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Intradermal Delivery of IL-12 Naked DNA Induces Systemic NK Cell Activation and Th1 Response In Vivo That Is Independent of Endogenous IL-12 Production


In this study four murine IL-12 naked DNA expression plasmids (pIL-12), containing both the p35 and p40 subunits, were shown to induce systemic biological effects in vivo after intradermal injection. Three of the four IL-12 expression vectors augmented NK activity and induced expression of the IFN-γ and IFN-γ-inducible Mig genes. Both IL-12 p70 heterodimer and IFN-γ proteins were documented in the serum within 24 h after intradermal injection of the pIL-12o plasmid, which also induced the highest level of NK activity in the spleen and liver among the IL-12 constructs. Interestingly, both p40 mRNA expression at the injection site and serum protein levels followed a biphasic pattern of expression, with peaks on days 1 and 5. Subsequent studies revealed that the ability of intradermally injected pIL-12o to augment NK lytic activity was prevented by administration of a neutralizing anti-IL-12 mAb. Finally, injection of the pIL-12o into BALB/c IL-12 p40–/– mice also resulted in a biphasic pattern of IL-12 p70 appearance in the serum, and induced IFN-γ protein and activated NK lytic activity in liver and spleen. These results demonstrate that injection of delivered naked DNA encoding the IL-12 gene mediates the biphasic systemic production of IL-12-inducible genes and augments the cytokine function of NK cells in lymphoid and parenchymal organs as a direct result of transgene expression. The results also suggest that these naked DNA plasmids may be useful adjuvants for vaccines against infectious and neoplastic diseases. The Journal of Immunology, 1999, 163: 1943–1950.

IL-12 was initially identified and isolated as an NK cell stimulatory factor (1). Compared with other cytokines, it has a unique 70-kDa heterodimeric structure composed of two covalently linked p35 and p40 subunits, both of which are required for biological activities (1, 2). IL-12 is produced principally by APC, such as monocytes, macrophages, and dendritic cells. In addition to this stimulatory effect on NK cells, IL-12 activates cytotoxic T cells (3–5), differentiates CD4+ lymphocytes (6, 7), plays an important role in regulating the balance between the type 1 and type 2 response of Th lymphocytes (8, 9), primes macrophages for nitric oxide production (10), and possesses IFN-γ-dependent antiangiogenic activity (11, 12). These diverse biological effects make IL-12 an attractive candidate as a therapeutic agent for cancer and infectious diseases. Systemic administration of IL-12 protein alone (2, 13–15) or in combination with IL-2 (16–18) significantly suppressed the growth of a variety of established mouse tumors and prolonged the survival of tumor-bearing mice. IL-12 also has efficacy as an adjuvant for vaccination against cancer (19), and intratumoral delivery of adenovirus containing the IL-12 gene can cause regression of some established tumors in mice (20–23). The targeted inactivation of both alleles of the IL-12 p40 gene impairs the production of IFN-γ and the induction of a delayed-type hypersensitivity response (24) and renders mice susceptible to infection by Leishmania major (24).

Despite these interesting therapeutic implications for IL-12, the best approaches for delivery of IL-12 in vivo remain to be determined. Most studies have been performed using systemic delivery of the rIL-12 protein. Although the pharmacodynamics of IL-12 are more favorable than those of many other cytokines, repeated administration on a daily basis is required for maximal therapeutic activity in mice (25, 26). In addition, the repeated bolus administration of the recombinant proteins can cause undesirable side effects (27, 28). Alternative approaches for IL-12 delivery also have some limitations. For example, virus-mediated gene delivery can result in the subsequent generation of neutralizing Ab, which limits the duration that active immunotherapy is effective. In the case of cytokine-mediated retroviruses, integration of the virus genome into host chromosomes may be a concern for other deleterious effects.

The direct in vivo transfer of DNA without any carrier agents (referred to as naked DNA) was first described in 1990 as a novel form of gene therapy (29). The initial studies showed that muscle was a suitable target tissue for gene delivery (29, 30), but skin also was shown to be suitable as an alternative site for injection (31–34). Some initial success of naked DNA encoding therapeutic
proteins was documented by the induction of a host immune response against several infectious agents (31, 35, 36). Subsequently, naked DNA was also proven to induce local or systemic biological effects in vivo, including improvement of anemia by in vivo delivery of the erythropoietin gene (37) or recruitment of neutrophils into the site of IL-8 plasmid DNA injection (32). Major advantages for the in vivo use of highly pure plasmids include the relatively simple and inexpensive production compared with protein and the possibility that more chronic production may decrease the need for high systemic protein levels associated with bolus administration of cytokines, thereby reducing unfavorable side effects.

