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IL-6 is crucial for the induction of immune pathology and clinical disease in collagen-induced (3) or spontaneous (4) arthritis and in experimental autoimmune encephalomyelitis (EAE; Ref. 5, 6), which are murine models for the human autoimmune disorders rheumatoid arthritis and MS, respectively. Recent studies have identified a subpopulation of CD4+ Th cells, namely Th17 T-cells, that are key Ag-specific effector T cells in these murine models (4, 7–9). The demonstration that the formation of Th17 T-cells is driven by IL-6 together with TGF-β in vitro and in vivo (7, 10–12) explains, in part, why the murine autoimmune models are critically dependent on IL-6. However, in the case of EAE, studies to pin down the precise role of IL-6 in the induction of EAE have failed to identify a unifying mechanism. One study suggested that IL-6 is necessary for the induction of cerebrovascular adhesion molecules such as VCAM-1, which are crucial for leukocyte trafficking to the CNS in EAE (13); a second study concluded that local, perivascular-produced IL-6 is needed for the induction of EAE and not peripheral IL-6 (5), while a third study proposed that IL-6 is required for autoantigen-reactive CD4+ T cell proliferation during EAE (6). Finally, in a more recent report IL-6 was shown to be a dominant inhibitor of the conversion of conventional T cells to FoxP3-positive T regulatory (reg) cells with the loss of IL-6 resulting in an overwhelming T reg

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8 Abbreviations used in this paper: MS, multiple sclerosis; KO, knockout; EAE, experimental autoimmune encephalomyelitis; T reg, regulatory T cell; MOG, myelin oligodendrocyte glycoprotein; BBB, blood-brain barrier; WT, wild type; LFB, luxol fast blue; RPA, RNase protection assay; Ni, nonimmunized; Cl, BSA-CFA-immunized; Mi, MOG-CFA-immunized.

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response that prevents the induction of Th1 and Th17 effector cells in EAE (14).

During an autoimmune response, the production of IL-6 is known to arise from a large number of different cell types that include infiltrating leukocytes as well as tissue-resident cells (15). Adoptive transfer experiments in a spontaneous arthritis model (4) as well as in AEA (5) suggest that the local cytokine milieu may also play an important role in modulating the development and progression of disease. Due to the plurifunctional nature of IL-6, it is likely that in addition to its potential to control the activity of infiltrating self-reactive Th17 T cells, that this cytokine may influence the pathogenesis of autoimmunity at multiple levels, e.g., via the regulation of adhesion molecule expression on vascular endothelium (16). However, the degree to which localized, tissue-specific production of IL-6 may influence the development of autoimmune pathology within specialized tissues such as the CNS and the mechanisms that are involved remain largely unknown.

To begin to address these outstanding issues, in the present study we investigated myelin oligodendrocyte glycoprotein (MOG) immunization-induced EAE in a unique transgenic mouse model with CNS-restricted, astrocyte-targeted production of IL-6. The so-called GFAP-IL6 transgenic mouse has been characterized extensively (17–21) and shown to develop a progressive inflammatory encephalomyelitis with increased adhesion molecule expression by the cerebrovascular endothelium, loss of integrity of the blood-brain barrier (BBB), reactive astrogliosis and microgliosis, and induction of a number of acute phase response genes including alphal-antichymotrypsin and complement C3. Thus, this model afforded us the opportunity to examine the induction and evolution of an autoimmune response to a target tissue in which the microenvironment contained and was modified by IL-6.

Materials and Methods

Animals

Construction and characterization of the GFAP-IL6 transgenic mice with astrocyte-targeted production of IL-6 was described previously (19). The GFAP-IL6 transgenic and control nontransgenic littermates used in this study were produced by breeding of GFAP-IL6 (C57BL/6 strain) mice of an autoimmune response to a target tissue in which the microenvironment contained and was modified by IL-6.

Determination of IL-6 levels in different brain regions

GFAP-IL6 and WT littermate mice (n = 4 for both genotype groups) at 12 wk of age were euthanized and the cerebrum, cerebellum, and spinal cord were dissected on ice and each lysed and homogenized in complete RIPA lysis buffer (100 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 2 mM PMSF, 50 mM NaF, 1 mM NaVanadate, Protease Inhibitor Cocktail Set III (Calbiochem-Merck), and Phosphatase Inhibitor Cocktail Set II (Calbiochem-Merck)). The homogenates were centrifuged at 14,000 rpm for 20 min at 4°C. The protein in the cleared lysates was quantified using the BCA Protein Assay Kit (Thermo Scientific).

