IL-12-Deficient Mice Are Defective in IFNγ Production and Type 1 Cytokine Responses

Jeanne Magram,* Suzanne E. Connaughton,[†]
Rajeev R. Warrier,[†] Daisy M. Carvajal,[†]
Chang-you Wu,[†] Jessica Ferrante,*
Colin Stewart,[‡] Ulla Sarmiento,[§]
Denise A. Faherty,[†] and Maurice K. Gately[†]
*Department of Biotechnology
[†]Department of Inflammation/Autoimmune Diseases
[‡]Roche Institute of Molecular Biology
[§]Department of Toxicology and Pathology
Hoffmann-La Roche, Incorporated
340 Kingsland Street
Nutley, New Jersey 07110

Summary

IL-12 is a cytokine that can exert regulatory effects on T and NK cells and promote Th1 responses. To delineate further the physiologic role of IL-12 in immunity, mice deficient for this cytokine were generated. IL-12-deficient mice were impaired but not completely lacking in the ability to produce IFNγ following endotoxin administration and to mount a Th1 response in vivo, as measured by antigen-induced IFNγ secretion by immune lymph node cells in vitro. In contrast, secretion of IL-4 was enhanced, while proliferation and secretion of IL-2 and IL-10 were normal following antigen stimulation. DTH responses were significantly reduced in IL-12-deficient mice, but no defect in allogeneic CTL responses was observed. These results indicate that IL-12 plays an essential role in regulating IFNγ production and in facilitating normal DTH responses. However, other phenomena associated with Th1 responses and cell-mediated immunity, i.e., IL-2 secretion and CTL generation, were not compromised in the absence of IL-12.

Introduction

Interleukin-12 (IL-12) is a heterodimeric cytokine that has a variety of effects on T and natural killer (NK) cells. These include its ability to induce interferon-γ (IFNγ) secretion by T and NK cells, to act as a growth factor for activated T and NK cells, to enhance the lytic activity of NK/lymphokine-activated killer (NK/LAK) cells, and to facilitate specific cytolytic T lymphocyte (CTL) responses (reviewed by Trinchieri, 1994; Hendrzak and Brunda, 1995). Furthermore, and possibly most importantly, IL-12 plays a unique role in regulating the balance between the type 1 and type 2 subsets of T helper (Th) cells. Type 1 Th (Th1) cells produce IFNγ and IL-2 and promote primarily cellular immunity, whereas Th2 cells produce IL-4, IL-5, IL-10, and IL-13 and promote principally humoral immunity (Mosmann and Coffman, 1989; Scott, 1993). IL-12 facilitates Th1 responses by stimulating the differentiation of naive Th cells into Th1 cells (Manetti et al., 1993; Hsieh et al., 1993) and by serving

as a costimulus required for maximum IFNγ secretion by antigen-activated Th1 cells (Murphy et al., 1994).

The heterodimeric structure of IL-12 is unique among the cytokines. IL-12 consists of two disulfide-bonded subunits, called p40 and p35, referring to their molecular masses of 40 and 35 kDa, respectively (Kobayashi et al., 1989; Stern et al., 1990). The genes encoding the p40 and p35 subunits are unrelated and map to different human chromosomes (Sieburth et al., 1992). Both subunits are required for the biological activity of the cytokine (Wolf et al., 1991; Gubler et al., 1991); however, recent experiments have demonstrated that p40 can be secreted as a homodimer that behaves as an IL-12 antagonist (Gillessen et al., 1995). IL-12 is synthesized primarily by antigen-presenting cells, including macrophages, monocytes, and dendritic cells (D'Andrea et al., 1992; Macatonia et al., 1995). Expression of the individual subunit genes is regulated independently (Trinchieri, 1994), and synthesis of IL-12 heterodimer is invariably accompanied by production of a large excess of the p40 subunit (D'Andrea et al., 1992), although the significance of this is not yet known. The p35 gene is more ubiquitously expressed than the p40 gene in cell lines (Trinchieri, 1994); and in mice, the p35 gene is expressed in tissues that do not express the p40 gene, such as brain and liver (Schoenhaut et al., 1992).

To evaluate further the role of endogenous IL-12 in mediating immune responses, mice deficient in IL-12 production were generated using homologous recombination in embryonic stem (ES) cells. A mutation in the p40 gene was targeted, since expression of this gene appeared to be restricted to lymphoid tissues (Schoenhaut et al., 1992). Also, p35 in the absence of p40 is poorly secreted; however, the p40 subunit is efficiently secreted even in the absence of the p35 subunit (Wolf et al., 1991; Gubler et al., 1991). Therefore, mutations in the p40 gene would result in a lack of secreted IL-12 heterodimer, p40 monomer, and p40 homodimer.

IL-12-deficient mice developed normally, were fully fertile, and displayed no obvious defects in any organs upon necropsy. Nevertheless, these mice were deficient in their ability to produce IFNγ in response to several stimuli and to generate normal Th1 responses, as measured by antigen-induced IFNγ secretion. On the other hand, their ability to produce IL-2 and to mount allogeneic CTL responses was normal. These results demonstrate an important role for IL-12 in promoting some, but not all, facets of cell-mediated immunity associated with Th1 responses.

Results

Construction of IL-12-Deficient Mice

To introduce a null mutation into the IL-12 p40 gene, a replacement targeting vector was created as illustrated in Figure 1A. Upon homologous recombination, a portion of exon 3 (the second coding exon) was replaced with a *neo* cassette. The targeting vector was introduced into W9.5 ES cells (Szabo and Mann, 1994), and G418-resistant FIAU-resistant colonies were isolated. Southern blot analysis was used to distinguish wild-type and
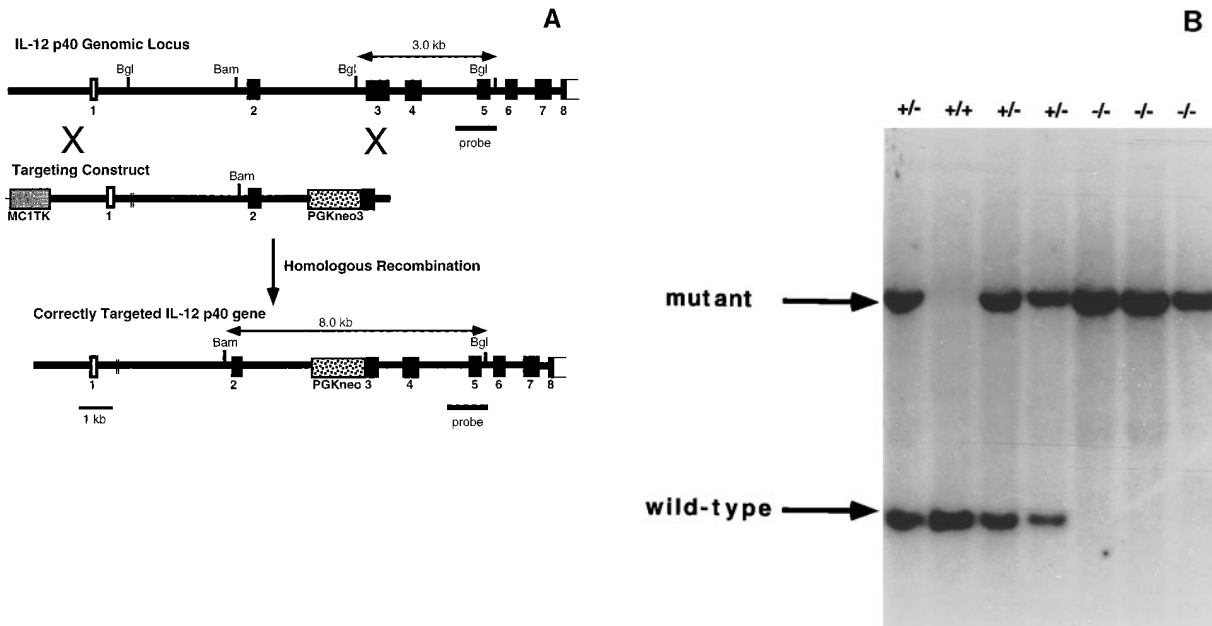
Figure 1. Disruption of the IL-12 p40 Gene

(A) IL-12 p40 targeting strategy. IL-12 p40 genomic structure is represented; closed boxes indicate coding exons, open box indicates a noncoding exon. The MC1–*tk* gene is indicated by the shaded box; the PGK–*neo* gene is indicated by the stippled box. BamHI (Bam) and BglII (Bgl) restriction endonuclease sites are indicated. The probe used for screening by genomic Southern blot analysis is indicated by the closed bar. The size and location of fragments predicted for wild-type and mutant alleles detected by this screen are indicated by double-headed arrows.

(B) Identification of IL-12 p40$^{-/-}$ mice. Genomic DNA isolated from tails was digested with BamHI and BglII, electrophoresed, transferred to membranes and hybridized with the indicated probe as described in the text. +/+, wild-type; +/−, heterozygous; −/−, homozygous IL-12 p40 mutant mice.

mutated IL-12 p40 alleles. Approximately 10% of the isolated ES cell colonies contained a correctly targeted mutation. Two of these clones (J6 and C3) were used to generate chimeric animals by injection into C57BL/6J blastocysts. Both clones gave rise to highly chimeric mice, which transmitted the mutation through the germ-line. Heterozygotes from each clone were intercrossed and produced mice homozygous for the IL-12 p40 muta-tion as shown in Figure 1B. Both clones gave compara-ble results and therefore will not be described sepa-rately.