In this study we constructed several IL-12 expression vectors that encode both murine p35 and p40. These plasmids were injected intradermally (i.d.) and shown to induce IL-12 mediated biological activity, including activation of NK cells, induction of IFN-γ, and the IFN-γ-inducible chemokine monokine induced by IFN-γ (MIG). These effects also were detectable in IL-12 p40−/− mice, showing that they were not mediated by endogenous production of normal host IL-12 or contaminating endotoxin. Overall, these results show that in vivo transferred pIL-12 DNA can induce the expected systemic bioactivities of IL-12 and suggest that this form of gene therapy is efficient and safe for IL-12 delivery.

Materials and Methods

Animals

Pathogen-free female BALB/c mice between 6–8 wk of age were obtained from the Animal Production Area, National Cancer Institute-Frederick Cancer Research and Development Center. These mice were housed under specific pathogen-free conditions and provided sterilized mouse chow and water ad libitum. BALB/c IL-12 p40−/− mice (38) were donated by Dr. Jean Magram, Hoffmann-La Roche (Nutley, NJ) and maintained as a small breeding colony in our own animal facility. Animal care was provided in accordance with the procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell lines and reagents

The hybridoma C17.8 for anti-mouse IL-12 p70 was a gift from Dr. Gior- gio Trinchieri (Wistar Institute of Anatomy and Biology, Philadelphia, PA). YAC-1 and P815 cells were maintained in vitro in RPMI 1640 containing 10% FCS and 2 mM glutamine.

Plasmids

The expression vector CMV-B, which encodes the β-galactosidase gene, was obtained from Clontech (Palo Alto, CA). Four constructs of murine plLI-12 were generated as follows. First, the pcDNA3.1 plasmid (Invitro- gen, Carlsbad, CA) was modified by inserting an SV40 intron between the CMV enhancer/promoter and multicloning site. As shown in Fig. 1, the p35 and p40 subunits of murine IL-12 were each driven by a separate CMV enhancer (see Fig. 1, the p35 and p40 subunits of murine IL-12 were each driven by a separate CMV enhancer/promoter, and individual expression cassettes were oriented in either the CMV, CMV enhancer and promoter; i, SV40 intron: p35, mouse IL-12 p35 subunit; p40, mouse IL-12 p40 subunit; BHG, bovine growth hormone polyadenylation site; neo, SV40 early promoter and origin followed by neomycin resistance gene and polyadenylation signal. All the plasmids contain the same backbone from pcDNA3.1+1, which includes the replication origin and ampicillin resistance gene.

Detection and quantification of murine IL-12 and IFN-γ by ELISA

Blood was collected at various times from mice after injection of plasmid DNA, and the serum was assayed for murine IL-12 and IFN-γ by ELISA kits purchased from Endogen (Woburn, MA) and R&D Systems (Minn- apolis, MN), respectively.

Assessment of NK cell activity in leukocytes isolated from liver and spleen

At various times after plasmid injection, mice were euthanized, and blood, liver, and spleen were harvested. The livers were perfused with HBSS, and mononuclear cells were prepared as previously described (18). Briefly, three or four livers were dissociated on a stomacher (Tekmar, Cincinnati, OH) and centrifuged at 500 × g. The resuspended pellet was filtered with nylon gauze, overlaid on Lympholyte M (Cedar Lane Laboratories, Ontario, Canada), and centrifuged at 2600 × g for 30 min. The leukocyte layer was recovered, and the cells were washed and counted. Various numbers of leukocytes were then cocultured for 4 h with 1 × 106 51Cr-labeled YAC-1 or P815 target cells in 96-well microplates.

