been continued interest in identifying safe adjuvants that stimulate TH1 immunity. Currently approved adjuvants such as alum are poor stimulators of TH1 immunity, which is believed to be a crucial component of host defense against viruses (47). There is interest in the use of DNA vaccination to induce protective immunity in neonatal animals. Siegrist and colleagues (48) reported that immunization of newborn animals with DNA plasmids encoding measles virus hemagglutinin resulted in enhancement of IFN-γ and IgG2a production. In addition, Bot et al. (49) demonstrated that DNA vaccination of newborn mice with a plasmid encoding the influenza hemagglutinin gene conferred protective immunity against lethal challenge of adults with influenza virus. There is evidence to suggest that the augmented effects seen with DNA vaccination are due to IL-12, because it has been found that treatment of adult mice with synthetic oligodeoxynucleotides containing CpG motifs stimulates IL-12 production (50). In a recent study, the incorporation of CpG DNA as an adjuvant for hepatitis B virus surface Ag was evaluated in young mice (51). CpG coadministered with Ag at birth had some effects on humoral and cell-mediated responses, but augmented responses were only observed in 7-day-old immunized mice. We have now shown that IL-12 administered at birth serves as a safe and powerful viral vaccine adjuvant to induce protective TH1 immunity.

Acknowledgments

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References


