IL-12 is considered a critical proinflammatory cytokine for autoimmune diseases such as multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE). IL-12 is a heterodimer composed of a p35 subunit and a common p40 subunit shared by other cytokines. Both IL-12 p40−/− and p35−/− mice fail to produce IL-12 p70 heterodimer. However, in contrast to p40−/− mice, p35−/− mice are highly susceptible to the induction of EAE, establishing that IL-12 p70 is not essential for the development of EAE. When compared with wild-type mice, both p40−/− and p35−/− mice show deficiencies in primary IFN-γ responses by lymph node cells. Expression profiling of the inflamed CNS revealed that Th2 cytokines such as IL-4 and IL-10 are upregulated in p35−/− mice, whereas LT-α and TNF-α levels are reduced. These studies show that a molecule other than IL-12 p70, which uses the p40 subunit, fulfills the functions previously attributed to IL-12 with regard to the development and pathogenesis of this autoimmune disease.


Introduction
IL-12 is an important inflammatory mediator in cell-mediated immunity (1). IL-12 is a 70-kDa heterodimer secreted protein consisting of two disulfide-linked subunits designated p40 and p35. It is produced mainly by antigen-presenting cells upon activation through Toll-like receptors and by interactions with T cells. Engagement of the IL-12 receptor complex on T cells has been shown to be critical in Th1-mediated autoimmune diseases in which neuroantigen-reactive lymphocytes infiltrate the CNS, mediate the development of inflammatory lesions, and in some models, trigger the demyelination of axons leading to progressive paralysis (6). In humans, elevated IL-12 levels have been reported in progressive multiple sclerosis (7, 8), and increased frequencies of IL-12–secreting monocytes appear to correlate with active brain lesions detected by magnetic resonance imaging (9). Finally, IL-12 has been functionally implicated in the development of EAE by the observations that αIL-12 (αp70) blocks disease development in mice and IL-12 p40−/− mice are resistant to EAE induction.

It has recently been reported that the IL-12 p40 subunit is also a component of other molecules, including p40 homodimers and IL-23 (10). Therefore, studies that have implicated IL-12 based solely on alterations in p40 function or expression may be misleading due to the use of the p40 subunit by cytokines other than IL-12. In fact, discordant immune responses have been observed between p35−/− and p40−/− mice, bringing into question the essentiality of IL-12 in these particular models (11–14). In this report, the reevaluation of IL-12 subunits in EAE has revealed that p35−/− mice are susceptible, but p40−/− mice are resistant to EAE. Therefore, while p40 is absolutely critical for the development of EAE, IL-12 is completely dispensable.

Methods
Peptide, antibodies, and ELISA. MOG35–55 peptide (MEGVWYRSPFSRVHLVRNGK) was obtained from Research Genetics (Huntsville, Alabama, USA). ELISAs for IL-2, IL-4, IFN-γ, and IL-12 p70 were performed with kits purchased from Pharmingen (San Diego, California, USA), following the manufacturer’s guidelines. All antibodies for flow cytometry (CD45, CD11b, CD8α, CD4, and GR-1) were preconjugated with either phycoerythrin, FITC, PerCP, or biotin, and were purchased from Pharmingen.

Induction of EAE. Female C57BL/6 mice were obtained from NCI Laboratories (Frederick, Maryland, USA). Homozygous IL-12 p35−/− and IL-12 p40−/− C57BL/6 mice were originally purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) and were bred in-house under pathogen-free conditions.

Female C57BL/6, IL-12 p40−/−, and p35−/− mice 5–8 weeks old were immunized subcutaneously with 200 μg of MOG35–55 peptide emulsified in CFA supplemented with 5 mg/ml of Mycobacterium tuberculosis (H37RA; Difco Laboratories, Detroit, Michigan, USA). The mice received intraperitoneal injections with 250 ng pertussis toxin (Sigma-Aldrich, St. Louis, Missouri, USA) at the time of immunization and...
48 hours later. After 7 days, the mice received an identical booster immunization with MOG/CFA without pertussis toxin. Clinical disease usually commences between day 16 and day 20 after immunization.

Clinical evaluation. The mice were scored four times per week as follows: 0, no detectable signs of EAE; 0.5, limp distal tail; 1, complete limp tail; 1.5, limp tail and hind limb weakness; 2, unilateral partial hind limb paralysis; 2.5, bilateral partial hind limb paralysis; 3, complete bilateral hind limb paralysis; 3.5, complete hind limb paralysis and unilateral forelimb paralysis; 4, total paralysis of both forelimbs and hind limbs; 5, death. Mice scoring greater than 4 but less than 5 were euthanized.