RT-PCR

Total RNA was prepared from snap-frozen spleens using Trizol (Life Technologies). cDNA were synthesized with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) primed with an oli- go(dt)12–18 primer (Pharmacia, Piscataway, NJ) in the presence of 0.2 mM dNTP and 10 U of RNase inhibitor (Pharmacia). cDNA (250 ng) was used to amplify the IFN-γ and Mig genes. The reaction was performed as 30 cycles at 94°C for 30 s, 55°C for 60 s, and 72°C for 60 s. The sequences for primers are: IFN-γ sense, 5′-TGGCGCCTGCTTCATGACAAATGA 3′; IFN-γ antisense, 5′-TGAAATGGCTGCGGCGCGATCGT-3′; Mig sense, 5′-GATCAAAACGCTGCTGATCC-3′; Mig antisense, 5′-GGCT GGTTGATAGACACAGATG-3′; actin sense, 5′-CACTTGAGAGGGAATCAGTG-3′ and actin antisense, 5′-ACTGTGTTGGCCATAGAGGTC-3′.

Ten microliters of PCR product was resolved in a 1% agarose gel along with a 100-bp m.w. marker (Life Technologies).

Northern blot analysis

Total RNA was prepared at various times as described above from the skin at the site where DNA was injected. Five micrograms of total RNA was run
in 1% agarose gels containing 1× MOPS buffer and transferred to a nylon membrane Hybond N (Amersham, Arlington Heights, IL). The RNA blot was prehybridized in Easy Hyb (Boehringer Mannheim, Indianapolis, IN) and hybridized overnight in the same buffer containing 100 ng/ml of digoxigenin-labeled murine IL-12 p40 antisense riboprobe. The membrane was then washed twice with 0.1% SDS and 0.1× SSC at 65°C for 15 min, and chemiluminescence was detected with CSPD as a substrate according to the manufacturer’s handbook (The DIG System User’s Guide for Filter Hybridization, Boehringer Mannheim).

Results

Intradermal gene expression

To confirm that i.d. injected DNA actually leads to expressed genes in vivo, we initially investigated the expression of the i.d. injected β-galactosidase gene, CMV-β, as a model system. Twenty-four hours after the i.d. injection of 20 µg of CMV-β-β-galactosidase activity was readily demonstrated at the site of injection (data not shown). Histological analysis of the site revealed the activity was mainly in the smooth muscle of the s.c. tissue (Fig. 2).

Systemic augmentation of NK activity by various IL-12 gene-encoding plasmids

These studies were performed to compare the abilities of the various IL-12 plasmids to augment mouse NK activity in vitro. In preliminary screening studies, leukocytes were isolated from liver and spleen 4 days after the i.d. injection of control or IL-12 expression vectors, and NK-mediated lytic activity was measured against YAC-1 target cells. In these studies pIL-12o, in which the p35 and p40 expression cassettes were placed in opposite orientations in a single retroviral vector (Fig. 1) gave the highest NK cell activity in the liver and spleen, followed by pIL-12 IRES, pIL-12o, and pIL-12o in the order of NK activity induced (data not shown). The results suggested that the use of separate expression cassettes for p40 and p35 produced higher NK cell activity than that obtained employing the IRES strategy, particularly when the p40 and p35 expression cassettes were placed in opposite orientations. The results also suggested that the presence of the neomycin expression cassette may have some inhibitory effect on IL-12 production and NK activity in vivo. Therefore we constructed the pIL-12o in which the p35 and p40 genes were oriented in the opposite direction from each other and where both were under separate control of CMV promoters, but the neomycin gene expression cassette was deleted. The study shown in Fig. 3 compares the NK-augmenting effects of the neo- and neo+ constructs. NK lytic activity induced by pIL-12o was comparable to that obtained with IL-12o and was 4- and 3-fold higher than levels induced by the IRES vector in the liver and spleen, respectively. Because there was no apparent benefit to expressing neomycin at the site of gene delivery, the IL-12o plasmid was selected for further study to exclude any concerns about whether any observed in vivo biological effects could be related to the expression of neomycin in vivo. None of the mice that received single injections of either pcDNA control vector or the pIL-12 constructs showed any gross toxicities.