To confirm that mice homozygous for the targeted mutation were unable to produce biologically active IL-12, IL-12 bioassays were performed. The results are shown in Table 1. As expected, mice homozygous for the IL-12 p40 mutation do not produce biologically active IL-12. However, using an enzyme-linked immunosorbent assay (ELISA) that detects p40 monomer and dimer, as well as IL-12 heterodimer, a small amount of immunore-active protein (<4% of the wild-type level) could be de-tected. Using Northern blot analysis and reverse tran-scriptase polymerase chain reaction with a large number of primers spread across the coding sequence, it was determined that this protein was translated from an mRNA that resulted from removal of the mutated exon 3 by a precise splice. This determination was possible due to previous characterization of the genomic struc-ture of the IL-12 p40 gene (Chizzonite et al., 1996, Mur-phy et al., 1995), and this conclusion was confirmed

by sequencing the product resulting from polymerase chain reaction using primers in exons 2 (5′-GCTAAC CATCTCCTGGTTTGC-3′) and 4 (5′-CCAGCCATGAG CACGTGAACC-3′; data not shown). This splice would result in an mRNA encoding a p40 protein with a deletion of 89 aa and would therefore be expected to be biologi-cally inactive. To confirm that this mutant p40 protein could not contribute to the formation of biologically ac-tive IL-12 heterodimer, the mutant p40 was coexpressed with p35 in COS cells, and supernatant fluid was col-lected and analyzed in an IL-12 bioassay. Even when expressed at higher levels, this immunoreactive protein was unable to mediate biological activity (U. Gubler,

Table 1. IL-12 p40$^{-/-}$ Mice Fail to Produce Biologically Active IL-12

Mice	IL-12 Bioactivity (pg/ml)
IL-12 p40$^{+/+}$	1060
IL-12 p40$^{-/-}$	<5

Thioglycollate-elicited peritoneal exudate cells (PEC) obtained from IL-12 p40$^{-/-}$ mice and control littermates were stimulated to pro-duce IL-12 by culture in the presence of LPS and IFN-γ. Levels of biologically active IL-12 in the supernates were measured using a phytohemagglutinin-activated lymphoblast proliferation assay. The results shown were generated using PEC from 2 IL-12 p40$^{-/-}$ mice assayed individually and pooled PEC from 2 wild-type mice. These data are representative of three separate experiments.
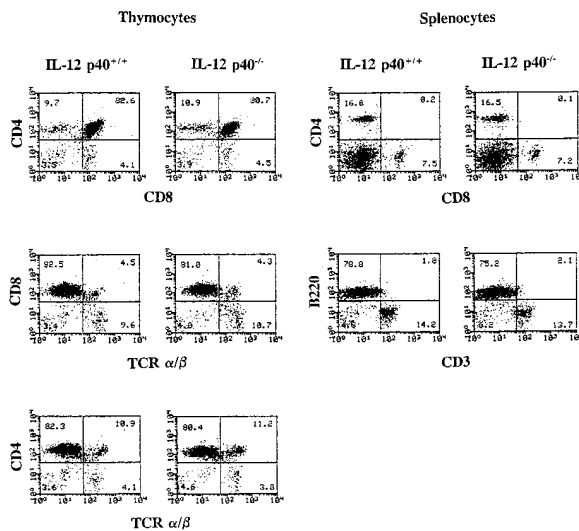
Thymocytes   **Splenocytes**

Figure 2. Flow Cytometric Analysis of Lymphocyte Subset Distributions in IL-12 p40$^{-/-}$ and Wild-Type Mice

Cells from indicated tissues were obtained from 6- to 7-week-old IL-12 p40$^{-/-}$ mice and wild-type littermate controls, stained with MAbs, and analyzed using flow cytometry. Representative comparisons of CD4, CD8, TCRα/β, CD3, and B220 markers on thymocytes and splenocytes are shown. Numbers in each quadrant indicate the percent of total cells in that quadrant. The data shown are for individual mice and are representative of results obtained for 4–5 mice per group from two separate litters.

J. F. and M. K. G., data not shown). Therefore, these mice cannot produce biologically active IL-12.

IL-12-Deficient Mice Display Normal Development

Mice homozygous for the IL-12 p40 targeted mutation (IL-12 p40$^{-/-}$ mice) were detected at the expected Mendelian frequency (Figure 1B; data not shown). No abnormalities were detected in organs of heterozygous or homozygous animals upon gross necropsy or histopathological examination. Further analysis of hematology and clinical chemistry parameters revealed no differences between wild-type and IL-12 p40$^{-/-}$ mice (data not shown). Mice carrying one or two copies of the mutated IL-12 p40 allele were also of normal size and weight, and both sexes were fully fertile.

It was previously reported that IL-12 could influence mouse thymocyte proliferation and development in vitro (Godfrey et al., 1994). Likewise, both p35 and p40 IL-12 mRNAs were detected in fetal and adult thymus and in thymic epithelial cell lines (Schoenhaut et al., 1992; Godfrey et al., 1994). Thus, it seemed possible that IL-12 might influence T cell development in the thymus. However, flow cytometric analyses of thymocytes, splenocytes, and lymph node cells from 6- to 7-week-old C57BL/6 wild-type and IL-12 p40$^{-/-}$ mice revealed no abnormalities in expression of CD3, CD4, CD8, T cell antigen receptor (TCR) α/β, B220, F4/80, or major histocompatibility complex (MHC) class II antigen in IL-12 p40$^{-/-}$ mice (Figure 2; data not shown). Hence, IL-12 does not appear to be required for normal thymocyte

maturation or seeding of mature T cells to peripheral lymphoid tissues.

NK Cell Function in IL-12-Deficient Mice

IL-12 was initially identified on the basis of its ability to enhance the lytic activity of NK cells (Kobayashi et al., 1989) and LAK cells (Stern et al., 1990). It was, therefore, of interest to determine whether IL-12 p40$^{-/-}$ mice displayed normal NK lytic activity. In five separate assays involving a total of 18 wild-type C57BL/6 mice and 20 C57BL/6 IL-12 p40$^{-/-}$ mice, the mean NK lytic activity of IL-12 p40$^{-/-}$ splenocytes was about 66% of the mean NK lytic activity of wild-type splenocytes (IL-12 p40$^{-/-}$, 0.84 ± 0.14 lytic U/10^6 splenocytes; wild-type, 1.27 ± 0.17 lytic U/10^6 splenocytes; p < 0.05 by Mann-Whitney test). However, when splenocytes from wild-type or IL-12 p40$^{-/-}$ mice were cultured with IL-2 (5, 50, or 500 U/ml) for 3 days, the IL-2-activated NK cell lytic activities of wild-type and IL-12 p40$^{-/-}$ splenocytes were essentially identical at all concentrations of IL-2 used (data not shown).

It was previously reported that treatment of mice with neutralizing anti-IL-12 antibodies reduced the production of IFNγ in response to endotoxin lipopolysaccharide (LPS) in vivo. Anti-IL-12-treated mice injected with LPS displayed serum IFNγ levels that were 16%–22% of the levels observed in control LPS-injected mice (Heinzel et al., 1994). It was not clear whether the residual IFNγ secretion in anti-IL-12-treated mice was due to incomplete neutralization of IL-12 or, alternatively, to IFNγ production via an IL-12-independent mechanism. To examine this, we administered LPS to C57BL/6 wild-type and IL-12 p40$^{-/-}$ mice and measured serum IFNγ levels 6 hr later by ELISA. Serum IFNγ levels, which were undetectable prior to LPS administration, were substantially reduced in IL-12 p40$^{-/-}$ mice as compared with wild-type mice (Figure 3). In four separate experiments using a total of 22 wild-type and 27 IL-12 p40$^{-/-}$ mice, the LPS-induced IFNγ levels in IL-12 p40$^{-/-}$ mice were 17% ± 5% of the levels seen in wild-type mice. Hence, the production of IFNγ in response to LPS was largely, but not entirely, IL-12 dependent in this model. Treatment of mice prior to LPS administration with a dose of anti-asialo GM1 (ASGM-1) antibody that was previously shown to deplete NK cells selectively in vivo (Gately et al., 1988) reduced LPS-induced IFNγ production by > 85% in both wild-type and IL-12 p40$^{-/-}$ mice (data not shown). Thus, IFNγ produced in response to LPS in these experiments appeared to be largely NK cell–derived.

