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Astrocyte-Targeted Expression of IL-12 Induces Active Cellular Immune Responses in the Central Nervous System and Modulates Experimental Allergic Encephalomyelitis[1]

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The role of IL-12 in the evolution of immunoinflammatory responses at a localized tissue level was investigated. Transgenic mice were developed with expression of either both the IL-12 subunits (p35 and p40) or only the IL-12 p40 subunit genes targeted to astrocytes in the mouse CNS. Glial fibrillary acidic protein (GF)-IL-12 mice, bigenic for the p35 and p40 genes, developed neurologic disease which correlated with the levels and sites of transgene-encoded IL-12 expression. In these mice, the brain contained numerous perivascular and parenchymal inflammatory lesions consisting of predominantly $CD4^+$ and $CD8^+$ T cells as well as NK cells. The majority of the infiltrating T cells had an activated phenotype ($CD44^{high}$, $CD45Rb^{low}$, $CD62L^{low}$, $CD69^{high}$, VLA-4 ^{high}, and $CD25^+$). Functional activation of the cellular immune response was also evident with marked cerebral expression of the IFN-γ, *TNF*, and *IL-1*$\alpha\beta$ genes. Concomitant with leukocyte infiltration, the CNS expression of immune accessory molecules was induced or up-regulated, including ICAM-1, VCAM-1, and MHC class II and B7-2. Glial fibrillary acidic protein-p40 mice with expression of IL-12 p40 alone remained asymptomatic, with no inflammation evident at any age studied. The effect of local CNS production of IL-12 in the development of experimental autoimmune encephalomyelitis was studied. After immunization with myelin oligodendrocyte glycoprotein-peptides, GF-IL-12 mice had an earlier onset and higher incidence but not more severe disease. We conclude that localized expression of IL-12 by astrocytes can 1) promote the spontaneous development of activated type 1 T cell and NK cellular immunity and cytokine responses in the CNS, and 2) promote more effective Ag-specific T cell dynamics but not activity in experimental autoimmune encephalomyelitis. *The Journal of Immunology,* **2000, 164: 4481–4492.**

Interleukin-12 is a key regulator of cellular immunity in both innate and adaptive immune responses (for reviews, see Refs. 1–3). This cytokine is unique in being a heterodimer consisting of a p35 and a p40 subunit. For the secretion of bioactive IL-12, both subunits have to be produced in the same cell. Macrophages, dendritic cells, and polymorphonucleated cells are the major cellular sources of IL-12, which exerts its effects on T cells, NK cells, and B cells. Stimulation of $CD4^+$ T cells by IL-12 during Ag presentation induces their differentiation from a Th 0 to a Th1 phenotype. Further effects of IL-12 include the activation of NK cells, stimulation of T cell and NK cell proliferation, and enhancement of the cytolytic function of $CD8^+$ and NK cells. Both T cells as well as NK cells produce a number of immune effector molecules upon IL-12 stimulation, in particular IFN-γ. Since IFN-γ is a potent inducer of IL-12 production in phagocytic cells, the IL-12/IFN-γ system represents a positive feedback mechanism that initiates and maintains innate and adaptive immune responses in inflammation (1–3). Accordingly, IL-12 has been shown to be a pivotal factor in the immune response against infectious agents, including parasites, viruses, and bacteria, and in tumor rejection.

Expression of IL-12 in the CNS is found in a number of immunoinflammatory states including multiple sclerosis (MS)[5] (4), experimental allergic encephalomyelitis (EAE) in rats (5) and in mice (6), and in murine endotoxemia (7, 8). Besides being produced by CNS-infiltrating immune cells, IL-12 can be produced by neural cells including microglia (7–10) and astrocytes (8, 11). The demonstration of cerebral IL-12 induction in endotoxemic mice is particularly notable considering two studies that demonstrated that IL-12 unmasks latent autoimmune encephalitis in resistant mice and that microbial products, including LPS, can facilitate the induction of EAE by an IL-12-dependent pathway (12, 13). The significance of IL-12 in CNS inflammation is further underlined by the finding that in EAE treatment with Abs against IL-12 ameliorates disease symptoms (14, 15), whereas mice with a disruption of the *IL-12* gene do not develop EAE (15).

The consequences of local CNS production of IL-12 for the development of spontaneous immunoinflammatory responses and in modulating Ag-targeted immune responses are unknown. Therefore, we developed transgenic mice in which the expression of IL-12 was targeted to astrocytes under the transcriptional control of the glial fibrillary acidic protein (GFAP) promoter. We show

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[5] Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental allergic encephalomyelitis; GFAP, glial fibrillary acidic protein; MOG, myelin oligodendrocyte glycoprotein; hGH, human growth hormone; MBP, myelin basic protein; RPA, RNase protection assay.

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that astrocyte-targeted expression of the heterodimeric *IL-12* genes but not the *IL-12* p40 gene alone can initiate and maintain, in an otherwise unmanipulated mouse, a severe neurological disease with activated type 1-like $CD4^+$ and $CD8^+$ T cell and NK cellular immune and cytokine responses in the CNS. Moreover, localized expression of IL-12 may facilitate more effective recruitment and infiltration of the CNS by Ag-specific T cells in EAE.

Materials and Methods

Generation of GFAP-IL-12 transgenic mice

The coding sequences for the murine *IL-12* p35 gene (nucleotide 14–819, GenBank accession no. M86672) and for the *IL-12* p40 gene (nucleotide 1–1055, accession no. M86671) were amplified by RT-PCR from mouse spleen RNA. The PCR primers consisted of 20 nucleotides specific for the cytokine gene flanked by *Bam*HI (+strand) and *Not*I (−strand) restriction sites, respectively. The following oligonucleotide primers were used: p35 (+) CCGTCGACTCCTGGGAAAGTCTGCCGGCTA, p35 (−) AAGGATC CTCCTATCTGTGTGAGGAGGGCG, p40 (+) CCGTCGACGCACATCA GACCAGGCAGCTCG, and p40(−) AAGGATCCCCAACGTTGCATC CTAGGATCG.

After digestion with the appropriate restriction enzymes, the amplified fragments were cloned into a vector (pIC-hGH, kindly provided by Dr. Jan Allison, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) containing a human growth hormone (hGH) polyadenylation signal. To confirm the identity of the amplified fragments, overlapping sequence analysis of both DNA strands was performed. Correct constructs consisting of the published p35 or p40 coding sequence along with the downstream hGH polyadenylation signal were then inserted into a cassette consisting of the mouse GFAP promoter and an SV40 splice donor/acceptor site upstream and the genomic sequence for GFAP downstream (16). The resulting fusion gene constructs were excised from the vector and purified, and an equimolar mixture of the p35 and the p40 constructs was microinjected into fertilized eggs of $(C57BL/6J \times SJL)F_1$ hybrid mice. Transgenic offspring were identified by PCR analysis of tail DNA. The PCR primers were designed to generate fragments of 800 bases for the p40 and 450 bases for the p35 transgene. By using a downstream primer homologous to the hGH polyadenylation signal, amplification was restricted to transgenic p35 and p40 sequences.

RNA isolation

Organs were removed and immediately snap frozen in liquid nitrogen and stored at −80°C pending RNA extraction. $Poly(A)^+$ RNA was isolated according to a previously published method (17). Total RNA was extracted with Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's protocol.

RNase protection assays

RNase protection assays for the detection of cytokine RNAs were performed as described previously (18). The RNA samples were hybridized with labeled cytokine probe sets ML11 (19) and ML26 (kindly provided by Dr. Monte Hobbs, The Scripps Research Institute) or a probe set containing two probes for the IL-12 p40-hGH and p35-hGH transgene. The latter probes included a target site (see below) in the respective IL-12 subunits and 75 bp of the downstream hGH transgene. Thus, these probes allowed for the differentiation between expression of transgenic vs endogenous IL-12 mRNA since transgenic mRNA hybridized >297 bp (IL-12 p40-hGH) and 279 bp (IL-12 p35-hGH) whereas endogenous IL-12 mRNA hybridized at 222 bp (IL-12 p40) and 204 bp (IL-12 p35). In all probe sets, a fragment of the RPL32-4A (20) gene served as an internal loading control.

Immunoblot analysis

Cerebellum was removed from symptomatic GF-IL12 mice, 4-mo-old GF-p40 mice, or wild-type littermates and solubilized in lysis buffer (500 ml PBS containing 1.0% Triton X-100 (Sigma, St. Louis, MO), EDTA (1 mM), leupeptin (1 mg/ml; Sigma), aprotonin (5 mg/ml; Sigma), and PMSF (50 mg/ml; Sigma), pH 7.5. Following solubilization, the samples were clarified by centrifugation at 10,000 × *g* for 10 min. An aliquot was then removed for protein assay using a commercially available kit (Bio-Rad, Hercules, CA) and the remainder of the lysate was frozen at −20°C pending analysis. For electrophoretic fractionation, an equal volume of lysate (containing 25 μg of protein) or protein standards (Bio-Rad) was mixed with 2× electrophoresis buffer containing SDS (4%), Tris-HCl (125 mM, pH 6.8), glycerol (20% v/v), and bromophenol blue (0.1%) and fractionated on a 10% Tris-glycine gel at 150 V. Following electrophoresis, the samples were transferred to nitrocellulose, blocked, and then incubated with a rat mAb against murine IL-12 p40 (21) (kindly provided by Dr. Trinchieri, Wistar Institute, Philadelphia, PA).

