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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’Toole, § and Dennis W. Metzger2*§

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.
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 alun (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% normal 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 alun. 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 75% 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′-TGAACGGCTA CACACTGATCTTGG-3′ and 5′-CGACTCCTTTCTGCCTTCCTAG-3′; IL-10-5′-ATGCAGACTTTAGGATCTGGGT-3′ and 5′-ATTTCCGAGAGAGCTACAACGGT-3′; and HPRT-5′-GTTGG ATACAAGGCAGACTTIGGT-3′ and 5′-GATTCAACTTGCGCTA TCTAAGGC-3′. PCR amplification was performed by mixing 2 µl of cDNA, 0.25 mM dNTPs (Invitrogen, San Diego, CA), 0.8 µM primer, and 2.5 U of Taq DNA Polymerase (Life Technologies) in a final volume of 50 µl in 60 mM Tris-HCl, pH 8.5, 15 mM (NH4)2 SO4, and 0.4 mM MgCl2. The mixtures were incubated at 95°C for 5 min and then subjected to the following amplification profile: 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C for a duration of 35 cycles. This was followed by a final extension for 10 min at 72°C. The PCR products were separated on a 5% agarose gel, stained with ethidium bromide, and visualized by UV transillumination. RNAse protection assay

Cytokine mRNA levels were determined using the RiboQuant multikuprobe RNAse protection assay system (PharMingen, San Diego, CA) according to the manufacturer’s instructions. Briefly, 10 µg of total RNA were hybridized to 32P-labeled RNA probes overnight at 56°C. The nucleic acid was treated with RNAse for 45 min at 30°C to digest single-stranded RNA, and the RNAse-treated nucleic acid was 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 GAPDH, and relative cytokine mRNA levels were expressed as arbitrary values.

Enzyme-linked immunospot (ELISPOT) analysis

ELISPOT assays were used to enumerate the frequency of cytokine-secreting cells in spleens of adult mice primed at birth with 1 µg of H1N1 subunit influenza vaccine ± IL-12 and boosted in vitro with H1N1. Nicotrocellulose-backed microtiter plates (Millipore, Bedford, MA) were coated overnight with 100 µl of anti-IL-2 (2 µg/ml; clone JES6-1A2, PharMingen), anti-IFN-γ (4 µg/ml; clone R4-6A2, PharMingen), or anti-IL-4 (5 µg/ml; clone 1B11) capture Ab. Following washing, the wells were blocked with 1% BSA in PBS. Spleen cells were incubated (105 cells/well) with 0.1 µg of H1N1 at 37°C in 5% CO2 for 24 h (IL-2 and IFN-γ assays) or 48 h (IL-4 assays). The plates were subsequently washed and incubated overnight at 4°C with 100 µl of biotinylated detection Ab (0.5 µg/ml anti-IL-2 [clone JES6-5H4, PharMingen], 0.5 µg/ml anti-IFN-γ [clone XMG1.2, PharMingen], or 1 µg/ml anti-IL-4 [clone BVD6–2G2]). The plates were then washed and incubated with streptavidin-alkaline phosphatase (Life Technologies) for 2 h at room temperature. Spots were visualized by the addition of nitroblue tetrazolium 5-bromo-4-chloro-3-indolyl phosphate substrate and counted with the aid of a dissecting microscope. Statistical significance was determined using a two-tailed Student’s t test.

Detection of Ab and isotype levels by ELISA

Anti-H1N1 levels in serum were determined by ELISA essentially as described (15, 27) with minor modifications. Briefly, microtiter plates (Nalge Nunc International, Rochester, NY) were coated overnight with 1 µl of H1N1 in PBS. The plates were washed with PBS containing 0.3% Brij-35 (Sigma, St. Louis, MO) and blocked for 1 h at room temperature with PBS containing 5% FCS (HyClone Laboratories, Logan, UT) and 0.1% Brij-35. Serial dilutions of serum were added, and the plates were incubated for 2 h at room temperature. The plates were washed and incubated with goat anti-mouse whole Ig, IgM, IgG1, or IgG2A conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). After incubation for 1 h, the plates were washed and p-nitrophenyl phosphate substrate was added to obtain color development. Plates were read at 405 nm with an ELISA microplate reader (Bio-Tek Instruments, Winooski, VT). In all cases, appropriate working dilutions and isotype specificities of the secondary Ab conjugates were determined using purified myeloma proteins of known isotypes (Sigma). Statistical analyses were performed using the Mann-Whitney U test. Data were considered statistically significant if values of p < 0.05 were obtained using 50% end point titers.

Virus challenge

For protection studies, 1-day-old mice were immunized i.p. with 1 µg of H1N1 subunit influenza vaccine mixed with alum. The mice also received 1 µg of IL-12 in PBS-NMS or PBS-NMS only. Some mice received only PBS-NMS (no H1N1 subunit vaccine). Approximately 4–5 wk after primary immunization, viral challenge was performed using infectious A/PR8/34 influenza virus (provided by Dr. Doris Bucher) administered intranasally to anesthetized mice in 40 µl of sterile PBS. The mice were then weighed daily and monitored for morbidity and mortality. Survival data were analyzed using a log-rank test and compared using χ2 analysis.

Passive transfer of sera

For passive transfer experiments, sera were obtained from adult mice that had been primed at birth with PBS-NMS only, vaccine only, or vaccine plus IL-12. Normal adult recipients were injected i.p. with 100 µl of a 1:5 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 newborns 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

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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.

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

<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.
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/PR/8/34 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).

**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 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.

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/PR/8/34 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).
FIGURE 7. Passive transfer of serum from mice immunized with the subunit influenza vaccine plus IL-12 at birth confers enhanced protection against influenza virus challenge. Sera were collected from mice immunized with the H1N1 subunit influenza vaccine plus IL-12 (▲), vaccine plus PBS (●), or PBS vehicle only (□). Pooled serum was diluted 1:5 in sterile PBS and injected i.p. at a dose of 0.1 ml/mouse. All mice (8 per group) were then challenged intranasally 6 h later with 10^7 pfu of A/PR/8/34 influenza virus. The differences in survival between mice receiving serum from animals immunized with vaccine and IL-12 and those from animals immunized with vaccine and PBS were significant at p > 0.05.

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 infection. 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