Induction of IFN-γ and Mig genes in the spleen

Because many of the biological effects of IL-12 are known to be mediated via induction of IFN-γ and subsequent induction of other IFN-γ-inducible genes, we studied the inducibility of the IFN-γ and Mig genes. Portions of the spleens were obtained from mice treated with the pIL-12o and/or pIL-12o plasmids alone or with pcDNA vector. Twenty-four hours after the i.d. injection of pIL-12o, modest induction of IFN-γ gene expression was demonstrated in the spleen by RT-PCR (Fig. 4A). Mig gene expression also was observed in the spleen (Fig. 4B) 4 days after the injection of pIL-12o and pIL-12o.

Kinetics of NK lytic activity and IL-12 production induced by pIL-12o

The previous data demonstrated that NK cell activity was induced by pIL-12o by day 4. However, it was unclear whether this was the optimal time for detecting augmented NK activity and how this related to the production of IL-12. Therefore, a more detailed kinetic evaluation of these events was performed at various times between 1–14 days after i.d. plasmid injection using the same dose of DNA described above. As shown in Fig. 5 under this time scheme, only at 5 days after pIL-12o injection was there a clear increase in NK activity in the liver and spleen.

The data in Fig. 6 depict the kinetics of IL-12 and IFN-γ in sera pooled from three mice per group at various times after injection of 50 µg of pIL-12o or pcDNA. As expected, the serum levels of these two cytokines peaked well before the detectable augmentation of NK activity (Fig. 5). Specifically, there was detectable IL-12 (Fig. 6A) and IFN-γ protein (Fig. 6B) by 24 h after DNA
injection. Surprisingly, the IL-12 protein rose again 96 h after injection, thereby displaying a biphasic profile of IL-12 p70 protein in the serum after the single i.d. injection of pIL-12⁻²⁻.

Expression of p40 gene at the site of DNA injection

To investigate the mechanism for the biphasic production of IL-12 protein, tissue was harvested from the injection site at various times after the injection of pIL-12⁻²⁻ and analyzed by Northern blot for p40 gene expression (Fig. 7). These results showed that by 24 h there was a pronounced induction of the p40 gene, which was then down-regulated by day 3 and re-elevated on day 5, consistent with the protein data presented in Fig. 6 above. The gene re-expression initially detected on day 5 remained until day 7. No p40 expression was detected after pcDNA injection, demonstrating that the gene expression observed above was directly due to transcription of the injected pIL-12⁻²⁻. Overall, these results suggest that the biphasic expression of IL-12 protein occurred because of a biphasic expression of the IL-12 gene.

Inhibition of NK activity by anti-IL-12 mAb

To confirm that the systemic NK activity elicited by pIL-12⁻²⁻ was directly dependent on the production of IL-12 protein, mice were pretreated i.p. with 25 μg of either C17.8 rat mAb, which specifically neutralizes the murine IL-12 p70 heterodimer, or control rat Ab beginning 24 h before the injection of 50 μg of pIL-12⁻²⁻ or pcDNA injection. Spleens and livers were then harvested, and isolated leukocytes were tested for NK activity. In this experiment, because of a limited availability of leukocytes from the liver, a single E:T cell ratio was employed to test NK activity, while the assay for splenic NK activity was conducted as in the previous experiment. Mice treated with control Ab and pIL-12⁻²⁻ exhibited the expected increase in NK activity, while those treated with C17.8 Ab and pIL-12⁻²⁻ showed no augmentation of NK activity (Fig. 8).

The i.v. administration of pIL-12⁻²⁻ also results in detectable levels of serum IL-12 and IFN-γ

The data presented above cumulatively support the ability of a locally injected IL-12 expression plasmid to induce systematically
detectable cytokine levels and biological effects. However, it is possible that direct localization of the expression plasmid in major organs and the focused production of IL-12 in such sites might have more potent biological effects in specific organs. Therefore, we investigated whether i.v. injected plasmid DNA could augment NK activity in the spleen and liver. This approach resulted in very potent induction of NK activity by 2 days after iv injection of the IL-12 plasmid (Fig. 9). This augmented NK activity was retained at 96 h, and the levels of NK activity achieved in the liver were quite high compared with those observed after i.d. injection (Fig. 3).