Histology, in situ hybridization, and immunohistochemistry

For routine histology, brains and spinal cords were removed, fixed in 4% buffered paraformaldehyde, and embedded in paraffin. Sections (8 μm) were processed according to standard procedures and used for routine staining and in situ hybridization (see below). An Alizarin Red S technique modified from a previous study (22) was used for identification of calcium. Briefly, after deparaffinization, brain sections were incubated in Alizarin Red S (Sigma) solution (2% in H_2O), rinsed with acetone, coverslipped, and examined by bright field microscopy.

In situ hybridization was performed as described previously (23, 24) with ^{35}S-labeled antisense and sense RNA probes transcribed from the p40 and the p35 cDNAs that were used for the generation of the transgenic constructs cloned into the pGEM-4Z plasmid (Promega, Madison, WI). For the analysis of *IFN-γ* gene expression, the probe used was a ^{33}P-labeled single-stranded antisense RNA to murine IFN-γ. A murine IFN-γ cDNA (kindly provided by Genentech, South San Francisco, CA) was subcloned in the pGEMEX-1 (Promega) transcription vector. Dual-label in situ hybridization and immunohistochemistry were performed as described previously (25). A rabbit anti-human CD3 Ab (Dako, Carpinteria, CA) was used to immunostain murine T cells.

For immunophenotyping and cellular adhesion molecule immunostaining, mice were killed, and organs were removed and immediately snap frozen in isopentane and stored at −70°C until sectioning. Sagittal cryomicrotome cut serial sections (10 μm) were air dried, fixed in cold (−20°C) acetone:methanol (1:1) for 45 s, and nonspecific binding was blocked by incubating the sections for 30 min in PBS containing 3% rabbit and 3% goat serum. Sections were then incubated for 2 h at room temperature in rat mAbs to identify leukocytes (CD45 from PharMingen, San Diego, CA), lymphocytes (CD4, CD8, and B220 from PharMingen), activation markers (B7-2 from PharMingen; MHC-class II, clones M5/114 and Mac-1, and clone TIB 126 from American Type Culture Collection, Manassas, VA), and cellular adhesion molecules (mucosal addressin cell adhesion molecule-1 and VCAM-1 from PharMingen and ICAM-1 hybridema clone YN11.1 kindly provided by Dr. F. Takei, Toronto, Ontario, Canada). All Abs were used at a final concentration of 5 μg/ml diluted in the blocking buffer. Bound Ab was detected using a biotinylated anti-rat Ab (Southern Biotechnology Associates, Birmingham, AL) followed by avidin-labeled HRP (Sigma). Staining employed 3′,3′-Diaminobenzidine (Sigma) as substrate. Before mounting, sections were counterstained with Mayer's hematoxylin and dehydrated in graded ethanols.

FACS characterization of CNS-infiltrating leukocytes

Leukocytes were isolated as described previously (26, 27), with minor modifications. Briefly, mice were sacrificed by halothane inhalation and perfused with 20 ml PBS unless stated otherwise. Brains were rapidly removed and mechanically dissociated by sequentially forcing the tissue through 210- and 70-mm nitex meshes. The cell suspension was enzymatically digested with DNase I (28 U/ml; Sigma) and collagenase (0.2 mg/ml; Sigma) for 1 h at 37°C in a shaking incubator in HBSS without serum. After quenching the digestion with the addition of 10% FBS (final concentration), the cell suspension was separated on a discontinuous 1.033/1.088/1.122 Percoll gradient. Leukocytes were collected from the interfaces at the 1.033 Percoll fraction. Myelin and cell debris separated above the gradient.

Biotin-conjugated, FITC-conjugated, and PE-conjugated Abs against mouse CD4, CD8, CD25, CD44, CD45RB, CD62L, CD69, B220, and VLA-4 (PharMingen) were reacted with the cells isolated from the Percoll gradient or lymph node cell suspensions. For the detection of NK cells, a rabbit anti-rat asialo GM1 Ab (Cederlane, Hornby, Ontario, Canada) was used. After incubation with the Ab and washing, cells were reacted with FITC-anti-rabbit Ab (Sigma). Stained cells were then analyzed with a FACScan by using CellQuest acquisition software (Becton Dickinson, Mountain View, CA).

Induction of EAE

To reproducibly induce EAE in mice of the C57BL6/SJL genetic background, active immunization was achieved with a combination of myelin oligodendrocyte gycoprotein (MOG) peptides (Research Genetics, Huntsville, AL), known to be encephalitogenic in C57BL6 (MOG_{35-56}) (28) and SJL/J (MOG_{92-106}) (29) mice. On day 0, naive, 2–3-mo-old nontransgenic littermates or transgenic animals (i.e., well before onset of spontaneous immunity in GF-IL-12 mice) were immunized s.c. into the rear flanks with

Table I. *Characterization of GFAP-IL-12 transgenic founder mice and their offspring*

Mouse	Status	Genotype	Transgene Expression	Clinical Symptoms[a]	Histological Features[b]
G12A4	Founder	p35/p40	++	Death day 8	ND
G12H6	Founder	p35/p40	+++	++	+++
G12A3	Founder	p35/p40	+++	+++	+++
GF-IL-12	Founder	p35/p40	++	++	++
GF-IL-12[c]	Offspring:				
	Hemizygous	p35/p40	+–++	++	++
	Homozygous	p35/p40	++	+++	+++
GF-p40	Offspring	p40	++		

[a] Diminished size, hunched posture, ruffled fur, ataxia, muscle atrophia, reduced life span.
[b] Astrocytosis, cerebral infiltration by lymphocytes and NK cells, expression of cell adhesion molecules, cerebellar calcification, spongiosis of cerebellar white matter.
[c] Penetrance of the phenotype was variable between mice.

a 1:1 emulsion of 100 μl $MOG_{35–56}$ and $MOG_{92–106}$ synthetic peptides (50 μl of each at 3 mg/ml,) in 100 μl CFA (Sigma) supplemented with 4 mg/ml *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI). In addition, each mouse received an i.p. injection of 500 ng pertussis toxin (Sigma) on days 0 and 2. In an alternative approach, mice were immunized on day 0 as above with 200 μg of bovine MBP and 500 μg of *Mycobacterium tuberculosis* in incomplete Freund's adjuvant (Sigma), receiving 200 ng of pertussis toxin on days 0 and 3. This protocol gave similar results, but disease was not as reproducible as with MOG-EAE (data not shown).

Animals were observed for up to 6 wk on a daily basis and disease severity was expressed as grade 0, no disease; grade 0.5, partial loss of tail tonus; grade 1, complete tail atony; grade 2, hind limb paraparesis; grade 3, hind limb paralysis; grade 4, moribund; and grade 5, death. For statistical analysis, one-way ANOVA with Dunnett's *t* test was used.

Results

Transgenic expression of IL-12 in the CNS induces a dose-related neurological disorder

From 61 founder mice, 8 had integration of both the IL-12 p40 and IL-12 p35 transgenes (bigenic mice) whereas 1 had integration of the IL-12 p40 gene only (see Table I). Of the bigenic mice, one died at 8 days of age while two others, beginning at 3 wk of age, showed progressive signs including diminished size, hunched posture, ruffled fur, ataxia, and muscle atrophia. These mice had no offspring and had to be killed at 3 mo and 4 mo of age. Histological examination of the brain from these mice revealed severe meningoencephalitis and calcification in the cerebellum correlating with high expression of the IL-12 p35 and p40 transgenes in the cerebellum and at lower levels in the forebrain (Table I). Of the remaining founder mice, two stable transgenic lines were established: one (GF-IL-12 line) expressed both the p35 and the p40 transgenes whereas the other (GF-p40 line) expressed the p40 transgene only.

Mice of the GF-IL-12 line, hemizygous for the transgene, appeared to be healthy until around 4–6 mo of age when some of the animals developed a progressive disease similar to that of the founder bigenic mice with high expression of IL-12 described above. Although inheritance of the transgene followed a Mendelian fashion, about 15% of the GF-IL-12 mice up to 18 mo of age had no physical evidence of disease. In contrast, GF-IL-12 mice homozygous for the transgene all exhibited an earlier (2–3 mo of age) onset of neurological disease (Table I). No mice of the GF-p40 line (up to 18 mo of age) have shown any physical differences compared with wild-type control littermates.