IL-6 protein was measured using a Ready-Set-Go IL-6 ELISA Kit (eBioscience) performed as per the manufacturer’s instructions. The levels were corrected for the amount of total protein added (−100 μg) to the wells.

Induction of EAE and clinical evaluation

For the induction of EAE (see below), 3-mo-old male (n = 16 WT, n = 10 GFAP-IL6) and female (n = 10 WT, n = 17 GFAP-IL6) mice were used. GFAP-IL6 mice at this age did not show any physical signs of neurological disease. An additional cohort of WT and GFAP-IL6 (n = 3 per group) were used as nonimmunized controls. EAE was induced by active immunization with MOG35-55 peptide. On day 0, GFAP-IL6 and WT animals were injected s.c. into the hind flanks with an emulsion of 100 μl MOG35-55 (3 mg/ml) and 100 μl CFA (Sigma-Aldrich) supplemented with 4 mg/ml Mycobacterium tuberculosis H37RA (Difco). In addition, animals received an i.p. injection of 500 ng pertussis toxin (Sigma-Aldrich), which was repeated 2 days after immunization. As controls, another cohort of mice from each genotype (n = 4) was immunized similarly with the exception that MOG peptide was replaced with BSA.

Immunized animals were examined daily and scored for EAE. Initially, we observed that GFAP-IL6 mice showed reduced classical clinical signs (which typically reflect spinal cord involvement), while displaying non-classical clinical signs of ataxia. To better characterize the clinical evolution of the disease in the two genotypes used, an elaborated general clinical score that included the prototypical physical signs of EAE and an additional score for ataxia was used. The general clinical score was assessed for each animal according to the following criteria: 0 = no signs of disease, 1 = loss of tail tonus, 1.5 = moderate hind limb paraparesis, 2 = severe hind limb paraparesis, 3 = hind limb paralysis, 4 = tetraplegia, and 5 = death. For each animal, we determined the time to disease onset (clinical score ≥1 or severe ataxia ≥1), time to peak disease, peak-score, cumulative score (sum of all scores from disease onset to day 50), outcome (final score at day 50), and grade of remission (difference between peak score and outcome). To score for ataxia, a previously reported protocol (22) was used as follows: ataxic signs were scored using a cumulative scale of 4 points, giving 1 point to each of these four physical signs: splayed legs, dragging weight on the trunk rather than on the legs, wobbling, and falling from side to side.

Histology

At peak disease, mice were euthanized under halothane anesthesia and the brain and spinal cord removed immediately and fixed in 4% paraformaldehyde. After embedding in paraffin, sections (5-μm) were cut and deparaffinized in xylene and dehydrated through a series of increasing alcohol solutions. For some processed sections, pretreatment was performed with protease K (Sigma-Aldrich, 10 μg/ml, 15 min at 37°C) to unmask Ag. Slides were then incubated for 1 h at room temperature with primary Abs (anti-CD3) or biotinylated tomato lectin (Sigma-Aldrich). After washing in PBS, a biotinylated secondary Ab (Vector Laboratories; 1:200, 45 min) and HRP-coupled streptavidin (Vector Laboratories; 1:200, 30 min) was used. Nova red (Vector Laboratories) was applied as the immunoperoxidase substrate according to the manufacturer’s instructions. Sections were counterstained with hematoxylin (Sigma-Aldrich).

Dual-label staining on paraffin sections was performed using an additional primary Ab of a different species, which was detected with a secondary alkaline phosphatase-coupled Ab and Vector blue (Vector Laboratories) as the second color substrate. Counterstaining was omitted for dual label immunohistochemistry. For apoptotic cell counting a TUNEL assay (Roche) was performed on paraffin slides according to the manufacturer’s instructions.

Analysis of demyelination and cell counting

Sections were stained either with H&E for routine histological analysis or with luxol fast blue (LFB) for myelin evaluation. Conventional stained sections were examined by a DM4000B bright field and fluorescence microscope (Leica). Bright field images were acquired using a Leica DFC480 camera and Leica Firecam 1.7.1 software (Leica). To quantify infiltrated areas of longitudinal spinal cord sections, images of H&E stains were examined with Image analysis software AnalySIS 3.0 (Softimaging). The total area of the spinal cord cross-section and infiltrated areas were measured by an observer blinded to the sample identity. Histological analyses were performed on three sections (50 μm apart) of each brain from three mice per group.