Flow cytometry. Mice were euthanized with CO₂, and spinal cords were removed by flushing the spinal column with sterile HBSS. The brain was dissected to isolate the brain stem. Both tissues were homogenized and strained through a 100-µm nylon filter (Fisher Scientific Co., Pittsburgh, Pennsylvania, USA). After centrifugation, the cell suspension was resuspended in 37% isotonic Percoll and underlaid with 70% isotonic Percoll. The gradient was centrifuged at 600 g for 25 minutes at room temperature. The interphase cells were collected and washed extensively prior to staining. For flow cytometry, the cells were stained with primary antibodies for 30 minutes at 4°C, washed, and incubated with streptavidin-conjugated allo-phycocyanin or PerCP (Pharmingen) for 15 minutes. The cells were washed and analyzed with a FACS Calibur flow cytometer using CellQuest software (Becton, Dickinson and Co., San Jose, California, USA). Postacquisition analysis was performed using WinMDI 2.8 software (Scripps Research Institute, La Jolla, California, USA).

Histology. Mice were euthanized with CO₂. The spinal column was removed and fixed in 10% buffered formalin. The spinal cord was dissected and embedded in paraffin prior to staining with hematoxylin and eosin to assess infiltration.

Recall responses and in vitro IL-12 production. Mice were primed by flank injections of 100 µg keyhole limpet hemocyanin/CFA (KLH/CFA) (Calbiochem-Novabiochem Corp., La Jolla, California, USA). After 5 days, the axillary and inguinal lymph nodes (LNs) were removed and homogenized. LN cells (5 × 10⁶) were placed as triplicates in a 96-well plate and pulsed with different amounts of KLH or irrelevant protein (grade VI chicken ovalbumin; Sigma-Aldrich) as a control. After 48 hours, cells were pulsed with [³H]thymidine (NEN Life Science Products Inc., Boston, Massachusetts, USA) and incubated for an additional 15 hours before cells were harvested. Thymidine incorporation was assessed using a FilterMate harvester and a TopCount NXT microplate scintillation and luminescence counter (both from Packard BioScience Co., Meriden, Connecticut, USA). Sister cultures were harvested 48 hours later for cytokine analysis, and culture supernatants were analyzed by ELISA for IFN-γ, IL-2, and IL-4 (Pharmingen).

To measure IL-12 production in vitro, spleens were removed and homogenized. Red blood cells were lysed, and 5 × 10⁶ splenocytes were stimulated with 1 µg/ml LPS (Sigma-Aldrich) and 10 ng/ml IFN-γ (Pepro-Tech Inc., Rocky Hill, New Jersey, USA) in 96-well plates. Culture supernatants were harvested and analyzed for IL-12 p70 by ELISA (Pharmingen).

Real-time PCR. RNA was extracted from spinal cords of mice with EAE as described (15). Briefly, the spinal column was flushed with ice-cold HBSS and the cords were homogenized in Trizol reagent (Invitrogen Corp., San Diego, California, USA). RNA was extracted following manufacturer instructions. The samples were treated with DNase using DNA-free (Ambion Inc., Austin, Texas, USA), and 5 µg of RNA was transcribed into cDNA using oligo(dT) primers and the SuperScript II MMLV-RT kit (Invitrogen Corp.). Twelve nanograms per well of cDNA was transferred into iCycler 96-well plates (Bio-Rad Laboratories Inc., Hercules, California, USA), and SYBR Green PCR master mix (Applied Biosystems, Foster City, California, USA) was added following the manufacturer’s instructions. PCR was performed on an iCycler with an optical unit (Bio-Rad Laboratories Inc.). The PCR conditions were: 5 seconds of denaturing at 95°C followed by 40 cycles of 15 seconds at 95°C, 45

Table 1
EAE in experimental mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Incidence</th>
<th>Mean day of disease onset</th>
<th>Mean maximal clinical score</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>6/6</td>
<td>19.8</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>p35−/−</td>
<td>5/5</td>
<td>19.4</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>p40−/−</td>
<td>0/6</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data shows a disease report of the experiment shown in Figure 1 above. ND, no disease. *Of diseased mice.
seconds at 63°C, and 15 seconds at 72°C. Amplicon accumulation was measured during the extension phase. The primers used were (5′–3′): β-actin (AGAGGGAAATCGTGCGTGAC; CAATA-GTGATGACCTGGCCGT), CD3Eta (ACAAGATGGCAGAAGCCTACA; ACTGCTTGGAAGTGGCTGTC), IFN-γ (GCATTCATGAGTATTGCCAAG; GGTGGACCACTCGGATGA), IL-4 (ACAGGAGAAGGGA-CGCCAT; GAAGCCCTACA-AGACGAGCTCA), TNF-α (CATCTTCTCAAAATTCGAGTACAA; TGGGAGTAGACAAGGTAACCC), IL-2 (CCTGAGCAGATGGAGAATAC; TCCAGAACATGCCGAG), IL-10 (GGTTGCCAAGCCTTATCGGAG; ACCTGCTCCACTGCCTTG), and LT-α (GACTCTCTGGTGTCCCTTC; ACAAGGTGAGCAGCAGGTTT). Reaction efficiency for each primer was 90–100%, based on the slope generated by standard curves using specific amplicons. A standard curve was generated for each 96-well plate using a plasmid containing the β-actin amplicon. Data were analyzed using the iCycler analysis software, version 2.3.