Transgene-encoded IL-12 RNA and protein expression in the CNS of GFAP-IL-12 mice

Levels of *IL-12* gene expression in the CNS of mice from the GF-IL-12 and GF-p40 lines were analyzed by RNase protection assay (RPA) (Fig. 1*A*). No detectable IL-12 p40 RNA expression was noted in the brain of any wild-type mouse examined. In contrast, the expression of the IL-12 p40 RNA was readily detectable in brain from hemizygous GF-IL-12 and GF-p40 mice. Similar, very low levels of IL-12 p35 RNA expression were detectable in brain from both wild-type and GF-p40 mice. However, expression of IL-12 p35 RNA was clearly increased in brain from the GF-IL-12 transgenic animals. In situ hybridization to detect expression of the IL-12 subunit transcripts combined with immunostaining for GFAP to identify astrocytes demonstrated that expression of both the IL-12 p40 (Fig. 1*B*) and IL-12 p35 (Fig. 1*C*) transcripts was localized to astrocytes. To evaluate the pattern and specificity of GFAP promoter-driven expression of the targeted transgenes, RPA analysis, using probes that were specific to the transgene, was performed on RNA extracts from eye, brain, spinal cord, spleen, thymus, liver, kidney, muscle, heart, and lung. This strategy allowed for the discrimination between transgenic and endogenous IL-12 transcripts and demonstrated that only CNS tissues expressed the transgene (Fig. 1*D*). Most prominent expression of the transgene-encoded IL-12 p40 and p35 genes was observed in the cerebellum and eyes, whereas little or no detectable expression was found in the spinal cord. Moreover, in the cerebellum of GF-IL-12 mice, induction of endogenous IL-12 p35 and IL-12 p40 mRNAs was observed.

To determine whether the IL-12 transcripts detected above were translated to protein, protein lysates from the brain were analyzed by immunoblotting with a mAb against the IL-12 p40 protein. With the exception of a common and presumably nonspecific band at ~35 kDa, no detectable immunoreactive species were observed in brain lysates from wild-type mice (Fig. 1*E*). In contrast, brain lysates from both the hemizygous GF-IL-12 and the GF-p40 mice contained a prominent 40-kDa species corresponding in size to the IL-12 p40 monomer (21, 30). In addition, brain lysates from the GF-IL-12 mice also contained very low levels of a 70-kDa species which likely corresponded to the IL-12 p35/p40 heterodimeric protein. A molecular species of ~140 kDa of unknown identity also present in the GF-IL-12 specimens may correspond to a dimer of the IL-12 p70.

Neurological signs are associated with marked immunoinflammatory cell infiltration and degenerative disease in the CNS of GFAP-IL-12 mice

Routine histological examination of the brain from symptomatic GF-IL-12 mice revealed severe inflammatory and degenerative neurological disease affecting many regions of the brain, with greatest severity in the cerebellum and least severity in the spinal cord. In cerebellar meninges, perivascular sites, and parenchyma, large accumulations of mononuclear cells were present (Fig. 2*A*). In regions affected by inflammation, hypertrophy and proliferation

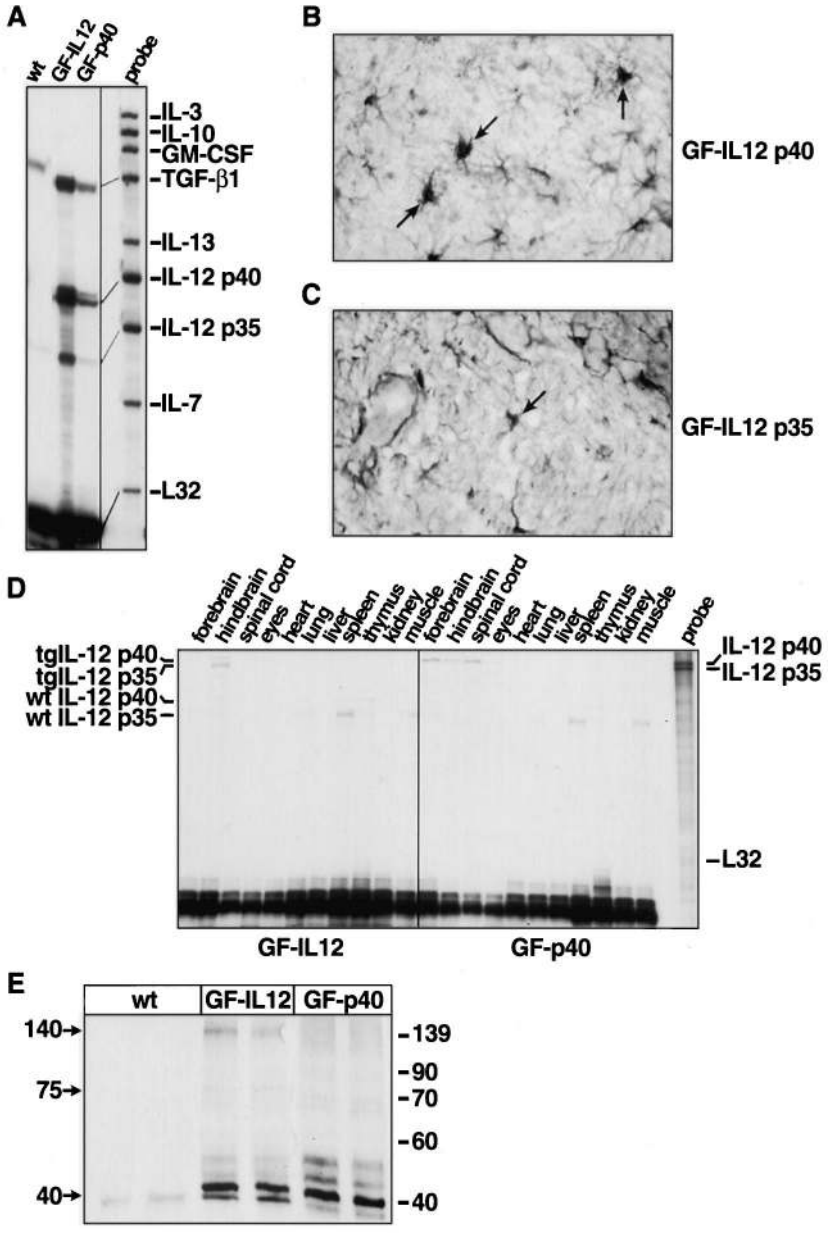

FIGURE 1. Analysis of IL-12 subunit gene expression in GF-IL-12 and GF-p40 transgenic mice. Cytokine mRNA expression was detected by RPA (*A*) using the ML-26 probe set as described in *Materials and Methods*. Samples were poly(A)$^+$ RNA (2 μg) from brain of a wild-type (wt), a symptomatic GF-IL-12 (6 mo of age), or GF-p40 mouse (2 mo of age). Localization of the IL-12 p40 (*B*) and IL-12 p35 (*C*) RNA expression to astrocytes immunostained for GFAP (arrows). Original magnification, both panels ×660. Transgene vs endogenous IL-12 p40 and p35 mRNA expression (*D*) was distinguished using target-specific probes generated as described in *Materials and Methods*. Organs were removed from symptomatic GF-IL-12 and GF-p40 mice and poly(A)$^+$ RNA was prepared for analysis by RPA as described in *Materials and Methods*. Immunoblot analysis of IL-12 p40 expression in brain from wild-type, GF-IL-12 or GF-p40 mice (*E*). Organs were homogenized in lysis buffer and proteins were separated unreduced by SDS-PAGE as described in *Materials and Methods*. After transfer, nitrocellulose membranes were incubated with a rat mAb C17.8 (21) to murine IL-12 p40. After washing, the bound Ab was detected using commercially available chemiluminescence kits (enhanced chemiluminescence kit; Amersham Pharmacia Biotech, Piscataway, NJ).

of the vascular endothelium was marked. Marked calcification affecting the granular cell layer of the cerebellum was also commonly found (Fig. 2*B*). In advanced lesions, gross degenerative changes were seen involving disruption and loss of gray matter (Fig. 2*A*) and adjacent white matter. As revealed by immunostaining for neurofilament protein, when compared with wild-type controls (Fig. 2*C*), brain from symptomatic GF-IL-12 mice showed a loss of various neuronal populations including Purkinje cells (Fig. 2*D*), granular and molecular layer neurons, and hippocampal neurons (Fig. 2*F*). Immunohistochemistry also revealed prominent, widespread reactive astrocytosis (Fig. 2*H*) with significant up-regulation of GFAP-immunoreactivity and astrocyte hypertrophy in the regions of the inflammatory degenerative lesions, the cerebellum, the brainstem, and in the hippocampus (data not shown). Striking pathological alterations were also observed in the brain of younger homozygous GF-IL-12 mice and were indistinguishable for those seen in hemizygous symptomatic GF-IL-12 mice.