T Cell Function in IL-12-Deficient Mice

IL-12 has been shown to play an important role in promoting Th1 responses in a number of mouse models both in vitro and in vivo (reviewed by Trinchieri, 1994; Hendrzak and Brunda, 1995). Therefore antigen-induced cytokine production and T cell proliferation in wild-type and IL-12 p40$^{-/-}$ mice were compared. For this purpose, mice were immunized with keyhole limpet hemocyanin (KLH) in complete Freund's adjuvant (CFA) or with alum-precipitated KLH combined with heat-killed Corynebacterium parvum as adjuvant. Draining lymph nodes were
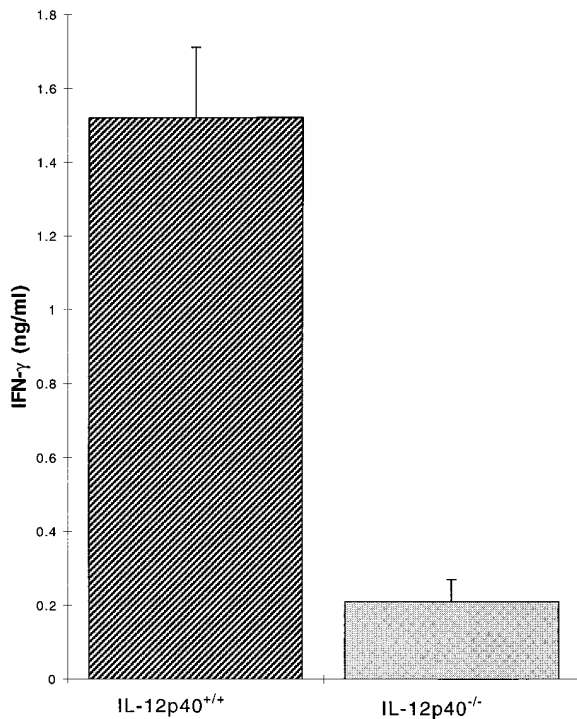
Figure 3. LPS-Induced IFNγ Production In Vivo in IL-12 p40$^{-/-}$ and Wild-Type Mice

IL-12 p40$^{-/-}$ mice and wild-type C57BL/6J controls were injected with LPS intraperitoneally and bled six hours later. Serum levels of IFNγ were measured using an ELISA. IFNγ levels were undetectable (< 0.15 ng/ml) prior to administration of LPS. The numbers given represent averages of 4 wild-type and 6 IL-12 p40$^{-/-}$ mice. Similar results were obtained in three additional experiments with comparable numbers of mice.

harvested 4–7 days later; and proliferation and cytokine production by lymph node cells (LNC) cultured with KLH were examined. LNC from KLH-immune IL-12 p40$^{-/-}$ mice showed a marked deficiency in their ability to secrete IFNγ when cultured with KLH, whereas antigen-induced IL-4 production by KLH-immune IL-12 p40$^{-/-}$ LNC was enhanced, and antigen-induced LNC proliferation was normal (Figure 4). In 13 experiments in which wild-type and IL-12 p40$^{-/-}$ mice were immunized with KLH in CFA, the ratio of IFNγ produced by IL-12 p40$^{-/-}$ KLH-stimulated LNC compared with wild-type KLH-stimulated LNC was 0.12 ± 0.02 (mean ± 1 standard error), and the ratio of IL-4 produced was 2.9 ± 0.4. In five experiments using mice immunized with alum-precipitated KLH plus C. parvum, the ratios of antigen-induced IFNγ and IL-4 production for IL-12 p40$^{-/-}$ LNC compared with wild-type LNC were 0.024 ± 0.003 and 5.2 ± 0.7, respectively. Three experiments in which LNC were depleted of CD4$^+$, CD8$^+$, or asialo GM1$^+$ cells by treatment with the corresponding antibody plus complement prior to culture with KLH indicated that 73%–93% of the IFNγ produced by wild-type LNC, >90% of the IFNγ produced by IL-12 p40$^{-/-}$ LNC, and >90% of the IL-4 produced by both wild-type and IL-12 p40$^{-/-}$ LNC were derived from CD4$^+$ T cells (data not shown). Thus, these experiments indicated that IL-12 p40$^{-/-}$ mice were

deficient, but not entirely lacking, in their ability to generate a type 1 cytokine response, as measured by antigen-induced IFNγ production, and made an enhanced type 2 cytokine response, as monitored by antigen-stimulated secretion of IL-4.

In addition to IFNγ, IL-2 is considered to be a type 1 cytokine (Scott, 1993; Paul and Seder, 1994). Although IL-10 has been considered a type 2 cytokine (Scott, 1993; Paul and Seder, 1994), administration of rIL-12 to normal mice was shown to induce the production of IL-10 (Morris et al., 1994). Likewise, as a result of in vitro priming of human peripheral blood mononuclear cells in the presence of IL-12, secretion of IL-10, as well as IFNγ, was enhanced following secondary stimulation (Chehimi et al., 1996). We therefore compared the abilities of KLH-immune LNC from wild-type and IL-12 p40$^{-/-}$ mice to produce IL-2 and IL-10 in response to KLH. For mice immunized with KLH in CFA, the ratio of cytokine produced by IL-12 p40$^{-/-}$ KLH-stimulated LNC compared with wild-type KLH-stimulated LNC was 1.5 ± 0.1 for both IL-2 and IL-10 (Figure 4). Hence, endogenously produced IL-12 appears to play little role in the regulation of IL-2 or IL-10 production, in contrast with the dramatic effects it exerts on IFNγ production.

In vitro studies have suggested that IFNγ acts as a costimulus with IL-12 in promoting Th1 responses (Seder et al., 1993; Schmitt et al., 1994). To evaluate the role of IFNγ in promoting Th1 responses in KLH-immunized wild-type and IL-12 p40$^{-/-}$ mice in vivo, mice were treated with neutralizing rat anti-mouse IFNγ monoclonal antibody (MAb) or with recombinant mouse IFNγ. In vivo treatment with anti-IFNγ MAb dramatically reduced IFNγ production by KLH-immune wild-type and IL-12 p40$^{-/-}$ LNC, whereas IL-4 production was enhanced (Figure 5). Thus, IFNγ was essential for the residual IL-12-independent type 1 cytokine response in IL-12 p40$^{-/-}$ mice, as well as for normal type 1 cytokine responses in wild-type mice. To determine whether NK cells, as a possible source of IFNγ, played a role in promoting the type 1 cytokine responses observed in these studies, wild-type and IL-12 p40$^{-/-}$ mice were treated with anti-ASGM-1 antibody to deplete NK cells in vivo (confirmed by depletion of splenic NK lytic activity). In three experiments, anti-ASGM-1 treatment of mice immunized with KLH caused a modest inhibition (41% ± 15%) of KLH-induced IFNγ production by wild-type LNC but did not significantly inhibit (5% ± 12%) KLH-induced secretion of IFNγ by IL-12 p40$^{-/-}$ LNC (data not shown). Thus, NK cells did not appear to contribute to the residual IL-12-independent type 1 cytokine response in IL-12 p40$^{-/-}$ mice.

Treatment of KLH-immunized wild-type mice with recombinant mouse IFNγ caused a dose-dependent enhancement of the type 1 cytokine response, as monitored by IFNγ secretion; whereas the ability of KLH-immune IL-12 p40$^{-/-}$ LNC to produce IFNγ in response to KLH was only modestly increased (Figure 6A). Treatment of IL-12 p40$^{-/-}$ mice with as much as 20 μg/day rIFNγ failed to reconstitute IFNγ production by KLH-immune IL-12 p40$^{-/-}$ LNC to the level seen for KLH-immune LNC from vehicle-treated wild-type mice (Figure 6A), whereas treatment of IL-12 p40$^{-/-}$ mice with only 10 ng/day of rIL-12 restored the type 1 cytokine
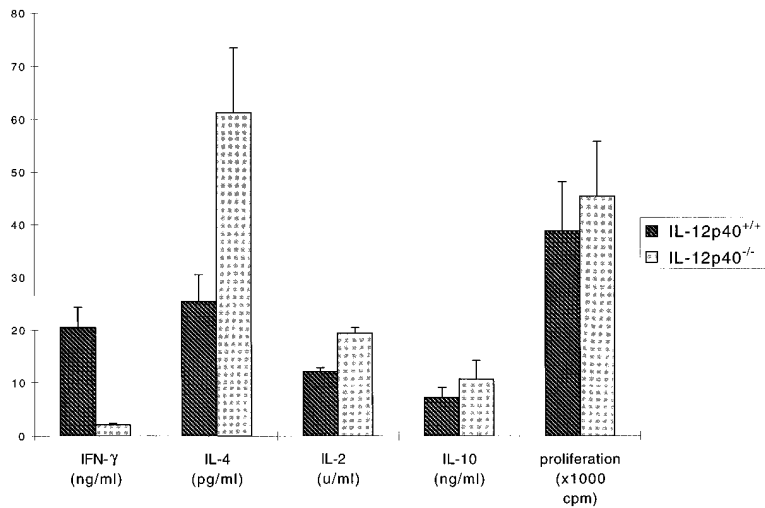
Figure 4. Comparison of Antigen-Induced Cytokine Production and Proliferation by Immune LNC from IL-12 p40$^{-/-}$ and Wild-Type Mice

IL-12 p40$^{-/-}$ mice on the C57BL/6 genetic background and wild-type C57BL/6 controls were immunized with KLH in CFA, and lymph nodes were harvested 4 days later. LNC were cultured in the presence of 100 µg/ml KLH and analyzed for their ability to proliferate and produce IFNγ, IL-4, IL-2, and IL-10. The values indicated for IFNγ, IL-4, IL-2, IL-10, and proliferation are the mean and standard error from multiple independent experiments. Although IFNγ and IL-4 were measured in each of the 10 experiments, IL-2 , IL-10, and proliferation were only measured in six, three, and four experiments, respectively. LNC cultured in the absence of KLH did not secrete measurable amounts of any of the 4 cytokines, and spontaneous [³H]thymidine incorporation in the absence of KLH was 7480 ± 520 cpm and 7960 ± 580 cpm for wild-type and IL-12 p40$^{-/-}$ mice, respectively. Experiments using IL-12 p40$^{-/-}$ mice on the BALB/c genetic background and wild-type BALB/c controls gave comparable results (data not shown).

response to KLH to normal levels (Figure 7A). Thus, although the residual IL-12-independent type 1 cytokine response in IL-12 p40$^{-/-}$ mice was largely IFNγ dependent, it could not be reconstituted to normal levels by administration of large amounts of rIFNγ, emphasizing the essential role of IL-12 in facilitating an optimal type 1 cytokine response. Furthermore, administration of rIFNγ diminished and administration of rIL-12 abolished measurable IL-4 secretion (see Figure 6B; data not shown). Differential regulation of IFNγ and IL-2 production was also evident in these experiments in which wild-type mice immunized with KLH were treated with rIFNγ or rIL-12. Whereas rIFNγ and rIL-12 both caused a dose-dependent increase in the levels of KLH-induced IFNγ secretion, a dose-dependent decrease in IL-2 levels was observed (see Figure 6C; Figure 7B).