Results
Development of EAE in IL-12–deficient mice. In the course of studying the role of IL-12 in the development of EAE, discordant disease development was noted when comparing p35−/− with p40−/− mice. In order to comprehensively evaluate the susceptibility of IL-12 p40−/− and p35−/− mice to EAE, mice were immunized with MOG35–55 peptide emulsified in CFA, and the course of the clinical disease was measured. As expected (16), p40−/− mice did not show any clinical disease (Figure 1 and Table 1). In contrast to p40−/− mice, p35−/− mice were highly susceptible to EAE, and when compared with wild-type (WT) mice, the p35−/− mice showed clinical symptoms that were significantly more severe. Histological analysis of the inflamed CNS tissue revealed that p35−/− mice develop severe inflammation (Figure 2c). Cytofluorimetric analysis of the infiltrates indicated that p35−/− mice have higher numbers of infiltrating lymphocytes (CD45hi/CD11b−) than do WT mice (34% vs. 28%). Furthermore, within the infiltrating lymphocyte population, p35−/− mice showed an increased percentage of CD8 T cells compared with the WT (34% vs. 20%) (Figure 3). There were otherwise no overt differences in the cellular composition of the infiltrates. The disease susceptibility and histological analysis of the p35−/− mice and disease resistance of the p40−/− mice establish that a p40-containing cytokine distinct from IL-12 is essential for the development of this disease.

Both p35−/− mice and p40−/− mice display a marked failure to induce antigen-driven IFN-γ secretion. Based on antibody blocking studies and studies in p40−/− mice, IL-12 has been implicated in the polarization of Th cells into encephalitogenic Th1 cells. Given the discordant behavior of the p35−/− and p40−/− mice with regard to the development of EAE, studies were extended to evaluate whether each of these mice could generate Th1-type responses. First, the inability of LPS/IFN-γ–stimulated splenocytes from both the p35−/− and p40−/− mice to produce IL-12 p70 protein was established (Figure 4a). In order to confirm the role of IL-12 in Th1 skewing, primed T cells from p40−/−, p35−/−, and WT mice were rechallenged in vitro and IFN-γ production was measured. Although all three
groups were virtually indistinguishable with regard to the levels of antigen-induced proliferation (Figure 4b) and IL-2 secretion (Figure 4c), both p35−/− and p40−/− animals showed severe defects in stimulating IFN-γ secretion (Figure 4d) compared with WT. Levels of IL-4 were at the limit of detectability and were similar among all groups (not shown). The data confirm that both subunits of IL-12 are required to generate bioactive IL-12 and to polarize IFN-γ-secreting cells.

The inflamed CNS tissue in p35−/− mice displays a Th2 bias. To assess the cytokine expression profile of CNS-infiltrating cells, spinal cords were harvested from WT, p35−/−, and p40−/− mice 24 days after immunization, and cytokine expression within the inflamed tissue was analyzed by real-time PCR. Cytokine mRNA expression levels were not included from p40−/− mice due to lack of infiltrating cells. Overall, when comparing p35−/− mice with WT mice, levels of TNF-α were significantly reduced, whereas IL-4 and IL-10 levels were increased (Figure 5). IFN-γ and LT-α levels were both consistently reduced in p35−/− mice, yet not to the same extent as TNF-α. The data indicate that the inflamed CNS tissue of p35−/− mice displays a Th2 bias.

Discussion
The role of IL-12 in EAE has been addressed based on the differential susceptibility of p35−/− mice and p40−/− mice to the development of EAE. The data show that the p35−/− animals develop severe EAE when immunized with MOG peptide, whereas p40−/− mice are resistant to disease development. Thus, IL-12 p70 cannot be required for the development of EAE. In contrast, IL-12 p70 was shown to be required for Th1 differentiation by the fact that neither the p35−/− nor the p40−/− mice could support the differentiation of IFN-γ-producing T cells during a primary immune response. The notion that EAE is an IL-12-dependent disease was based on studies using neutralizing αIL-12 antibodies and studies in p40−/− mice (reviewed in refs. 1, 3, and 17). Studies have since shown that antibodies raised against IL-12 p70 and used to inhibit IL-12 block the common p40 subunit used not only by IL-12 but also by IL-23 (10) and p40 homodimers. Hence, neutralizing of disease by these antibodies does not allow one to discriminate between IL-12 and other molecules using p40 in mediating disease development. Prior to the realization that the p40 subunit was a common cytokine subunit, the role of IL-12 in disease development was also implicated by the finding that IL-12 p40−/− mice were completely resistant to EAE (5). The data presented herein confirm that the p40 subunit is critical for disease development and T cell encephalitogenicity; however, this cannot be attributed to IL-12 p70, since p35−/− mice were susceptible to EAE.