A survey of the brain from younger (2–3-mo-old) asymptomatic hemizygous GF-IL-12 mice indicated no significant pathological alterations compared with control littermates (data not shown). In addition, examination of the peripheral organs (liver, kidney, and pancreas) from symptomatic GF-IL-12 mice failed to reveal any abnormalities (data not shown). Finally, no overt histological alterations were observed in the brain from GF-p40 mice.

Infiltrating mononuclear cells are predominantly activated T lymphocytes and NK cells in the GF-IL-12 mice

Immunohistochemical staining with the pan-leukocyte marker CD45 on brain from symptomatic hemizygous GF-IL-12 mice confirmed the presence of large numbers of infiltrating leukocytes present in meningeal, perivascular, and parenchymal locations (Fig. 3*E*). Immunophenotyping of these cells revealed the presence of predominantly CD8$^+$ (Fig. 3*F*) and CD4$^+$ lymphocytes (Fig. 3*G*) with only a few B220$^+$ B lymphocytes (data not shown) present. Lymphocytic infiltrates also contained some Mac-1$^+$ microglia/macrophages and activation of resident microglia was also prominent (Fig. 3*H*). There was some variation in the degree of

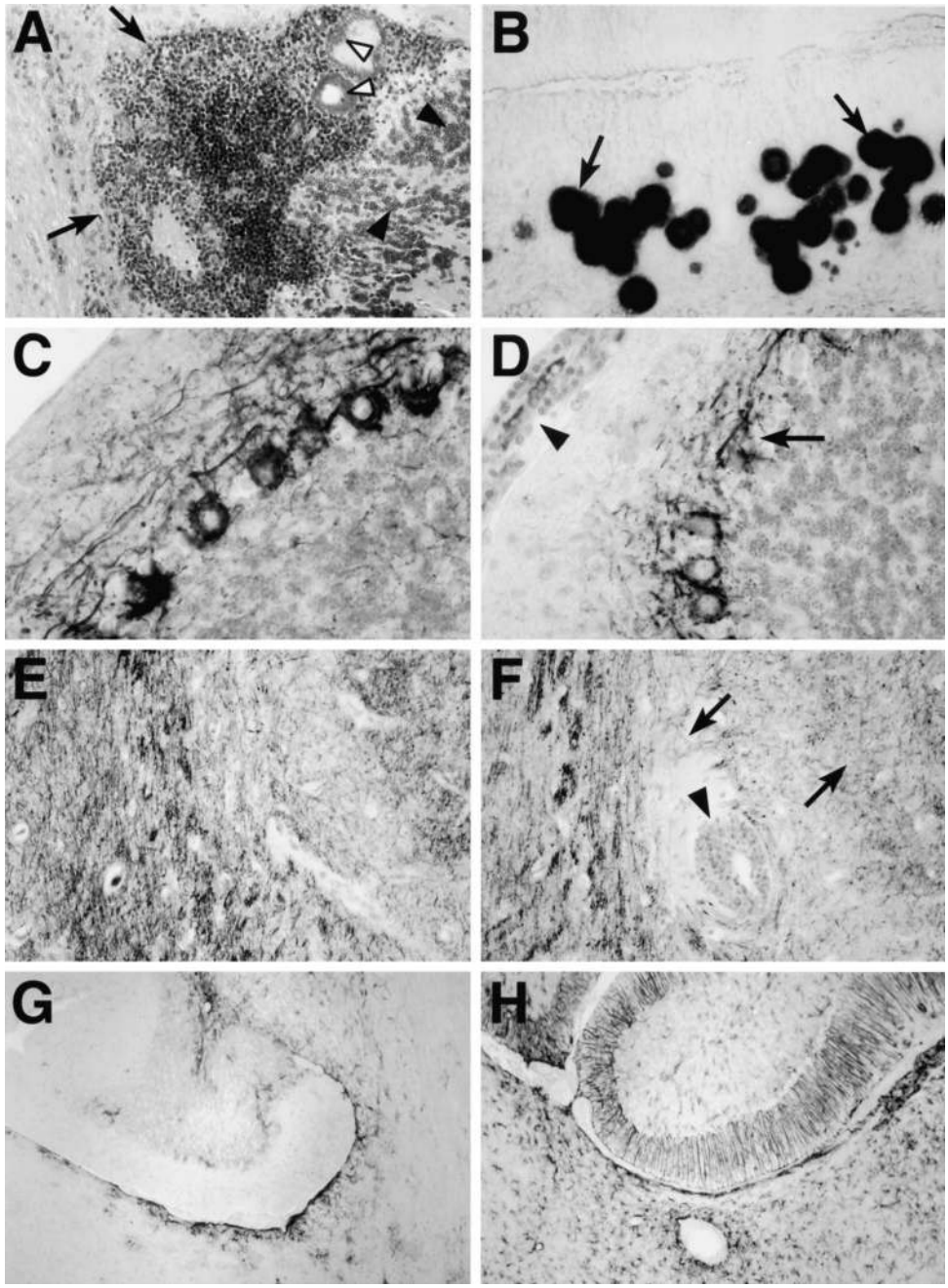

FIGURE 2. Pathologic alterations in the cerebellum of GF-IL-12 transgenic mice. Luxol fast blue stain of tissue from a symptomatic (age 6 mo) hemizygous mouse (*A*). Note the large inflammatory focus (arrows) containing darkly stained mononuclear cells and hypertrophy and thickening of the vascular nedothelium (open arrowheads). In addition, marked disruption of the adjacent granule cell layer can be seen (filled arrowheads). Original magnification, ×400. In the same specimen, areas of calcium mineralization were prominent and stained strongly with Alizarin Red S (arrows, *B*). Original magnification, ×400. Neurodegeneration was also evident as revealed by immunohistochemistry for neurofilament, here showing disruption and loss of Purkinje cells (arrows, *D*) and hippocampal neurons (arrows, *F*) in the specimen from the GF-IL-12 mouse compared with a wild-type littermate (*C* and *E*). Original magnification, both panels ×800. The adjacent meninges (*D*) and blood vessel (*F*) also show the accumulation of large numbers of inflammatory cells (arrowheads). Compared with wild-type littermates (*E*), diffuse reactive astrocytosis was seen throughout the brain of the GF-IL-12 mice (*F*). Astrocytes were visualized by GFAP immunostaining. Original magnification, both panels ×125.

infiltration by immune cells in different brain regions. The immunohistological alterations were most severe in the cerebellum while the hippocampus and the pons revealed lower numbers of infiltrating cells. The other parts of the brain, e.g., cortex, striate body, thalamus, and spinal cord contained the lowest level of lymphocytic and macrophage infiltration and activation. Examination of brains from 12-mo-old GF-p40 mice showed there was no significant expression of any of these immunophenotypic markers when compared with age-matched wild-type littermates.

Flow cytometric analysis of CNS-infiltrating cells from the brains of hemizygous GF-IL-12 mice was performed to determine the activation state of these cells (Fig. 4). Little or no lymphocytes could be isolated from the brain of nontransgenic control mice. In contrast, lymphocytes were readily isolated for further analysis from the brain of symptomatic GF-IL-12 mice. Similar to the immunohistochemical staining noted above, flow cytometric analysis of the isolated brain leukocyte population revealed that the majority of cells were $CD4^+$ and $CD8^+$ T cells with much fewer $B220^+$ B cells. When compared with naive lymph node T cells from wild-type littermates, the brain-infiltrating T cells from the symptomatic GF-IL-12 mice displayed an activated, memory phenotype: $CD44^{high}$ and $CD62L^{low}$ (Fig. 4*A*). The brain-infiltrating T cells were further analyzed and found to display increased expression of several markers of lymphocyte activation including CD25 (IL-2 receptor), the early activation marker CD69, and VLA-4 and decreased expression of CD45RB (Fig. 4*B*). This surface marker phenotypic profile clearly indicates that the T cells accumulating in the brains of the symptomatic GF-IL-12 mice were activated.