The production of type 1 cytokines has been shown to promote predominantly cell-mediated immunity (Scott, 1993; Paul and Seder, 1994). We thus examined the ability of IL-12 p40$^{-/-}$ mice to mount two types of cell-mediated immune responses: CTL responses and delayed-type hypersensitivity (DTH) reactions. CTL responses were elicited by immunizing mice with allogeneic splenocytes in the footpad and measuring the lytic activity of cells harvested from draining lymph nodes 4 days later. For immunization of IL-12 p40$^{-/-}$ mice, allogeneic IL-12 p40$^{-/-}$ splenocytes were used to prevent possible IL-12 production by the cells used as immunogen. In three separate experiments, IL-12 p40$^{-/-}$ mice were found to make normal CTL responses. The ratio of the lytic activity generated in IL-12 p40$^{-/-}$ mice to that observed for wild-type mice was 1.4 ± 0.1 (mean ± 1 standard error of three experiments; data not shown). As was previously reported in experiments using wild-type mice (Gately et al., 1994), the lytic activity of LNC from alloimmune wild-type and IL-12 p40$^{-/-}$ mice was mediated by antigen-specific CD8^{+} CTL (data not shown). In contrast with their ability to make normal

CTL responses, IL-12 p40$^{-/-}$ mice were deficient in the generation of DTH reactions. Wild-type and IL-12 p40$^{-/-}$ mice were immunized with methylated bovine serum albumin (MBSA), and DTH reactions were elicited by injection of MBSA into the footpads 7 days later. In four separate experiments, the specific footpad swelling reaction at 48 hr after challenge was inhibited by 47% ± 3% in IL-12 p40$^{-/-}$ as compared with wild-type mice. Similar inhibition was observed when footpad swelling was measured at 24 or 72 hr (Figure 8). Histologic examination of the DTH reaction sites in wild-type and IL-12 p40$^{-/-}$ mice revealed a qualitatively similar histological profile characterized by edema and a cellular infiltrate composed predominantly of neutrophils, but in the IL-12 p40$^{-/-}$ mice a reduction in the severity of edema, cellular infiltrate, and myonecrosis was observed (data not shown).

Discussion

This paper describes the generation and characterization of IL-12-deficient mice. These mice were viable, fertile, of normal size and weight, and had no gross abnormalities, indicating that IL-12 is not required for normal mouse development. This result is consistent with the hypothesis that IL-12 acts specifically on cells of the immune system. Previous in vitro studies had suggested a possible role for IL-12 in thymopoiesis (Godfrey et al., 1994). However, flow cytometry experiments measuring macrophage, T, and B cell markers revealed no significant differences between IL-12 p40$^{-/-}$ and wild-type control mice. These results suggest that IL-12 is not required for normal ontogeny of the immune system. Prior in vitro studies also demonstrated the ability of IL-12 to synergize with kit ligand, IL-3, and other hematopoietic growth factors to support the proliferation and differentiation of hematopoietic progenitor cells (Jacobsen et al., 1993; Bellone and Trinchieri, 1994).
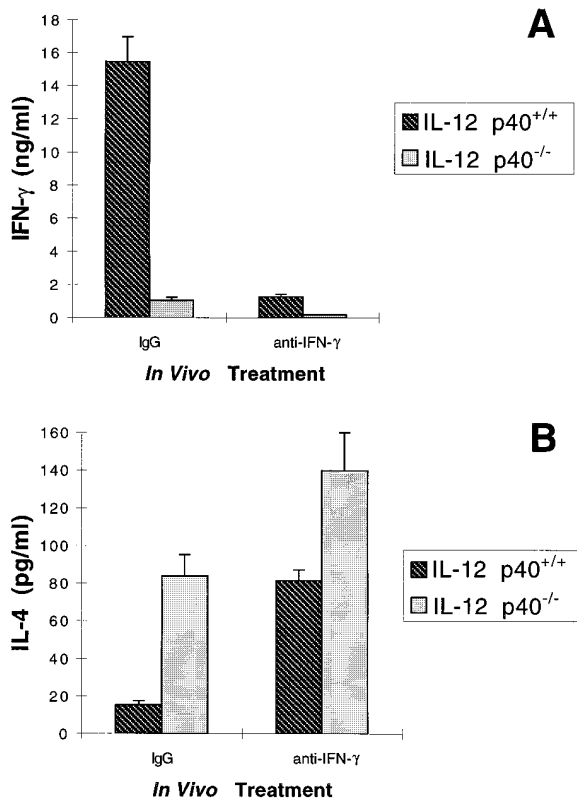
A

IFN-γ (ng/ml)

■ IL-12 p40⁺/⁺
□ IL-12 p40⁻/⁻

IgG   anti-IFN-γ
In Vivo Treatment

B

IL-4 (pg/ml)

■ IL-12 p40⁺/⁺
□ IL-12 p40⁻/⁻

IgG   anti-IFN-γ
In Vivo Treatment

Figure 5. Effects of In Vivo Administration of Neutralizing Anti-IFNγ MAb on Cytokine Production by Antigen-Stimulated Immune LNC In Vitro

IL-12 p40⁻/⁻ mice on the C57BL/6 genetic background and wild-type C57BL/6 controls (5 mice per treatment group) were injected with 1 mg of a neutralizing anti-IFNγ MAb or an IgG isotype control antibody intraperitoneally 1 day prior to immunization. Lymph nodes were harvested 4 days after immunization, and LNC were cultured in the presence of 100 μg/ml KLH followed by analysis of (A) IFNγ and (B) IL-4 levels. The mean and standard error are shown. The results seen in this experiment were representative of three separate experiments.

Nevertheless, no hematologic abnormalities were observed in IL-12 p40 ⁻/⁻ mice, suggesting that IL-12 is not required for normal hematopoiesis.

In an assay of NK cell lytic activity, splenocytes from IL-12 p40⁻/⁻ mice displayed approximately 66% the activity of splenocytes from wild-type mice. This difference was statistically significant, but it seems unlikely to be of biological significance. Furthermore, when NK cell activity was measured following in vitro incubation of splenocytes with IL-2, no difference was observed between splenocytes from IL-12 p40⁻/⁻ and wild-type mice. It is of interest to note that similar results were seen in IFNγ-deficient mice (Dalton et al., 1993).

The primary defects observed in IL-12 p40⁻/⁻ mice relate to the production of IFNγ. Following LPS administration, serum IFNγ levels in IL-12 p40⁻/⁻ mice were, on the average, 17% of the levels seen in wild-type mice. In this model, IFNγ was produced predominantly by NK cells. Similarly, Th1 responses as measured by antigen (KLH)-induced secretion of IFNγ by immune LNC from IL-12 p40⁻/⁻ mice were <15% of the responses observed

A

IFN-γ (ng/ml)

■ IL-12 p40⁺/⁺
□ IL-12 p40⁻/⁻

0   2   20
rIFN-γ, μg/dose

B

IL-4 (pg/ml)

■ IL-12 p40⁺/⁺
□ IL-12 p40⁻/⁻

0   2   20
rIFN-γ, μg/dose

C

IL-2 (u/ml)
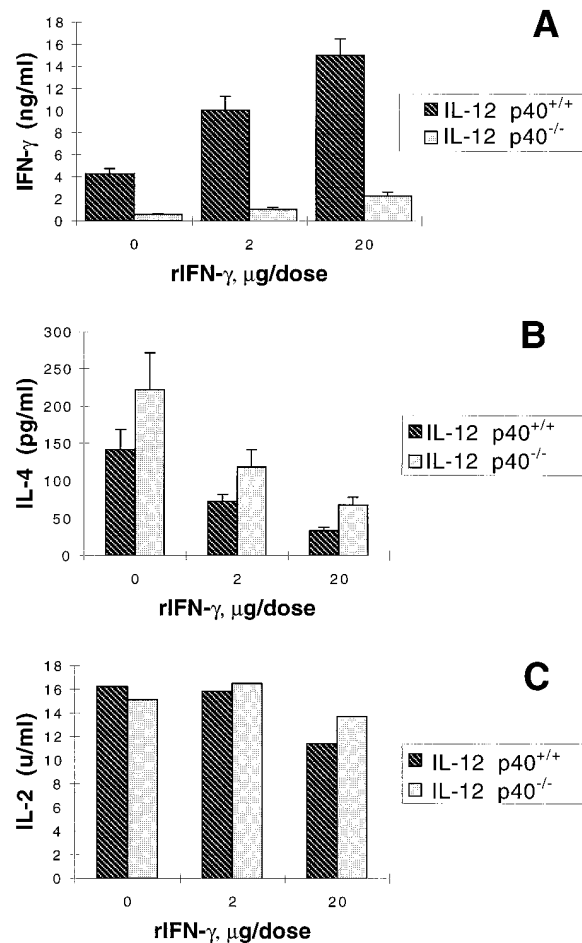
■ IL-12 p40⁺/⁺
□ IL-12 p40⁻/⁻

0   2   20
rIFN-γ, μg/dose

Figure 6. Effects of In Vivo Administration of rIFNγ on Cytokine Production by Antigen-Stimulated Immune LNC In Vitro

IL-12 p40⁻/⁻ mice on the BALB/c genetic background and wild-type BALB/c controls (5 mice per treatment group) were given 2 or 20 μg of rIFNγ or a comparable volume of vehicle intraperitoneally daily for 4 days starting on the day of immunization with KLH. Lymph nodes were harvested 4 days after immunization, and LNC were cultured in the presence of 100 μg/ml KLH followed by analysis of (A) IFNγ, (B) IL-4, and (C) IL-2 levels. The mean and standard error for IFNγ and IL-4 are shown; IL-2 data were obtained from pooled supernatants. Similar results were obtained in a second experiment using IL-12 p40⁻/⁻ mice on the C57BL/6 genetic background and wild-type C57BL/6 controls (data not shown).

in wild-type mice. In this model, IFNγ appeared to be secreted primarily by CD4⁺ T cells, and the induction of IFNγ secretion was specific, since little or no IFNγ was produced when LNC from mice immunized with CFA alone were cultured with KLH (M. K. G. and S. E. C., unpublished data). Additionally, splenocytes from IL-12 p40⁻/⁻ mice were found to be deficient in their ability to produce IFNγ in vitro in response to LPS or heat-killed Listeria monocytogenes (R. R. W. and M. K. G., unpublished data).