RNase protection assay (RPA)

The cerebellum and spinal cord were collected (as described above) and frozen in liquid nitrogen pending isolation of the RNA. Total RNA was isolated using TRIZol (Sigma-Aldrich). For RPA (see below), 5 μg of total RNA was used in each analysis.

The construction and characterization of the multiprobe sets used in the RPA, namely for host-response (iNOS, Mac1, A20, ICAM 1, EB22/5, CCL-12/MCP-5, IFN-γ, IL-6, RAANTE/CCL-5), has been described elsewhere (20, 23, 24).

IL-17 mRNA determination by quantitative real-time-PCR

Total RNA isolated as described above was reverse-transcribed into cDNA by using SuperScript II Reverse Transcriptase (Invitrogen). Real-time quantitative PCR assays were performed using SYBRgreen. The composition
of the reaction mixture was as follows: 2 μl of cDNA corresponding to 300 ng of total RNA, 100 nM of each primer, 1 × SYBR Green PCR Master Mix (Applied Biosystems) in a total volume of 25 μl. Samples were analyzed simultaneously for GAPDH mRNA as the internal control, and quantitative expression values were extrapolated from separate standard curves. Each sample was assayed in triplicate and normalized to GAPDH. Primer sequences used to amplify the IL-17A and GAPDH cDNA were: IL-17 sense 5′-AAG CGACAGGCGCATCAGCC-3′ and IL-17 anti-sense 5′-GGAAACGTTTAG GTACTTCTGAG-3′; GAPDH sense 5′-TCAACACGGCTGCACTGTG-3′ and GAPDH anti-sense 5′-GACTCCACGACTTCAGC-3′.

Flow cytometry analysis

The cerebellum from animals with peak disease was excised and placed in ice-cold PBS buffer solution before extrusion through a coarse 100-μm metal cell strainer. The subsequent homogenate was digested for 60 min in PBS with deoxyribonuclease (0.005 g/ml, Sigma-Aldrich) and collagenase IV (0.05 g/ml Sigma-Aldrich). Digested samples were incubated at 37°C in a humidified atmosphere of 5% CO₂, and were resuspended every 15 min. Digestion was stopped with 10% FCS and the homogenate was passed through a 70-μm cell strainer (BD Biosciences). The pellet obtained after 10 min centrifugation at 340 × g was dissolved in 30% Percoll (GE Healthcare). Subsequently, the 30% Percoll homogenate mix was layered over 80% Percoll. Leukocytes were collected from the 30%/80% interface after centrifugation (1140 × g) for 25 min at room temperature. The collected cells were washed in PBS and blocked with CD16/CD32 (BD Biosciences) Ab. Viable cells were counted using trypan blue exclusion. Isolated leukocytes were incubated with fluorochrome-conjugated Abs to detect CD8, CD4, Ly6G/GR1, CD11b, CD11c, CD45, B220, and the appropriate isotype controls (all from BD Biosciences). Samples were run on a FACSAria (BD Biosciences) and analyzed with Flow Jo (Tree Star).

Statistical analysis

For all clinical evaluations, a nonparametric U test was performed following the recommendations described elsewhere (25). For histological, IL-6 protein, RNA levels and FACS analyses, a two-way ANOVA was performed. In all cases, a statistical analysis software package (Statistical Program for Social Sciences v 13.0; SPSS) was used. p < 0.05 was considered significant throughout all analyses.

Results

IL-6 is present in the cerebellum but not detectable in the cerebrum or spinal cord from GFAP-IL6 mice

Previous characterization of the histopathological alterations in the CNS of the GFAP-IL6 transgenic mice used in the current study revealed that changes including loss of BBB integrity and gliosis were present at 3 mo age and were localized predominantly in the cerebellum compared with other brain regions (18, 19). Because the presence of IL-6 in different brain regions of these mice has not been studied, it was necessary to determine the levels of IL-6 in lysates of the cerebrum, cerebellum, and spinal cord from WT or GFAP-IL6 mice. In lysates of all three CNS tissues from WT mice, the level of IL-6 was close to the detection limit of the assay (Fig. 1). Similarly, IL-6 was barely detectable in lysates of cerebrum and spinal cord from GFAP-IL6 mice. However, in the cerebellum of GFAP-IL6 mice, IL-6 was detectable at significantly higher levels than the other brain regions. These findings indicated that, in the GFAP-IL6 mice, the transgene-encoded IL-6 production is largely restricted to the cerebellum and thus correlates with the previously reported neuropathology that is predominant in this region of the CNS (18, 19).