In addition to T cells, NK cells represent another significant cellular target for the effects of IL-12. Additional analysis of the brain-infiltrating leukocytes was performed using an Ab that recognizes the NK cell marker asialo GM1 and revealed the presence of a significant number of asialo GM1-positive cells (Fig. 4*C*). These findings suggest that in addition to lymphocytes,

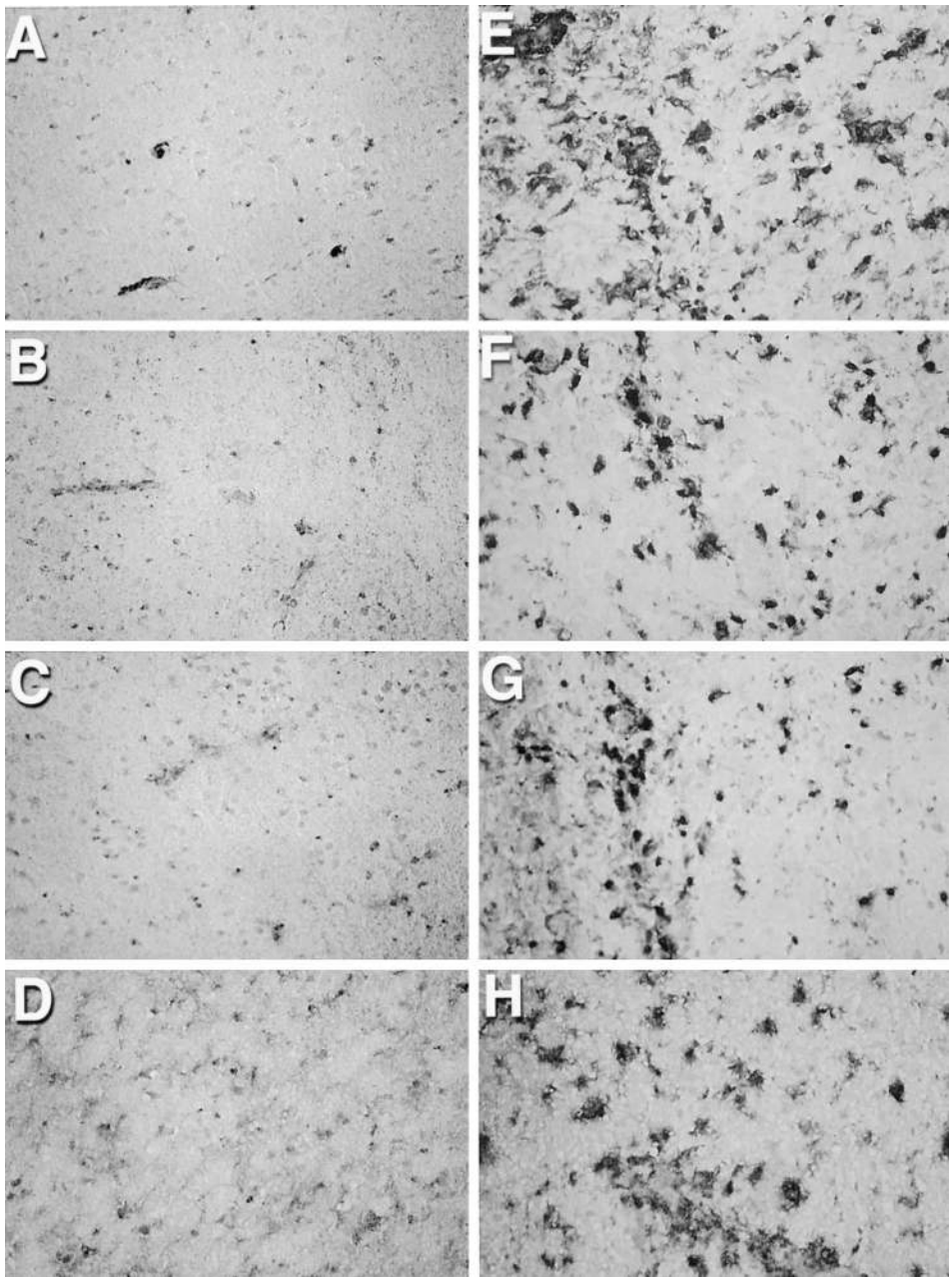

FIGURE 3. Immunophenotypic characterization of infiltrating leukocytes in brain from GF-IL-12 transgenic mice. Immunohistochemistry was performed as described in *Materials and Methods*. Sections from a wild-type control (*A–D*) or a symptomatic GF-IL-12 mouse (*E–H*) were immunostained for CD45 (*A* and *E*), CD8 (*B* and *F*), CD4 (*C* and *G*), or Mac-1 (*H*). Original magnification, all panels ×250. Large numbers of leukocytes surround blood vessels and infiltrate the brain parenchyma in the brain from the transgenic animal.

the brain-infiltrating leukocyte population in the GF-IL-12 mice contained NK cells.

Expression of the IFN-γ and some other cytokine genes is induced in the CNS of GFAP-IL-12 transgenic mice

IL-12 is a major inducer of proinflammatory cytokine and in particular IFN-γ gene expression in T cells and NK cells (31). We therefore examined cytokine gene expression in the brain of GFAP-IL-12 transgenic mice using multiprobe RPA analysis (Figs. 1*A* and 5*A*). No significant proinflammatory gene expression was detectable in brains from wild-type control mice (Fig. 5*A*). By contrast, expression of proinflammatory cytokine genes corresponding to IL-1α and β, TNF, and IFN-γ was induced in brains from symptomatic GF-IL-12 mice (Fig. 5*A*) as well as in brains from the high expressor bigenic founder mice (data not shown). These findings suggested there is functional activation of the CNS-infiltrating T cell and/or NK cell populations. The presence of IFN-γ gene expression is consistent with the known action of IL-12 to induce this cytokine from T cells and NK cells. To further delineate the source for IFN-γ gene expression, in situ hybridization for IFN-γ RNA was combined with immunohistochemical staining for the T cell marker CD3 (Fig. 5, *B* and *C*). The results show that the majority of IFN-γ RNA-expressing cells were localized to the immunoinflammatory cell infiltrates and included $CD3^+$ cells (Fig. 5*B*). However, IFN-γ RNA-expressing cells that did not stain for CD3 (Fig. 5*C*) were also identified, indicating that a non-T cell source of this cytokine was also present in the brain. These IFN-γ-positive non-T cells likely correspond to NK cells.

Interestingly, in addition to the proinflammatory cytokines, expression of the counter regulatory cytokine TGF-β1 was also increased in brains from the GFAP-IL-12 transgenic animals (Fig. 1*A*). In contrast to the GF-IL-12 mice, no significant alteration in the expression of any cytokine gene was observed in the brain from GF-p40 mice (Fig. 5*A*).

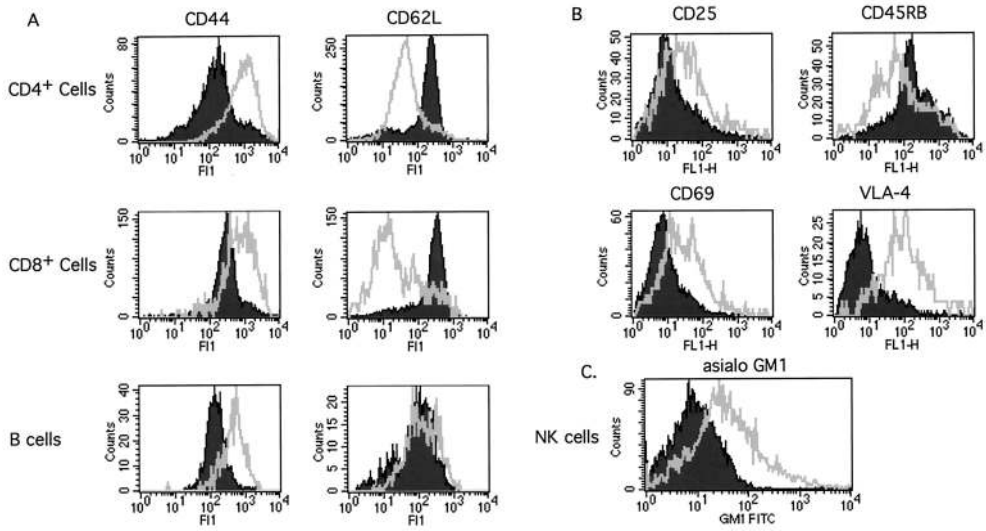

FIGURE 4. Flow cytometric analysis of mononuclear cells isolated from the lymph node of wild-type control or brain of symptomatic GF-IL-12 mice. *A*, $CD4^+$ and $CD8^+$ cells infiltrating the GF-IL-12 brains display a memory phenotype. Expression of CD44 and CD62L by $CD4^+$, $CD8^+$, and $B220^+$ B cells infiltrating the CNS of GF-IL-12 brains (open histograms) is compared with naive cells from the lymph nodes of wild-type controls (filled histograms). *B*, Infiltrating T cells display an activated phenotype. Expression of CD25, CD45RB, CD69, and VLA-4 by $CD3^+$ cells infiltrating the CNS of GF-IL-12 brains (open histograms) is compared with naive unactivated cells from the lymph nodes of wild-type controls (filled histograms). *C*, Asialo GM1-positive cells are found in the leukocyte infiltrates of symptomatic mice (open histogram). Filled histogram depicts level of expression on cells from wild-type controls.