In the model of KLH-induced Th1 responses, further studies were undertaken to evaluate the factors contributing to IFNγ production in the absence of IL-12. Depletion of IFNγ by administration of neutralizing anti-IFNγ MAb in vivo ablated antigen-induced IFNγ production
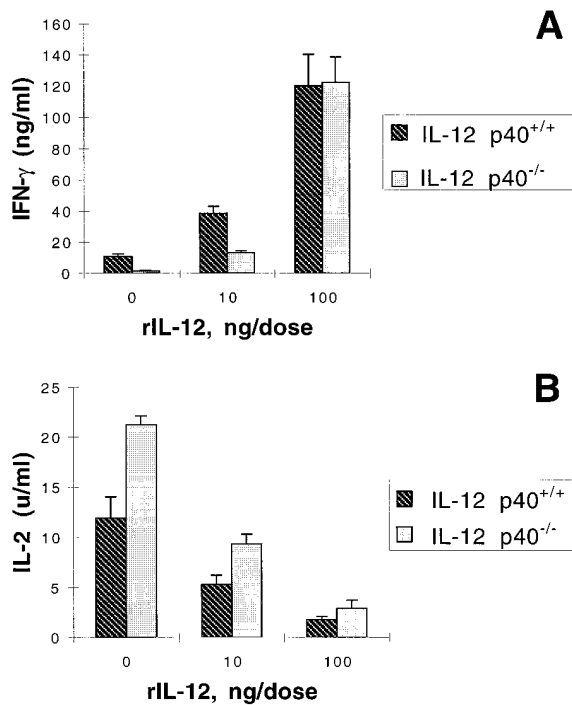
Figure 7. Effects of In Vivo Administration of rIL-12 on Cytokine Production by Antigen-Stimulated Immune LNC In Vitro

IL-12 p40$^{-/-}$ mice on the C57BL/6 genetic background and wild-type C57BL/6 controls (4 mice per treatment group) were injected with 10 or 100 ng of rIL-12 or a comparable volume of vehicle intraperitoneally daily for 4 days starting on the day of immunization with KLH. Lymph nodes were harvested 4 days after immunization, and LNC were cultured in the presence of 100 μg/ml KLH followed by analysis of (A) IFNγ and (B) IL-2 levels. The mean and standard error are shown. The results observed in this experiment were representative of three separate experiments.

by immune IL-12 p40$^{-/-}$ LNC and substantially reduced IFNγ production by wild-type LNC. In contrast, large doses of rIFNγ (20 μg/day) were not able to reconstitute IFNγ production by IL-12 p40$^{-/-}$ LNC to wild-type levels. These results are consistent with recent observations suggesting that the primary role of IFNγ in promoting Th1 responses in vivo is to serve as a costimulus required for optimal IL-12 production by antigen-presenting cells (Dighe et al., 1995). Administration of as little as 10 ng/day rIL-12 was able to restore the Th1 response to KLH in IL-12 p40$^{-/-}$ mice to wild-type levels. In the absence of IL-12, a number of potential inducers could contribute to the mechanism by which low levels of IFNγ are produced, including a recently discovered cytokine called IFNγ-inducing factor (Okamura et al., 1995) and IFNα (Manetti et al., 1995). Varying production of such factors in response to different immunization regimens may account for differences in the level of IL-12-independent IFNγ production observed. For example, for mice immunized with KLH together with C. parvum as adjuvant, IFNγ production by IL-12 p40$^{-/-}$ LNC was inhibited by 98% compared with wild-type controls; whereas for mice immunized with KLH in CFA, IFNγ production was inhibited by only 88%.

It was previously reported that IL-12 can both promote

the differentiation of naive Th cells into Th1 cells (Hsieh et al., 1993) and serve as a costimulus required for maximum antigen-induced IFNγ secretion by already differentiated Th1 cells (Murphy et al., 1994). Thus, in our model of Th1 responses to KLH, IL-12 may have been required for the differentiation of Th1 cells in vivo following immunization, for antigen-induced IFNγ secretion in vitro, or for both. Treatment of wild-type mice with neutralizing anti-IL-12 antibodies in vivo during immunization or addition of anti-IL-12 to LNC cultures both led to reduced secretion of IFNγ. Maximum inhibition, resulting in levels of IFNγ secretion similar to those seen in IL-12 p40$^{-/-}$ mice, required antibody treatment both in vivo and in vitro (M. K. G. and H. Yang, unpublished data). These results indicate a requirement for IL-12 in our model both during the differentiation of Th1 cells in vivo and during antigen-induced IFNγ production by Th1 cells in vitro.

In contrast with the defect in IFNγ production, KLH-immune LNC from IL-12 p40$^{-/-}$ mice displayed a moderately enhanced ability to produce IL-4. LNC from IL-12 p40$^{-/-}$ mice immunized with KLH in CFA, or with KLH plus C. parvum, displayed levels of KLH-induced IL-4 secretion 3- or 5-fold greater, respectively, than wild-type controls. Antigen-induced proliferation of KLH-immune IL-12 p40$^{-/-}$ LNC was normal. This result was confirmed in studies using purified KLH-immune CD4^{+} T cells cultured with KLH and irradiated, syngeneic, non-immune splenocytes as a source of antigen-presenting cells (M. K. G., S. E. C., and R. R. W., unpublished data). Previous experiments using mouse Th1 clones in vitro demonstrated an inhibitory effect of neutralizing anti-IL-12 antibodies on antigen-induced proliferation, as well as IFNγ secretion (Murphy et al., 1994; Kennedy et al., 1994). This apparent discrepancy with our results may be due to differences in the state of differentiation between Th1 clones and the Th cells studied in our experiments. Specifically, Th1 clones may represent more differentiated cells, which are more dependent upon the presence of IL-12 for maximum proliferation than Th1 cells examined 4 days after primary immunization.

Using IFNγ secretion as a measure of Th1 responses, IL-12 p40$^{-/-}$ mice appear to have a severe defect in their ability to generate such responses. However, IL-2 is also considered to be a type 1 cytokine (Scott, 1993; Paul and Seder, 1994), and yet its secretion is not diminished by IL-12 deficiency. Similarly, using an in vitro model, it was reported that IL-12 was required to prime cells for efficient IFNγ secretion but did not affect the secretion of IL-2 (Hsieh et al., 1993). Our observations that treatment of wild-type mice with rIFNγ or rIL-12 both led to dose-dependent increases in IFNγ secretion but no increase in IL-2 production provide further evidence that IL-2 and IFNγ secretion are regulated independently. These results are consistent with recent data from other laboratories, suggesting that production of individual cytokines within the group considered to be products of Th1 cells or within the Th2-derived group are independently regulated (Kelso, 1995; Bucy et al., 1995). This further demonstrates a need for analyses based on the production of different cytokines, rather than using a single cytokine as an indicator for a particular Th subset.
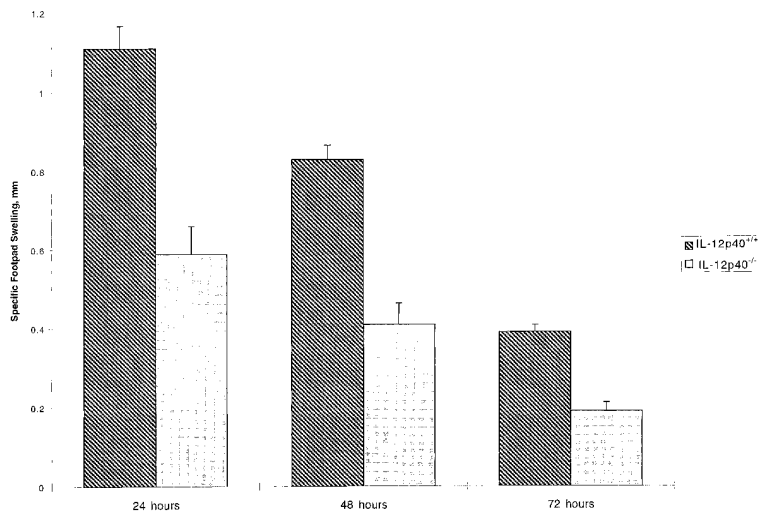
Figure 8. Measurement of a DTH Response in IL-12 p40$^{-/-}$ and Wild-Type Mice

We immunized 5 IL-12 p40$^{-/-}$ mice on the C57BL/6 genetic background and 5 wild-type C57BL/6 controls with MBSA. The mice were challenged by injection of MBSA or saline into the rear footpads 7 days later, and footpad swelling was measured at 24, 48, and 72 hr after challenge. Data are given as specific swelling calculated by subtracting the amount of swelling of control saline-injected footpads from the amount of swelling of MBSA-injected footpads. The mean and standard error are shown. Three additional experiments yielded similar results.