**NK cell activation by pIL-12o in p40−/− mice**

To completely exclude the possibility that any of the biological effects outlined above were due to the induction of endogenous IL-12 production rather than to direct transcription and translation of the gene product of the injected plasmid, we repeated the NK augmentation studies in IL-12 p40−/− mice. After i.d. injection of 50 μg of pIL-12o into the BALB/c p40−/− mice, IL-12 p70 was detected in the serum by 24 h (Fig. 10A). IFN-γ also was demonstrated in the serum by 24 h (Fig. 10B), and as seen in normal mice, the effect was biphasic, showing high levels at 24 h, a decline, and subsequent re-elevation by 4 days. This IFN-γ induction was confirmed by RT-PCR of spleen from the same treatment mice (Fig. 10C). The pIL-12o also augmented NK activity in the spleens of the p40−/− mice as previously observed in normal mice (Fig. 11) by day 4. However, in contrast to results in normal mice (Fig. 3), augmentation of NK activity in p40−/− mice occurred more rapidly (by 24 h) and persisted for 4 days. These results demonstrate that the injection of an appropriately constructed pIL-12 naked DNA plasmid can directly contribute enough IL-12 protein to mediate systemic production of IL-12-inducible genes and augment the cytotoxic function of NK cells in lymphoid and parenchymal organ sites.

**FIGURE 8.** Blockade of augmentation of NK lytic activity by pIL-12o using Abs against mouse IL-12. Twenty-five micrograms of C17.8 mAb or control IgG was injected i.p. daily starting on the day before the pIL-12o injection and continuing until the day before harvest. Fifty micrograms of pIL-12o was injected i.d. Four days later leukocytes from the liver (A) and spleen (B) were harvested, and NK lytic activity was measured in a 4-h 51Cr release assay. Because of a limited availability of leukocytes recovered from the liver, a single E:T cell ratio was employed for this NK lysis assay. Splenic NK activity was assayed as previously described.

**FIGURE 9.** The i.v. gene delivery of pIL-12o and pcDNA. Two hundred micrograms of DNA dissolved in PBS containing 5% glucose was injected into the tail vein of BALB/c mice. Four days later leukocytes were isolated from the liver and incubated for 4 h with 51Cr-labeled YAC-1 target cells.

**FIGURE 10.** Detection of IL-12 p70 (A) or IFN-γ (B) proteins in the serum of IL-12 p40−/− mice. Fifty micrograms of DNA was injected once i.d., and IL-12 p70 or IFN-γ was measured in the serum by ELISA on days 1, 2, and 4. IFN-γ gene induction 24 h after plasmid injection (C) is shown for spleens obtained from the same mice as those used for the protein analyses in A and B.

**FIGURE 11.** Induction of NK lytic activity induced by i.d. injection of pIL-12o in IL-12 p40−/− BALB/c mice. Leukocytes isolated from the spleens of the same mice that were monitored for serum cytokines in Fig. 10 were incubated with 51Cr-labeled YAC-1 cells for 4 h.
**Discussion**

IL-12 has proven to be a very active agent for immune modulation and for treatment of infectious and neoplastic diseases in animal models. However, much remains to be learned about the most appropriate manner in which to deliver this cytokine for maximal therapeutic benefit and with minimal toxicity. The studies presented herein focus on an approach using naked DNA to transfect the IL-12 gene into host cells in vivo. The design of the expression vectors used for our studies is complicated by the heterodimeric nature of IL-12, and we have made the assumption that a single vector, encoding both chains of the IL-12 protein, would be preferable to individual plasmids for p40 and p35. Because the p40 subunit is usually produced in large excess over the p70 dimer, but only the latter has biological activity (2), we speculated that a single plasmid encoding both genes would enhance the potential of obtaining mostly p70 and less free p40, which has been postulated to play a role in inhibiting p70 function (39, 40). Therefore, we constructed four IL-12 expression vectors based on this theory and tested these plasmids for their abilities to induce several characteristic indicators of systemic IL-12 activity. Specifically, we chose augmentation of NK activity and induction of IFN-γ gene expression and protein production as the parameters of choice for detecting systemic IL-12-induced bioactivities. We found that pIL-12o₁, in which the p40 and p35 subunits were driven by a separate CMV promoter/enhancer and where the expression cassettes were oriented in the opposite direction in the same construct, produced the highest NK cell activity in the liver and spleen. The degree of systemic augmentation of NK activity was higher than that achieved with other plasmids, including those in which p40 and p35 were both in the same orientation but driven by individual CMV promoter/enhancers or using an IRES plasmid in which the expression cassette was in a tandem orientation. Some previous reports also suggested that the coexpression of CMV enhancer and SV40 promoter for neomycin expression increased gene expression from the CMV enhancer (41). Therefore, in an attempt to obtain the most efficient in vivo construct possible, IL-12o₁, the neomycin expression cassette was removed from the pIL-12o₁ plasmid, and this pIL-12o₂ demonstrated NK activity at least comparable with that obtained using pIL-12o₁. In addition, pIL-12o₂ was superior to the pIL-12o₁ construct for induction of IFN-γ in vivo (data not shown). These results extend previous studies (34) that reported an augmentation of splenic NK activity by i.d. injection of IL-12 cDNA by demonstrating that the pIL-12 also can augment NK activity in the liver.