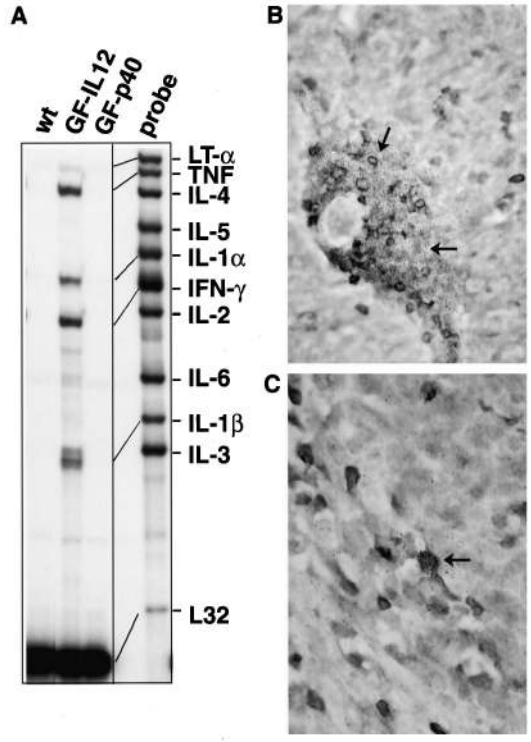

FIGURE 5. Analysis of cytokine gene expression in the brain of GF-IL-12 and GF-p40 transgenic mice. Cytokine mRNA expression was detected by RPA (*A*) using the ML-11 probe set as described in *Materials and Methods*. Samples were $poly^+$ RNA (2 μg) from brain of a wild-type (wt), a symptomatic GF-IL-12 (6 mo of age), or a GF-p40 mouse (2 mo of age). Localization of IFN-γ (*B* and *C*) RNA expression to non-$CD3^+$ perivascular leukocytes (*B*, arrows) and to $CD3^+$ leukocytes (*C*, arrows). Original magnification, both panels $\times$660.

Increased expression of cellular adhesion and immune accessory molecules in the CNS of the GF-IL-12 mice

To further elucidate the pathogenetic profile associated with the CNS immunoinflammatory lesions in the GF-IL-12 mice, the expression of a number of cellular adhesion molecules and immune accessory molecules was investigated. In wild-type control mice, low cerebral expression of the cellular adhesion molecules ICAM-1 and VCAM-1 (Fig. 6, *A* and *B*) was detectable by vascular endothelial cells. However, in GF-IL-12 mice, significant up-regulation in the expression of ICAM-1 and VCAM-1 (Fig. 6, *E* and *F*) was observed. In these mice, in addition to vascular endothelium, ICAM-1 was expressed by a variety of cells including infiltrating leukocytes and parenchymal ramified cells presumed to represent microglia, whereas VCAM-1 expression was restricted to the vascular endothelium. Expression of the cellular adhesion molecule mucosal addressin cell adhesion molecule-1 was not detectable in brains from wild-type or GF-IL-12 mice (data not shown).

In wild-type control mice, expression of the key immune accessory molecules, MHC class II and B7-2 (Fig. 6, *C* and *D*), was detectable only by perivascular microglial cells. In contrast, markedly up-regulated expression of these molecules was observed in the brain of the GF-IL-12 mice (Fig. 6, *G* and *H*). In the latter, both molecules were widely expressed including by infiltrating leukocytes, vascular endothelium, and parenchymal ramified cells presumed to be microglia.

Astrocyte-targeted expression of IL-12 favors a significantly earlier onset of EAE

IL-12 is known to be crucial for the development of the Th-1-mediated autoimmune disease EAE. To determine whether IL-12 produced locally in the brain could alter the development of EAE, groups of mice were immunized in the periphery with MOG-peptide Ag. Three separate experiments demonstrated that reproducible EAE could be induced in both wild-type and transgenic mice of the C57BL/6 $\times$ SJL hybrid background, by using the MOG-peptide immunization strategy (see *Materials and Methods*). The

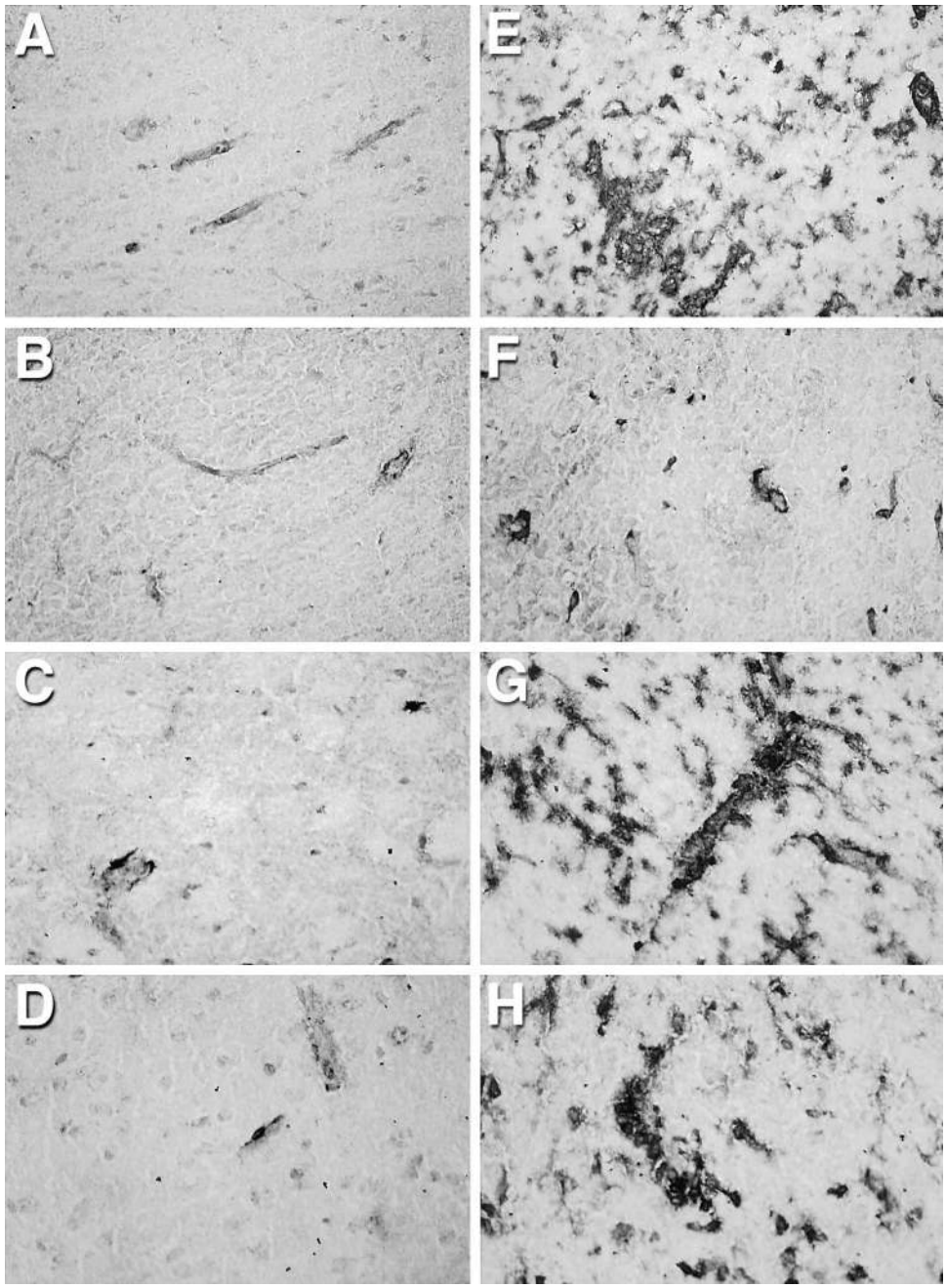

FIGURE 6. Analysis of adhesion molecule and immune accessory molecule expression in brain from GF-IL-12 transgenic mice. Immunohistochemistry was performed as described in *Materials and Methods*. Sections from wild-type control (*A–D*) or a symptomatic GF-IL-12 mouse (*E–H*) were immunostained for ICAM-1 (*A* and *E*), VCAM-1 (*B* and *F*), MHC class II (*C* and *G*), and B7-2 (*D* and *H*). Original magnifications: *A–F*, ×250 and *C–H*, ×660. Note in the transgenic specimens, with the exception of VCAM-1 whose expression was restricted to vascular endothelium, the expression of ICAM-1, MHC class II, and B7-2 was widely distributed and found on vascular endothelium, infiltrating immune cells, and ramified parenchymal cells presumed to be microglia.

disease course in all three animal groups was mildly relapsing-remitting. No statistically significant difference was observed between the three animal groups in either the progression or severity of disease (Fig. 7*A*). Differences were however seen among wild-type, GF-IL-12, and GF-p40 mice with respect to the onset of EAE. The disease onset was significantly ($p < 0.01$) earlier (mean day of onset, 8.5 ± 1.2) in GF-IL-12 mice compared with the wild-type controls (mean day of onset, 11.7 ± 1.7). In contrast, GF-p40 mice had a significantly ($p < 0.05$) delayed onset of EAE (mean day of onset, 13.5 ± 2.5) compared with the wild-type controls (Fig. 7*B*). Although the incidence of EAE was higher in the GF-IL-12 group, this did not reach statistical significance when compared with the wild-type or GF-p40 groups (Fig. 7*B*). Histological examination of brain and/or spinal cord removed from EAE-affected mice with peak clinical disease failed to reveal differences in the appearance of the inflammatory lesions between the wild-type and transgenic mice (data not shown).