In experiments in which wild-type or IL-12 p40$^{-/-}$ mice were immunized with KLH and then treated with rIL-12, a dose-dependent reduction in the levels of IL-2 was observed in cultures of IL-12 treated KLH-stimulated immune LNC (Figure 7B). The reduction in IL-2 levels observed in these experiments could have been due to decreased production of IL-2, to increased consumption of IL-2 by the activated LNC, or to both. To evaluate these possibilities, additional experiments were performed in which immune LNC from mice treated with 100 ng/day rIL-12 or with vehicle were incubated with KLH in the presence of saturating amounts of a neutralizing rat anti-mouse IL-2Rα MAb or control rat immunoglobulin G (IgG). In three experiments using wild-type mice and one using IL-12 p40$^{-/-}$ mice, the levels of IL-2 observed in cultures of LNC from rIL-12-treated mice were 8% $\pm$ 2% of the levels seen using LNC from vehicle-treated mice if the cultures contained control IgG, but 47% $\pm$ 8% if anti-IL-2Rα was included in the cultures (S. E. C., D. M. C., and M. K. G., unpublished data). These results suggest that the reduction in IL-2 levels observed in cultures containing LNC from rIL-12-treated mice was due, at least in part, to increased consumption of IL-2 by the activated LNC. This increased consumption of IL-2 might reflect rIL-12-induced up-regulation of IL-2R expression, as was previously observed in vitro (Yanagida et al., 1994).

Th1 cells promote cell-mediated immunity (Scott, 1993; Paul and Seder, 1994). Therefore, we evaluated cell-mediated immune responses in IL-12 p40$^{-/-}$ mice compared with wild-type controls. Although treatment of mice with exogenous rIL-12 was previously shown to enhance CTL responses (Gately et al., 1994), IL-12 p40$^{-/-}$ mice were capable of making normal CTL responses, indicating that endogenous IL-12 is not required for this response. This apparent discrepancy may be explained by the observation that doses of rIL-12 required to enhance CTL responses were $\geq$ 100 ng/ day, at least 10-fold greater than the dose of rIL-12 that reconstituted a normal Th1 response in IL-12 p40$^{-/-}$ mice. Hence, enhancement of CTL responses appears to be a pharmacologic effect that can be achieved when administering high doses of rIL-12 to mice but is not a physiologic effect of endogenous IL-12. The observation that IL-12 p40$^{-/-}$ mice make normal allogeneic CTL responses also contrasts with a prior report that anti-IL-12 antibody partially inhibited human allogeneic CTL responses in vitro (Chouaib et al., 1994). This difference could reflect either a species difference or a difference in the requirements for CTL responses in vivo as compared with in vitro. We have also observed partial inhibition of allogeneic human CTL responses by neutralizing anti-IL-12 antibodies in vitro (M. K. G. and A. Wolitzky, unpublished data), but we have not been able to inhibit allogeneic murine CTL responses in vitro using either anti-IL-12 antibodies or mouse p40 homodimer (M. K. G. and S. E. C., unpublished data). In contrast with their ability to make normal CTL responses, IL-12 p40$^{-/-}$ mice were deficient in the ability to generate DTH responses, as compared with wild-type mice. This result is consistent with a report that treatment of mice with neutralizing anti-IL-12 antiserum suppressed the induction of contact sensitivity in mice (Müller et al., 1995). Taken together, these results indicate that endogenous IL-12 plays a role in promoting some, but not all, types of cell-mediated immune responses.

Further studies using IL-12 p40$^{-/-}$ mice to evaluate the role of endogenous IL-12 in resistance to various infectious diseases and in the pathogenesis of autoimmune diseases are in progress. These studies have important clinical implications for the proposed use of rIL-12 or IL-12 antagonists in the treatment of such disorders.

Experimental Procedures

Reagents

Complete culture medium (CCM) used in these experiments consisted of RPMI 1640 (Whittaker Bioproducts) supplemented with 100 U/ml of penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.05

mM 2-mercaptoethanol (all from GIBCO BRL), and the indicated percentage of heat-inactivated fetal bovine serum (FBS) (Hyclone). Purified mouse rIL-12 was prepared as previously described (Gately et al., 1994). Goat anti-mouse IL-12 IgG (Tripp et al., 1994) was from Dr. J. Hakimi, Hoffmann-La Roche, Incorporated. Purified recombinant human IL-2 was supplied by Dr. F. Khan, Hoffmann-La Roche, Incorporated. MAbs AN-18 and XMG-1.2 to mouse IFNγ and purified recombinant mouse IFNγ were provided by Dr. G. Alber, F. Hoffmann-La Roche, Limited (Basel, Switzerland). Mouse IL-4 and IL-10 were from Pepro Tech, Incorporated, and Pharmingen, respectively. Rabbit anti-ASGM-1 antibody was from Wako Industries, and normal rabbit IgG, normal goat IgG, and Salmonella enteritidis LPS were from Sigma Chemical Company.

Construction of the Targeting Vector

Mouse IL-12 p40 genomic clones were isolated from a λ library derived from 129/Sv DNA (Stratagene). The inserts were digested with NotI and EcoRI and the resulting pieces subcloned into pBluescript KS(+) (Stratagene). Restriction mapping and further analysis were completed using standard techniques (Sambrook et al., 1989). The targeting vector was generated using four pieces of DNA: upstream and downstream flanking DNA, the PGK-1 *neo* gene (Soriano et al., 1991), and the pMC1–*tk* gene (Labow et al., 1994). An 8 kb EcoRI–EcoRI fragment containing exons 2 and 3 from the IL-12 p40 gene was further subcloned into pSP72 (Promega). This plasmid was then digested with BstXI, and the protruding ends were made blunt using T4 DNA Polymerase (Boehringer Mannheim) and dephosphorylated using calf alkaline phosphatase (Boehringer Mannheim). This results in an approximately 1.6 kb deletion into which an XhoI–XhoI fragment containing the PGK-1 *neo* gene was ligated following removal of the protruding ends using T4 DNA polymerase (Boehringer Mannheim) generating clone 8. In parallel, an XhoI–SalI fragment containing the pMC1–*tk* gene was subcloned into pBluescript KS(+). The resultant plasmid was digested with HindIII, and a HindIII–HindIII fragment containing exon 1 of the IL-12 p40 gene was cloned into this site in order to generate clone 10. The clone 8 insert containing the PGK-1 *neo* gene and portions of the IL-12 p40 gene was dissected from the vector using digestion with BglII and cloned into the BamHI site of clone 10, generating the targeting vector. This construct was linearized using NotI prior to introduction into ES cells.

Identification of Targeted ES Cell Clone

The W9.5 ES cell line was maintained on irradiated primary embryonic feeder cells as previously described (Abbondanzo et al., 1993) in media containing leukemia inhibitory factor and 15% fetal calf serum (GIBCO BRL). A confluent plate of ES cells was harvested and electroporated with 25 μg of linearized targeting vector using a Gene Pulser (Bio Rad). The cells were then selected in G418 (350 μg/ml, GIBCO BRL) and 1-(2-deoxy, 2-fluoro-β-d-arabinofuranosil-)-5-iodouracil (FIAU; 0.2 μM, Oclassen Pharmaceutical, Incorporated), and colonies picked after 10 days and expanded.

A Southern blot strategy was used to determine which of the colonies contained a correctly targeted event. Extraction of DNA from ES cells was performed as previously described (Laird et al., 1991). DNA was digested with BamHI and BglII, fractionated by agarose gel electrophoresis, transferred to nylon membranes by a Hoeffer electroblot apparatus, and cross-linked using ultraviolet light or a Stratalinker (Stratagene). Membranes were hybridized for 1 hr in Rapid-Hyb buffer (Amersham) at 65°C using a random primed probe. The probe was derived from a downstream region of the IL-12 p40 gene outside the targeted region and was labeled using a Prime-It kit as per the instructions of the manufacturer (Stratagene).

Generation of Chimeric Animals

Correctly targeted ES cell clones were karyotyped as described (Abbondanzo et al., 1993) and two clones, J6 and C3, which were shown to have a normal 40XY karyotype, were used to generate chimeric mice. Exponentially growing ES cell clones were injected into host C57BL/6J blastocysts, and the embryos were then transplanted into the uterine horns of pseudopregnant C57BL/6J × CBA/J F1 females. Chimeric males, as judged by agouti coat color, were mated to C57BL/6J, BALB/cByJ, and 129/Sv females (the strain from which W9.5 was derived). 129/Sv progeny carrying the mutant allele were intercrossed in order to generate mice homozygous for the mutation. C57BL/6J and BALB/cByJ progeny were backcrossed to the appropriate parental strain for a total of five backcrosses, and then intercrossed in order to generate mice homozygous for the mutation on specific genetic backgrounds.

In Vitro Generation and Assay of IL-12

To stimulate IL-12 production, thioglycollate-elicited mouse peritoneal exudate cells were cultured with 10 μg/ml LPS plus 200 U/ml mouse rIFNγ as previously described (Wilkinson et al., 1996). Bioactive IL-12 in the culture supernates was measured in a human phytohemagglutinin-activated lymphoblast proliferation assay as described (Gately et al., 1995). The specificity of the proliferative response was determined by comparing proliferation in cultures containing 10 μg/ml goat anti-mouse IL-12 IgG to proliferation in cultures containing 10 μg/ml normal goat IgG. The amount of IL-12 p40 in the supernates was measured by an ELISA as described previously (Gillessen et al., 1995), except that immunoplates were coated with 4 μg/ml affinity-purified goat anti-mouse IL-12 IgG.