The best cellular target for in vivo transfection remains unclear. In our initial experiments we chose the i.d. route to deliver naked DNA for two reasons. First, it is technically simpler than i.m. injection, and second, the previous literature demonstrates that i.d. transferred genes are expressed more quickly than those expressed after injection into muscle. Because we also plan to contrast naked DNA delivery with protein therapy for the treatment of rapidly growing established tumors in mice, we speculate that a process that results in more rapid gene expression in vivo could be more beneficial than one where initial gene expression is more prolonged, but delayed in its onset. As shown in Fig. 2, the injected DNA was expressed mainly in the i.d. smooth muscle, while in previous studies the activity of the i.d. injected gene product was visualized predominantly in the epidermis in the human and pig skin tissue (32). Thus, there may be a preference for a particular cell type for the DNA entry depending on the nature of the construct, the genes to be expressed, or the species to be injected. By Northern blot analysis, p40 gene expression was detectable up to 7 days at the site of the pIL-12o₁ injection, and mRNA expression was highest at 24 h, with another peak appearing 5 days later after a single injection of plasmid. Serum levels for IL-12 after pIL-12o⁻ injection paralleled the kinetics of this p40 gene expression, supporting the observed biphasic nature of p40 gene expression. This biphasic pattern of expression was surprising, and we speculated that the first peak represented IL-12 produced from the injected plasmid, while the second peak could be due to subsequent production of IFN-γ by T and NK cells (42) and subsequently induction of more IL-12 production by phagocytic cells (43). However, this is not the case, because the same biphasic gene expression is obtained after pIL-12o⁻ injection into p40⁻/⁻ mice, in which endogenous IL-12 p70 cannot be induced. Taking these three independent results (p40 Northern blotting, IL-12 and IFN-γ serum ELISAs in normal mice and p40⁻/⁻ mice) together, therefore, we conclude that i.d. pIL-12o⁻ injection results in a biphasic expression pattern for the transferred gene itself in vivo. Although previous studies using i.m. DNA injection revealed a gradual increase in protein expression by the transgene up to 14 days, with activity detected as long as 120 days (29, 44), we know of no reports of clear biphasic or intermittent expression. To date, the relatively short term (e.g., 1–7 days) kinetics of naked DNA in vivo have not been investigated in complete detail, and we plan to examine the mechanism for these biphasic effects.

Taking into consideration the expression data outlined above, it may not be surprising that the highest NK cell activity in liver and spleen was not observed until about 4 days after pIL-12o⁻ injection (i.d.) even though the blood level of IL-12 was highest at about 24 h. Recent data from our laboratory have shown that a single administration of recombinant mouse IL-12 protein (0.5 μg/ day) induced the highest NK activity in the liver compared with daily injection for 2–4 days (45). In these studies a decrease in NK activity after repeated administration of IL-12 was accompanied by a reduced number of NK cells. However, in the studies using the pIL-12o⁻ no decrease in the number of NK cells in the liver was observed even 4 days after pIL-12o⁻ injection (data not shown), suggesting a basic biological difference in the regulation of hepatic NK cells by exogenous IL-12 protein vs pIL-12o⁻. Interestingly, we noted in our previous studies with IL-12 protein administration that the ability of IL-12 to induce recruitment of NK cells to the liver is dependent on the production of IFN-γ (45). Therefore, IFN-γ induced by IL-12 may contribute to a recruitment of NK cells to at least some sites. In addition, the IFN-γ-inducible Mig gene, which serves as another indicator of systemic effects of IL-12, also was induced. Although this expression of Mig may have no direct relevance to induction of Th1 responses, it may play some role in the IFN-γ-dependent recruitment of NK cells induced by IL-12 (45). We are currently studying the role of IFN-γ-inducible genes in IL-12-induced leukocyte recruitment.