Discussion

IL-12 is a central mediator of innate and adaptive cellular immune responses where it activates NK cells and promotes $CD4^+$ and $CD8^+$ type 1 development via induction of IFN-γ (1–3). Mice deficient in IL-12 have impaired IFN-γ production and defective Th1 cell responses (32). Moreover, a number of autoimmune disease models demonstrate the critical role of IL-12 in the generation of Th1 immunity, including collagen-induced arthritis (33, 34), experimental colitis (35), insulin-dependant diabetes mellitus (36), and EAE (14, 15). Although autoimmune disease in these models was initiated by peripheral immunization with organ-specific self Ags, it is unclear to what extent, if any, the intrinsic environment of the target organ may further modulate the localized immune response. In the case of the CNS, it is known that the resident cells, microglia (7–10), and astrocytes (8, 11) can produce IL-12 and that expression of this cytokine is induced in the brain during EAE (6).

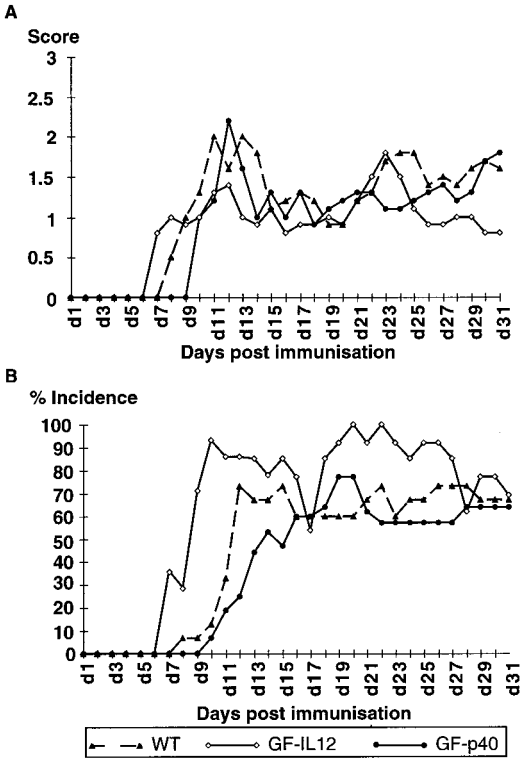

FIGURE 7. Disease severity and incidence of EAE in GF-IL-12 and GF-p40 transgenic mice. Wild-type (WT, $n = 15$), GF-IL12 ($n = 15$), and GF-p40 ($n = 15$) mice were immunized on day 0 with MOG-peptide in CFA and boosted with pertussis toxin to induce EAE as described in *Materials and Methods*. Clinical severity of EAE was similar in all groups of mice (*A*), but disease onset was earlier (day 7) in GF-IL-12 mice and later (day 10) in GF-p40 mice compared with wild-type mice (day 8). Whereas 90–100% of GF-IL-12 mice were sick from days 9 to 31, only 60–70% of the wild-type and GF-p40 mice had clinical symptoms (*B*).

The present study was conducted to address this issue as well as to gain better insights into the potential role of IL-12 in promoting spontaneous cellular immune responses at a localized tissue level. The findings establish that transgenic mice with astrocyte-targeted production of IL-12 but not the IL-12 p40 protein alone can initiate and maintain, in an otherwise unmanipulated mouse, a severe CNS immunoinflammatory disease with activation of a Th1 and cytotoxic T cell-like cellular and cytokine gene response. Additionally, consistent with its known actions, CNS production of IL-12 in these transgenic mice was also associated with evidence of an innate immune response with the presence of numerous NK cells in the brain. It has previously been documented that systemic administration of IL-12 (37–39) or transient gene therapy with, for example, an adenoviral vector expressing IL-12 (34) or IL-12-naked DNA (40) can markedly enhance cell-mediated, IFN-γ producing autoimmune, antitumor or antimicrobial cellular immune responses. Our studies here extend these findings to demonstrate for the first time that localized organ-restricted production of IL-12 can initiate, from an apparently previously unprovoked immune system, a vigorous and destructive immunoinflammatory response.

High expression of IL-12 in several founder generation mice was accompanied by severe meningoencephalomyelitis with premature death, whereas lower expression of IL-12 in the hemizygous GF-IL-12 line produced a much later onset (>4 mo age) of progressive CNS infiltration by immune cells that was not equally penetrant in all offspring. One reason for this might reside at the level of the transgene-encoded IL-12 p40 and IL-12 p35 subunit proteins. Other studies have shown that the secretion of IL-12 p40 monomer is in large excess over the IL-12 heterodimer and can result in the formation of IL-12 p40 homodimers that may act as natural antagonists of IL-12 (30, 41–43). This IL-12 inhibitory loop has been demonstrated in vitro (30, 42) and in different in vivo systems (44) and tumor models (45). Immunoblotting analysis of brain lysates from mice of the GF-IL-12 line suggested there was a large excess in the expression of the IL-12 p40 protein vs the IL-12 heterodimer. This might contribute to a complex interplay between the biological effects of the IL-12 heterodimer on the one hand and possible antagonistic effects mediated by the IL12 p40 protein on the other. However, the fact that mice homozygous for the IL-12 transgenes uniformly developed CNS immunoinflammatory disease with a much earlier age of onset (1–2 mo of age) indicates that doubling the dose of the transgene expression can overcome any competition by the excess IL-12 p40 and favors effector function by the IL-12 heterodimer.

Naive T cells lack responsiveness to IL-12 due to a lack of surface expression of the IL-12Rβ2 component of the IL-12 receptor (46). However, upon priming or activation, these cells acquire expression of IL-12Rβ2 and responsiveness to IL-12. Activated Th1 but not Th2 cells express IL-12Rβ2 and respond to IL-12 (47, 48). In contrast to naive T cells, memory T cells do not require initial priming and can respond to IL-12 directly (49). These observations raise the question of how it is that IL-12 released within the local milieu of the brain can provoke such a powerful immunoinflammatory assault. Part of the answer may lie in the fact that primed or activated T cells are known to move freely into the CNS (50). Although the numbers of such cells in the unmanipulated mouse would be expected to be quite low, circulating primed or activated or memory T cells and NK cells might spontaneously arise in the periphery as a consequence of differential exposure to immune stimulation by environmental factors. Having migrated into the CNS, these cells would then be responsive to IL-12 and begin to produce IFN-γ. IFN-γ is a notable positive modulator that further enhances IL-12 effects on a variety of immune cells including T cells, NK cells, and macrophages (51), and, in particular, can confer IL-12 responsiveness to naive T cells (49). In addition to direct effects on these leukocytes, IFN-γ also up-regulates the expression of a number of key molecules such as adhesion molecules and accessory molecules that facilitate T cell trafficking, Ag presentation, and activation both within (52, 53) and outside (54) the CNS. As we have shown in the present study, consistent with the hallmark action of IL-12, IFN-γ was expressed in the brain of the GF-IL-12 mice and this was associated with markedly increased expression of ICAM-1, VCAM-1, MHC class II, and B7-2. Therefore, IFN-γ may enhance the IL-12 responsiveness of incoming T cells and contribute further to the impetus of the immunoinflammatory cascade in the brain of the GF-IL-12 mice. Consequent to this proposed mechanism, it would be predicted that strategies that either increase or decrease peripheral immune responses would accelerate or reduce, respectively, the CNS immunoinflammatory disease in the GF-IL-12 mice. Recent experiments by us support this notion. Immunization of presymptomatic GF-IL-12 mice with CFA and pertussis toxin resulted in a dramatic acceleration of the onset of immunoinflammatory disease in the brain (S. Lassmann and I. Campbell, unpublished observations).