Lymphoid Cell Phenotype Analysis

Flow cytometric analysis of lymphoid cell phenotypes was performed as previously described (Gately et al., 1994). MAbs used for staining thymocytes were anti-TCR (αβ)–fluorescein isothiocyanate (FITC), anti-CD8–phycoerythrin (PE), and anti-CD4–biotin (all from PharMingen) followed by streptavidin–tricolor (Caltag Laboratories). Lymph node cells and splenocytes were stained using anti-CD3–FITC, anti-CD4–PE, anti-CD8–FITC, anti-B220–PE (all from PharMingen), and anti-F4/80–FITC (Harlan Bioproducts). Hamster IgG–FITC, rat IgG–PE, rat IgG–FITC, and rat IgG–biotin (PharMingen) were used as isotype controls.

Measurement of Resting and IL-2-Activated NK Cell Activity

NK cell lytic activity of fresh mouse splenocytes was determined using YAC-1 target cells as previously described (Gately et al., 1994). For measurement of IL-2-activated NK cell activity, splenocytes were cultured at 4×10^6 cells per 1 ml culture in the presence of 500, 50, or 5 U/ml human rIL-2 for 4 days at 37°C. Control cultures contained splenocytes without IL-2. The cells were then harvested and tested for NK lytic activity as described above. Lytic assays were performed in quadruplicate, and the spontaneous ^{51}Cr release ranged from 14%–18%.

Treatment of Cells with Antibody plus Complement

Pooled lymph node cells were washed and resuspended at 50×10^6 per ml in CCM with 10% FBS. The cells were then incubated for 45 min on ice with the following antibodies either alone or in various combinations: 10 μg/ml anti-CD4 (rat IgM MAb from Cambio), 50 μg/ml anti-CD8 (rat IgM MAb clone AD4 from Accurate Chemical and Scientific), or 25 μg/ml anti-ASGM-1 (Wako). After incubation for 45 min on ice, cells were washed and incubated with a 1:5 dilution of rabbit complement (Low Tox-M; Accurate Chemical and Scientific) for 45 min at 37°C. The extent of cell subset depletion was monitored by flow cytometry.

LPS-Induced IFNγ Production In Vivo

Mice were injected with 200 μg of LPS intraperitoneally. The mice were bled 6 hr later, and serum IFNγ levels were measured by ELISA. In some experiments, mice were depleted of NK cells by administration of 0.8 mg of rabbit anti-ASGM-1 intraperitoneally on the day prior to injection of LPS. Control mice received normal rabbit IgG (Sigma) 1 day prior to LPS.

ELISAs for IFNγ, IL-4, IL-10, and IL-2

ELISAs for measuring mouse IFNγ in sera and culture supernates were performed as previously described (Gately et al., 1994), except that the assays were developed using TMB Peroxidase (Kirkegaard and Perry), and absorbance was measured at 450 nm. The sensitivity of the assay was ~0.15 ng/ml. ELISAs for measuring mouse IL-4 and IL-10 were performed in a similar manner. For measurement of IL-4, plates were coated with 2 μg/ml of rat anti-mouse IL-4 (PharMingen), and captured IL-4 was detected using biotinylated

anti-mouse IL-4 (PharMingen) at 100 ng/ml. The sensitivity of this assay was ~15 pg/ml. To measure IL-10, plates were coated with 1 μg/ml of rat anti-mouse IL-10 (PharMingen), and captured IL-10 was detected using biotinylated anti-mouse IL-10 (PharMingen) at 500 ng/ml. The sensitivity of the IL-10 ELISA was ~0.4 ng/ml. Mouse IL-2 ELISAs were performed with a kit from Collaborative Biomedical Products and had a sensitivity of ~0.8 U/ml.

Induction of Th1 Responses In Vivo
For induction of Th1 responses in vivo, mice were immunized with KLH (Calbiochem), followed by culture of the immune LNC with KLH to elicit cytokine production in vitro. Mice were immunized subcutaneously at the base of the tail with 100 μg of KLH emulsified in CFA (GIBCO BRL) or with 100 μg alum-precipitated KLH together with 100 μg heat-killed C. parvum (Wellcome Reagents, Limited). On day 4, the subinguinal, axillary, and para-aortic lymph nodes were removed aseptically, passed through a wire mesh, washed, and cultured in CCM supplemented with 10% FBS and 0, 10, or 100 μg/ml of KLH. For measurement of cytokine production, LNC were incubated in 1 ml cultures in Costar 24-well plates at 6×10^6 cells/well. Culture supernates were harvested by centrifugation after 24 hr (for IL-2 ELISA) or 48 hr (for IFNγ, IL-4, and IL-10 ELISAs) and stored at 4°C until assayed. In all experiments, cytokine production by LNC cultured without KLH was assayed; however, since cytokine levels were routinely below the level of detection in such cultures, these results are not shown. For measurement of proliferative responses, LNC were plated in 96-well flat-bottomed microplates (Costar) at 6×10^6 cells/ml in 200 μl total volume. After incubation for 3 days at 37°C, cultures were pulsed with 0.5 μCi/well of [³H]thymidine for 5–6 hr, and incorporation of radiolabel into DNA was measured by liquid scintillation counting.

In Vivo Generation of CTL
CTL responses were induced by footpad injection of 10^7 allogeneic splenocytes, and CTL activity in the draining popliteal lymph nodes was measured 4 days later, as previously described (Gately et al., 1994). LNC populations were assayed for lytic activity at serial 2-fold dilutions, starting at an effector:target ratio of 160:1. Lytic assays were performed in triplicate, and the spontaneous ^{51}Cr release varied from 9%–16%.

DTH Reactions
Mice were sensitized to MBSA (Sigma) by intradermal injection of 50 μl of 5 mg/ml MBSA in CFA at two sites on the abdomen. On day 8 following immunization, the mice were challenged by injection of 20 μl of 5 mg/ml MBSA into one rear footpad, while the other rear footpad received a comparable volume of phosphate-buffered saline. Measurements of footpad swelling were taken at 24, 48, and, in some experiments, 72 hr after challenge by use of a micrometer (Mitutoyo Corporation). Measurements were made by an observer who was unaware of the identity of the mice. The magnitude of the DTH responses was determined from differences in footpad thickness between the antigen-and diluent-injected footpads. For examination of histology of the DTH reaction sites, feet were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin.

References

Abbondanzo, S.J., Gadi, I., and Stewart, C.L. (1993). Derivation of embryonic stem cell lines. Meth. Enzymol. 225, 803–823.

Bellone, G., and Trinchieri, G. (1994). Dual stimulatory and inhibitory effect of NK cell stimulatory factor/IL-12 on human hematopoiesis. J. Immunol. 153, 930–937.

Bucy, R.P., Karr, L., Huang, G.-Q., Li, J., Carter, D., Honjo, K., Lemons, J.A., Murphy, K.M., and Weaver, C.T. (1995). Single cell analysis of cytokine gene coexpression during CD4+ T-cell phenotype development. Proc. Natl. Acad. Sci. USA 92, 7565–7569.

Chehimi, J., Paganin, C., Frank, I., and Trinchieri, G. (1996). Interleukin-12 in the pathogenesis and therapy of HIV disease. Res. Immunol., in press.

Chizzonite, R., Gubler, U., Magram, J., and Stern, A. (1996). Interleukin 12. In Cytokines, A. Mire-Sluis and R. Thorpe, eds. (London: Academic Press), in press.

Chouaib, S., Chehimi, J., Bani, L., Genetet, N., Tursz, T., Gay, F., Trinchieri, G., and Mami-Chouaib, F. (1994). Interleukin 12 induces the differentiation of major histocompatibility complex class I–primed cytotoxic T-lymphocyte precursors into allospecific cytotoxic effectors. Proc. Natl. Acad. Sci. USA 91, 12659–12663.

Dalton, D.K., Pitts-Meek, S., Keshav, S., Figari, I.S., Bradley, A., and Stewart, T.A. (1993). Multiple defects of immune cell function in mice with disrupted interferon-γ genes. Science 259, 1739–1742.

D'Andrea, A., Rengaraju, M., Valiante, N.M., Chehimi, J., Kubin, M., Aste, M., Chan, S.H., Kobayashi, M., Young, D., Nickbarg, E., Chizzonite, R., Wolf, S.F., and Trinchieri, G. (1992). Production of natural killer cell stimulatory factor (interleukin-12) by peripheral blood mononuclear cells. J. Exp. Med. 176, 1387–1398.

Dighe, A.S., Campbell, D., Hsieh, C.-S., Clarke, S., Greaves, D.R., Gordon, S., Murphy, K.M., and Schreiber, R.D. (1995). Tissue-specific targeting of cytokine unresponsiveness in transgenic mice. Immunity 3, 657–666.

Gately, M.K., Anderson, T.D., and Hayes, T.J. (1988). Role of asialo-GM1-positive lymphoid cells in mediating the toxic effects of recombinant IL-2 in mice. J. Immunol. 141, 189–200.

Gately, M.K., Warrier, R.R., Honasoge, S., Carvajal, D.M., Faherty, D.A., Connaughton, S.E., Anderson, T.D., Sarmiento, U., Hubbard, B.R., and Murphy, M. (1994). Administration of recombinant IL-12 to normal mice enhances cytolytic lymphocyte activity and induces production of IFNγ in vivo. Int. Immunol. 6, 157–167.