To investigate whether the lack of p35 leads to a change in the inflammatory makeup compared with the WT, we used flow cytometry to quantify the number of infiltrating lymphocytes, macrophages, and granulocytes. In contrast to mice lacking IFN-γ that also develop severe EAE and display an increased number of GR-1+ neutrophils within the inflamed tissue (18), we cannot detect a major change in the infiltrating CD11b+ populations. However, there is an increased

**Figure 4**
p35−/− and p40−/− mice fail to generate Th1 immune responses. (a) Splenocytes from p35−/−, p40−/−, and WT mice were unstimulated (black bars) or were stimulated for 48 hours with IFN-γ and LPS (white bars). IL-12 p70 secretion was measured by ELISA. (b–d) p35−/−, p40−/−, and WT mice were immunized subcutaneously with KLH in CFA. After 5 days, recall responses of LN cells to either KLH (gray bars) or irrelevant ovalbumin (black bars) were measured by (b) [H]thymidine-uptake, (c) IL-2 ELISA, and (d) IFN-γ ELISA. Shown is a representative of at least two individual experiments.

**Figure 5**
Expression profile of inflamed spinal cords. RNA was isolated as described from inflamed spinal cords 24 days after immunization. Real-time RT-PCR analysis was performed by measuring amplicon accumulation using SYBR Green dye (white bars, WT; black bars, p35−/−). Data are expressed as arbitrary units based on the standard curve of triplicate wells ± SD. Values shown are representative of at least three individual experiments. Levels of β-actin mRNA were similar in both samples; other mRNA species levels were normalized to β-actin.
number of infiltrating lymphocytes – mostly CD8+ T cells in the p35−/− CNS – compared with the WT.

We further investigated the expression profile of the inflamed CNS with regard to Th1 and Th2 cytokines. Overall, in p35−/− mice, we see an increase in Th2 cytokines such as IL-4 and IL-10 and a decrease in TNF-α. In contrast to the in vitro recall responses, where we could not detect IFN-γ produced by p35−/− LN cells, the p35−/− CNS shows abundant IFN-γ message during EAE. This is in contrast to secondary lymphoid tissues (spleen), where we did not detect significant levels of IFN-γ. The CNS environment in p35−/− mice displays an increase in Th2 cytokines, indicating that a Th1 profile is not a requirement for encephalitogenicity. This is in accordance with the observation that Th2 cell lines generated from myelin basic protein–TcR transgenic mice can cause EAE in immunodeficient hosts (19).

The studies presented suggest that an activity associated with p40 other than IL-12 activity is required for the development of EAE. There are at least two cytokine complexes described that use p40 in addition to IL-12. First, there are p40 homodimers that can be found in mice during an immune response (20, 21). However, p40 homodimers have been shown to act as IL-12 receptor antagonists and are considered to have anti-inflammatory properties (22, 23). Thus, p40 homodimers are not likely to be responsible for the generation of encephalitogenic T cells in p35−/− mice. Nonetheless, one cannot exclude the possibility that p40 or p40 homodimers have agonistic activity, as suggested by Holscher et al. (24). Another potential explanation is that the newly discovered IL-23 (p40/p19) is responsible for the development of EAE in p35−/− mice. Indeed, transgenic mice that overexpress the p19 subunit ubiquitously display severe inflammation and multiorgan failure, indicating that IL-23 has strong proinflammatory properties (25). Similar to p35, p19 expression does not appear to be tightly regulated and is expressed in various tissues and by different cell types independent of activation and inflammation (10, 25). Expression of p40, however, is tightly regulated (3). Similar to IL-12, IL-23 is produced by activated macrophages and dendritic cells. IL-23 also binds the IL-12Rβ1 receptor subunit, but not IL-12Rβ2 (10). A dedicated IL-23 receptor that pairs with IL-12Rβ1 has just been identified (26). Nonetheless, IL-12 p70 is clearly not involved in the development of this disease, an important new finding that challenges contemporary dogma.

Over the years, studies using genetically deficient mice have convincingly shown that cytokines such as IFN-γ and TNF-α are dispensable for the development of EAE (27, 28). Yet both multiple sclerosis and EAE are considered Th1-mediated diseases, mostly due to the fact that IL-12 was considered necessary for disease development and pathology. We now can dismiss IL-12 as a critical mediator of this disease, and so the basic conceptual underpinnings of EAE as a Th1 inflammatory disorder need to be reconsidered.

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