The progression of the immunoinflammatory process in the CNS of GF-IL-12 mice was accompanied by considerable degenerative disease, highlighting the destructive potential that can be unleashed by activated cellular immune responses in this organ. In severe cases, almost complete obliteration of gray and white matter tissue was observed. As indicated above, accumulating evidence

indicates IL-12 may have a central role in the pathogenesis of a number of experimental $CD4^+$ Th1-mediated autoimmune disease models. Many of the pathogenetic features we have described in the brain of the GF-IL-12 mice are also found in EAE. First, the CNS-infiltrating T cell population in the GF-IL-12 mice consisted of a high number $CD4^+$ T cells, the majority of which were activated and expressed high levels of VLA-4. VLA-4 plays an important role in the trafficking of encephalitogenic $CD4^+$ T cells to the CNS and blocking this molecule significantly ameliorates EAE (55). Second, in the GF-IL-12 mice, the cerebral induction of IFN-γ gene expression and significant up-regulation in the expression of the TNF and IL-1 genes is indicative of a type 1 cytokine response. IFN-γ gene expression was localized to the infiltrating mononuclear cell population and in particular included $CD3^+$ T cells. Type 1 cytokine responses are characteristically found in the brain during EAE where IFN-γ and TNF expression is prominent (52). Third, in the GF-IL-12 mice, expression of a number of accessory molecules that play fundamental roles in T cell migration (ICAM-1 and VCAM-1) (56) and Ag presentation and proliferation (MHC class II, B7-2, and ICAM-1) (57) was increased markedly. With the exception of VCAM-1, which was largely restricted to vascular endothelium, the expression of ICAM-1, MHC class II, and B7-2 was found on a variety of cells including vascular endothelium, infiltrating leukocytes, and cells presumed to be resident microglia. Similar regulation and localization of these accessory molecules are found in EAE (58). In all, the characteristics of the infiltrating $CD4^+$ T cells and the local environment of the brain in the GF-IL-12 mice suggest a possible autoimmune pathogenesis. However, whether or not such an Ag-specific autoimmune process contributes to the development of CNS disease in the GF-IL-12 transgenic mice is unclear at this time and remains to be demonstrated by our ongoing studies.

A second mechanism that deserves comment in considering the immunopathogenesis of neurological disease in the GF-IL-12 mice is possible bystander destruction resulting from the IL-12-driven cellular immune response. Other than the $CD4^+$ T cells noted above, infiltrating $CD8^+$ T cells and NK cells have the potential to unleash considerable cytolytic potential (59). Alternatively, indirect toxicity might also contribute to the brain tissue injury and loss. Possible candidates here might include the cytokines IFN-γ and TNF whose expression was induced in the brain of the GF-IL-12 mice. These cytokines have been implicated as mediators of cellular injury during inflammatory responses in the CNS (60, 61).

The severe calcification in the cerebellum of the GF-IL-12 mice mirror recent observations in transgenic mice expressing IFN-α in astrocytes (62). Since no expression of the IFN-α or IFN-β genes was detectable by RPA in the CNS of the GF-IL-12 mice, it is plausible that IFN-γ may also be responsible for this pathologic feature. IFN-α and IFN-γ have many overlapping cellular actions and both utilize similar JAK/STAT signaling pathways (63). However, MBP-IFN-γ transgenic mice were not reported to have cerebral calcification (64, 65). On the other hand, prominent calcification of muscle tissue occurred in transgenic mice with expression of IFN-γ targeted to the neuromuscular junction (66). The basis for the differences between these transgenic models in terms of calcification is puzzling. However, with regard to the CNS, it might reflect a synergistic interaction between the IFN-γ and IL-12 in the GF-IL-12 mice and the utilization of different intracellular signaling pathways. At least in human NK and T cells, IL-12 and IFN-γ but not IFN-γ alone mediate the activation of STAT4 (67) and thus the combination of these cytokines might evoke cellular responses not exerted by IFN-γ alone. A better understanding of the contribution of IFN-γ to the CNS immune pathology induced by astrocyte expression of IL-12 should come from studies utilizing GF-IL-12 mice with targeted disruption of the IFN-γ gene.

In addition to the increased cerebral expression of the type 1 proinflammatory cytokine genes, a parallel increase in the expression of the gene for the counterregulatory cytokine TGF-β1 was also observed in the brain of the GF-IL-12 mice. Whether this increased TGF-β1 gene expression is mediated by IL-12 directly or is secondary to the increased expression of the other cytokines such as IFN-γ is unknown. In view of the well-described function of TGF-β to antagonize many of the proinflammatory actions of cytokines such as IFN-γ (68), it is reasonable to speculate that the increased TGF-β gene expression observed in the brain of the GF-IL-12 mice might constitute a protective response aimed at reducing the potentially harmful consequences of an otherwise unfettered inflammatory response. Our finding runs contrary to a previous report suggesting IL-12 may down-regulate TGF-β production (69). However, in this report, while Abs to either IL-12 or IFN-γ added to cultures of Ag-stimulated T cells markedly enhanced the production of TGF-β, addition of the cytokines themselves had no significant effect on TGF-β production. Clearly, there are great differences between the two models as well as in the technical approaches used; many of these likely account for the apparently disparate findings with regard to the effect of IL-12 on the expression of TGF-β.

Although, as noted above, under certain conditions the IL-12 p40 protein may act as a physiological antagonist of IL-12 action, the precise function of this molecule in vivo remains an enigma. IL-12 p40 homodimer binds with high affinity to the IL-12 receptor, a process originally found not to mediate a biologic response (41). However, recent studies showing that IL-12 p40 can stimulate rather than inhibit $CD8^+$ T cell differentiation (70) and is chemotactic for macrophages in vitro and in vivo (71) raise the possibility that this molecule may indeed have biologic effector functions. The present study provided an opportunity to further investigate the consequences of chronic IL-12 p40 production in vivo in a tissue-specific compartment. The results indicated no significant pathologic changes in the brain of GF-IL p40 transgenic mice with high levels of astrocyte expression of IL-12 p40 protein. However, these mice showed a delay of onset, but otherwise similar disease course for the development of MOG-induced EAE as wild-type animals. These findings would therefore suggest that at least in the CNS, IL-12 p40 lacks significant biologic effector function and does not switch off an established Ag-driven Th1-mediated immune response.

There is compelling evidence for a pivotal role of IL-12 in the pathogenesis of EAE. Stimulation of MBP-reactive T lymphocytes with IL-12 in vitro increases the severity of adoptively transferred EAE (72). Conversely, treatment of mice with Abs against IL-12 reduced EAE symptoms significantly (14). Furthermore, knockout mice that lack IL-12 production as a consequence of disruption of the IL-12 p40 gene are resistant to EAE (15). Expression of IL-12 has been reported in the CNS of mice with EAE (6). In clinical MS, IL-12 RNA expression was detected in MS plaques, raising the possibility that like EAE, IL-12 may be involved in the pathogenesis of the human demyelinating disease (4). Considering these observations, we used the GF-IL-12 mice to address the question of whether cerebral IL-12 expression could influence the development of EAE. This study revealed a significant acceleration in the onset of disease symptoms of EAE in these mice compared with either wild-type or GF-p40 mice. However, mean disease scores did not differ significantly between both transgenic lines and controls. Similar results were obtained using MBP as an immunogen (data not shown). These findings suggest that astrocyte expression

of IL-12 may influence the dynamics of the recruitment and infiltration by activated MOG-reactive $CD4^+$ T cells, perhaps favoring increased migration of these cells into the brain. Conversely, in the GF-p40 mice where a delay in the onset of EAE was observed, this process may be retarded. In either case, after infiltrating the CNS our data suggest that there is little further regulation of the effector functions of the MOG-reactive $CD4^+$ T cells by locally produced IL-12. In regard to this latter point, it should be noted that the activation and expansion of MOG-reactive $CD4^+$ T cells in EAE occurs in the periphery and is driven by IL-12 (15). IL-12 is also present in the CNS during EAE (6), and it is therefore possible that this endogenous source of IL-12 is sufficient to provide maximal maintenance stimulation of the MOG-reactive $CD4^+$ T cells in the brain. In addition, the increased TGF-β gene expression in the CNS of the GF-IL-12 mice might counteract to some extent any stimulatory actions of IL-12 on the infiltrating MOG-reactive $CD4^+$ T cells. A further consideration is that the EAE immunization schedule used in our study was optimized to give the highest incidence and clinical scores in the (C57BL/6 $\times$ SJL)F_1 hybrid strain background of the GF-IL-12 mice. The generation of vigorous Th1-immune responses can circumvent the need for downstream maintenance or effector stimulatory actions of IL-12 (3). It will therefore be of interest in future studies to determine whether the GF-IL-12 mice are more susceptible to EAE using strategies that employ suboptimal immunization conditions.

In conclusion, we have shown that cerebral expression of the IL-12 heterodimer but not of IL-12 p40 alone can induce a severe neuroimmunological disorder within the CNS. Specifically, IL-12 can initiate and maintain in the CNS of an otherwise naive mouse activated type 1-like $CD4^+CD8^+$ T cell and NK cellular immune and cytokine responses with many features resembling those found in EAE. Moreover, localized expression of IL-12 may facilitate more effective recruitment and infiltration of the CNS by Ag-specific T cells in EAE. The GF-IL-12 mice constitute a novel and interesting new model to dissect the role of IL-12 in the pathogenesis of CNS immune and autoimmune disorders as well as to study basic mechanisms of cellular immunity in the CNS.

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