Gately, M.K., Chizzonite, R., and Presky, D.H. (1995). Measurement of human and mouse interleukin 12. In Current Protocols in Immunology, Volume 1, J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, eds. (New York: John Wiley and Sons, Incorporated), pp. 6.16.1–6.16.15.

Gillessen, S., Carvajal, D., Ling, P., Podlaski, F.J., Stremlo, D.L., Familletti, P.C., Gubler, U., Presky, D.H., Stern, A.S., and Gately, M.K. (1995). Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. Eur. J. Immunol. 25, 200–206.

Godfrey, D.I., Kennedy, J., Gately, M.K., Hakimi, J., Hubbard, B.R., and Zlotnik, A. (1994). IL-12 influences intrathymic T cell development. J. Immunol. 152, 2729–2735.

Gubler, U., Chua, A.O., Schoenhaut, D.S., Dwyer, C.M., McComas, W., Motyka, R., Nabavi, N., Wolitzky, A.G., Quinn, P.M., Familletti, P.C., and Gately, M.K. (1991). Coexpression of two distinct genes is required to generate secreted, bioactive cytotoxic lymphocyte maturation factor. Proc. Natl. Acad. Sci. USA 88, 4143–4147.

Heinzel, F.P., Rerko, D.M., Ling, P., Hakimi, J., and Schoenhaut, D.S. (1994). Interleukin 12 is produced in vivo during endotoxemia and stimulates synthesis of gamma interferon. Infect. Immun. 62, 4244–4249.

Hendrzak, J.A., and Brunda, M.J. (1995). Interleukin-12: biologic activity, therapeutic utility, and role in disease. Lab. Invest. 72, 619–637.

Hsieh, C.-S., Macatonia, S.E., Tripp, C.S., Wolf, S.F., O'Garra, A., and Murphy, K.M. (1993). Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science 260, 547–549.

Jacobsen, S.E., Veiby, O.P., and Smeland, E.B. (1993). Cytotoxic lymphocyte maturation factor (interleukin 12) is a synergistic growth factor for hematopoietic stem cells. J. Exp. Med. 178, 413–418.

Kelso, A. (1995). Th1 and Th2 subsets: paradigms lost? Immunol. Today *16*, 374–379.

Kennedy, M.K., Picha, K.S., Shanebeck, K.D., Anderson, D.M., and Grabstein, K.H. (1994). Interleukin-12 regulates the proliferation of Th1, but not Th2 or Th0, clones. Eur. J. Immunol. *24*, 2271–2278.

Kobayashi, M., Fitz, L., Ryan, M., Hewick, R.M., Clark, S.C., Chan, S., Loudon, R., Sherman, F., Perussia, B., and Trinchieri, G. (1989). Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biological effects on human lymphocytes. J. Exp. Med. *170*, 827–845.

Labow, M.A., Norton, C.R., Rumberger, J.M., Lombard-Gillooly, K.M., Shuster, D.J., Hubbard, J., Bertko, R., Knaack, P.A., Terry, R.W., Harbison, M.L., Kontgen, F., Stewart, C.L., McIntyre, K.W., Will, P.C., Burns, D.K., and Wolitzky, B.A. (1994). Characterization of E-selectin-deficient mice: demonstration of overlapping function of the endothelial selectins. Immunity *1*, 709–720.

Laird, P.W., Zijderveld, A., Linders, K., Rudnicki, M.A., Jaenish, R., and Berns, A. (1991). Simplified mammalian DNA isolation procedure. Nucl. Acids Res. *19*, 4293.

Macatonia, S.E., Hosken, N.A., Litton, M., Vieira, P., Hsieh, C.-S., Culpepper, J.A., Wysocka, M., Trinchieri, G., Murphy, K.M., and O'Garra, A. (1995). Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. J. Immunol. *154*, 5071–5079.

Manetti, R., Parronchi, P., Giudizi, M.G., Piccinni, M.-P., Maggi, E., Trinchieri, G., and, Romagnani, S. (1993). Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. J. Exp. Med. *177*, 1199–1204.

Manetti, R., Annunziato, F., Tomasevic, L., Giannò, V., Parronchi, P., Romagnani, S., and Maggi, E. (1995). Polyinosinic acid: polycytidylic acid promotes T helper type 1-specific immune responses by stimulating macrophage production of interferon-α and interleukin-12. Eur. J. Immunol. *25*, 2656–2660.

Morris, S.C., Madden, K.B., Adamovicz, J.J., Gause, W.C., Hubbard, B.R., Gately, M.K., and Finkelman, F.D. (1994). Effects of IL-12 on in vivo cytokine gene expression and Ig isotype selection. J. Immunol. *152*, 1047–1056.

Mosmann, T.R., and Coffman, R.L. (1989). Heterogeneity of cytokine secretion patterns and functions of helper T cells. Adv. Immunol. *46*, 111–147.

Müller, G., Saloga, J., Germann, T., Schuler, G., Knop, J., and Enk, A.H. (1995). IL-12 as mediator and adjuvant for the induction of contact sensitivity in vivo. J. Immunol. *155*, 4661–4668.

Murphy, E.E., Terres, G., Macatonia, S.E., Hsieh, C.-S., Mattson, J., Lanier, L., Wysocka, M., Trinchieri, G., Murphy, K., and O'Garra, A. (1994). B7 and interleukin 12 cooperate for proliferation and interferon-γ production by mouse T helper clones that are unresponsive to B7 costimulation. J. Exp. Med. *180*, 223–231.

Murphy, T.L., Cleveland, M.G., Kulesza, P., Magram, J., and Murphy, K.M. (1995). Regulation of interleukin 12 p40 expression through an NF-κB half-site. Mol. Cell. Biol. *15*, 5258–5267.

Okamura, H., Tsutsui, H., Komatsu, T., Yutsudo, M., Hakura, A., Tanimoto, T., Torigoe, K., Okura, T., Nukada, Y., Hattori, K., Akita, K., Namba, M., Tanabe, F., Konishi, K., Fukuda, S., and Kurimoto, M. (1995). Cloning of a new cytokine that induces IFNγ production by T cells. Nature *378*, 88–91.

Paul, W.E., and Seder, R.A. (1994). Lymphocyte responses and cytokines. Cell *76*, 241–251.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning, A Laboratory Manual, Second Edition (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Schmitt, E., Hoehn, P., Huels, C., Goedert, S., Palm, N., Rüde, E., and Germann, T. (1994). T helper type 1 development of naive CD4+ T cells requires the coordinate action of interleukin-12 and interferon-γ and is inhibited by transforming growth factor-β. Eur. J. Immunol. *24*, 793–798.

Schoenhaut, D.S., Chua, A.O., Wolitzky, A.G., Quinn, P.M., Dwyer, C.M., McComas, W., Familletti, P.C., Gately, M.K., and Gubler, U. (1992). Cloning and expression of murine IL-12. J. Immunol. *148*, 3433–3440.

Scott, P. (1993). IL-12: initiation cytokine for cell-mediated immunity. Science *260*, 496–497.

Seder, R.A., Gazzinelli, R., Sher, A., and Paul, W.E. (1993). Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon-γ production and diminishes interleukin 4 inhibition of such priming. Proc. Natl. Acad. Sci. USA *90*, 10188–10192.

Sieburth, D., Jabs, E.W., Warrington, J.A., Li, X., Lasota, J., LaForgia, S., Kelleher, K., Huebner, K., Wasmuth, J.J., and Wolf, S.F. (1992). Assignment of NKSF/IL 12, a unique cytokine composed of two unrelated subunits, to chromosomes 3 and 5. Genomics *14*, 59–62.

Soriano, P., Montgomery, C., Geske, R., and Bradley, A. (1991). Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. Cell *64*, 693–699.

Stern, A.S., Podlaski, F.J., Hulmes, J.D., Pan, Y.-C.E., Quinn, P.M., Wolitzky, A.G., Familletti, P.C., Stremlo, D.L., Truitt, T., Chizzonite, R., and Gately, M.K. (1990). Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. Proc. Natl. Acad. Sci. USA *87*, 6808–6812.

Szabo, P., and Mann, J.R. (1994). Expression and methylation of imprinted genes during in vitro differentiation of mouse parthenogenetic and androgenetic embryonic stem cells. Development *120*, 1651–1660.

Trinchieri, G. (1994). Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. Blood *84*, 4008–4027.

Tripp, C.S., Gately, M.K., Hakimi, J., Ling, P., and Unanue, E.R. (1994). Neutralization of IL-12 decreases resistance to *Listeria* in SCID and C.B-17 mice: reversal by IFNγ. J. Immunol. *152*, 1883–1887.

Wilkinson, V., Warrier, R.R., Truitt, T.P., Nunes, P., Gately, M.K., and Presky, D.H. (1996). Characterization of anti-mouse IL-12 monoclonal antibodies and measurement of mouse IL-12 by ELISA. J. Immunol. Meth., in press.

Wolf, S.F., Temple, P.A., Kobayashi, M., Young, D., Dicig, M., Lowe, L., Dzialo, R., Fitz, L., Ferenz, C., Hewick, R.M., Kelleher, K., Herrmann, S.H., Clark, S.C., Azzoni, L., Chan, S.H., Trinchieri, G., and Perussia, B. (1991). Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. J. Immunol. *146*, 3074–3081.

Yanagida, T., Kato, T., Igarashi, O., Inoue, T., and Nariuchi, H. (1994). Second signal activity of IL-12 on the proliferation and IL-2R expression of T helper cell-1 clone. J. Immunol. *152*, 4919–4928.