Another intriguing finding of our study is that in p40⁻/⁻ mice, injection of pIL-12o⁻ induced more potent augmentation of NK activity and induced more IFN-γ than in normal mice, particularly in the second peak. The controlled production of the p40 subunit that is usually produced in large excess over the p35 subunit and can antagonize the biological effects of IL-12 p70 may explain the higher responsiveness to IL-12 translated from the expressed pIL-12o⁻ in p40⁻/⁻ mice.

In an effort to further optimize and understand the immuno-modulatory potential of the pIL-12o⁻, naked DNA delivery by the i.v. route also was investigated. A previous report found that the i.v. injection of naked DNA in PBS resulted in degradation within 5 min and the absence of any protein expression in various organs (46). However, in our studies i.v. injection of 200 μg of pIL-12o⁻...
effectively induced NK activity in both liver and spleen by 2 days after injection, and the augmented NK activity remained detectable at 4 days. Consistent with this observation are studies by Wang et al., who reported kallikrein gene expression in heart, lung, and liver even 3 wk after a single i.v. injection of 500 µg of DNA dissolved in PBS containing 5% glucose (47). Thus, the addition of 5% glucose, as used in our studies, may be useful for stabilizing the DNA for i.v. injection, although the mechanism involved in the stability has not been determined yet.

In vivo delivery of plasmid DNA is becoming more commonly used as a novel vaccination method (48), where i.m. or i.d. injection of Ag-coding DNA favors the effective development of Th1 responses (49), and a previous study has demonstrated that the injection of IL-12 cDNA can actually delay the growth of subsequently injected murine renal cancer cells. A recent finding also has been reported that particular unmethylated DNA sequences are often unmethylated and 20 times as common as in mammalian DNA, whose CpG motif is methylated in >80% of the cases. Thus, some mammalian DNA and, more preferentially, bacterial DNA also may target induction of a Th1 response by host leukocytes. In contrast, other approaches, such as DNA vaccination by the gene gun, preferentially induce Th2 responses (51). Although the gene gun requires 1/100th less DNA (usually 0.5–2 µg) compared with i.d. or i.m. DNA (50–200 µg) vaccination to elicit biological effects, it is also possible that the amount of DNA delivered by the gene gun technology is not sufficient to effectively trigger Th1 immune responses locally or systemically. Our experiments in normal and p40γ−/− mice demonstrate clearly that the i.d. injection of pIL-12o−/− DNA triggers a strong Th1 response that is independent of a secondary IFN-γ-induced or DNA-nonspecific production of endogenous IL-12. Thus, the i.d. delivery of pIL-12o−/− leads to effective transcription and translation of biologically active IL-12 p70 that systemically induces cytokines and host effector cell functions.

The effective induction of a Th1 response, as indicated by IFN-γ synthesis following pIL-12o−/− in our studies, may be a major advantage for the therapeutic use of IL-12 in vaccine approaches to infectious disease and cancer treatment. Ghosh et al. reported that T cells from mice bearing tumors for >1 mo gradually lose the Th1 phenotype (52), and the reversal or prevention of this effect may be important for maximizing the response to therapeutic vaccines. In addition to any systemic effects, the amount of IL-12 available at the tumor site contributes to both the type and the number of infiltrating leukocytes and the events leading to tumor regression (53). As for infection, a similar effect has been confirmed during vaccination against Schistosoma mansoni, in which vaccination with eggs and IL-12 prevents the subsequent pulmonary granuloma formation and tissue fibrosis that are associated with a Th2-dominated pattern of cytokine expression (54, 55). Also, as mentioned previously, IL-12 plays an important role in protecting against infection by L. major (24).

Overall, the IL-12 gene delivery approach described herein demonstrates the potency of appropriate DNA expression plasmids for the induction of systemic and local Th1-type responses. Such effects, in the absence of the practical limitations often observed during the use of viral vectors as gene delivery systems, suggest the considerable utility of this approach for vaccine-based prophylactic or therapeutic strategies in the treatment or prevention of infections and neoplastic diseases.

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