GFAP-IL6 mice develop an altered clinical course following MOG immunization

To determine whether or not astrocyte-targeted production of IL-6 influenced the clinical course of EAE, GFAP-IL6 and WT littermate mice were immunized with MOG35-55 peptide and observed over 50 days (Fig. 2). Nonimmunized (Ni)-WT and Ni-GFAP-IL6 mice were clinically indistinguishable (Fig. 2B). BSA-CFA-immunized (Ci) WT mice showed no physical changes compared with control animals while Ci-GFAP-IL6 mice showed a mild ataxia that developed late (~45 days) following immunization (Fig. 2B). As expected, MOG-CFA-immunized (Mi)-WT mice showed a typical EAE clinical course, with an onset of initial signs at 8.5 days, a peak of clinical signs around 15 days postimmunization, followed by a gradual reduction in clinical severity with incomplete remission at 50 days postimmunization (Fig. 2, A and C). However, in contrast to Mi-WT mice, Mi-GFAP-IL6 mice showed significantly reduced signs of classical EAE over the entire period of study, but showed an earlier onset of disease in the form of ataxia at 7.1 days (Fig. 2C). The Mi-GFAP-IL6 mice did not develop hind limb paralysis but, rather, exhibited signs of severe ataxia that prompted us to use a different clinical scoring system that allowed more accurate quantification of the degree of physical impairment in these animals. Based on this assessment compared with Mi-WT controls, Mi-GFAP-IL6 mice showed significantly increased ataxia over the entire period of study (Fig. 2B).

FIGURE 1. IL-6 production in GFAP-IL6 mice was restricted to the cerebellum. The IL-6 content in lysates from different CNS regions, namely cerebrum, cerebellum, and spinal cord, of WT and GFAP-IL6 mice was analyzed by ELISA as described in the Materials and Methods section. Significant levels of IL-6 were detected only in cerebellum of GFAP-IL6 mice (for statistical significance, *, p < 0.001 vs WT).

FIGURE 2. GFAP-IL6 mice developed an atypical course of disease characterized by severe ataxia without signs of limb paralysis. GFAP-IL6 (n = 23) and WT (n = 22) mice were immunized with 300 μg of MOG peptide emulsified in CFA (MOG-immunized, Mi-) and injected i.p. with 500 ng of pertussis toxin at days 0 and 2. A control cohort of WT and GFAP-IL6 mice (n = 4 for each group) received 300 μg of BSA (CFA immunized, Ci-). A group of naive (nonimmunized, Ni-) mice (n = 3 per genotype) was also used for comparisons. A, Clinical EAE evaluation in MOG immunized WT (Mi-WT) and GFAP-IL6 mice. B, Ataxia scores (assessed as described in the Materials and Methods section) in nonimmunized (Ni), CFA immunized (Ci), and MOG-immunized (Mi) WT and GFAP-IL6 mice. Note that ataxia was unable to be scored in Mi-WT at peak disease due to bilateral hind limb paralysis. C, Disease incidence, mortality, and average disease onset (either EAE score ≥1 or ataxia) scores in Mi-WT and Mi-GFAP-IL6 mice. For statistical significance, *, p < 0.05 vs WT.
In summary, GFAP-IL6 mice developed an atypical EAE disease after MOG immunization that suggested primary involvement of the cerebellum rather than the spinal cord.

The pathological lesions induced by MOG immunization in GFAP-IL6 mice are largely restricted to the cerebellum

To determine the basis for the atypical physical phenotype presented by the Mi-GFAP-IL6 mice, we examined the brain and the spinal cord by routine histology at disease peak (day 20 postimmunization). No significant difference in the histological appearance of the spinal cord was observed in Ci-WT mice (Fig. 3, A and B) compared with Ci-GFAP-IL6 mice (Fig. 3, E and F). By contrast, in Mi-WT mice in the white matter of the spinal cord, multifocal perivascular infiltrates were observed by H&E staining (Fig. 3C, arrows) and corresponded with extensive areas of demyelination as evidenced by the absence of LFB staining (Fig. 3D, arrows). However, in Mi-GFAP-IL6 mice far fewer infiltrates were observed (Fig. 3G) and correspondingly little, if any, demyelination was observed (Fig. 3H). Quantitative analyses of the lesion area as a proportion of the total white matter showed a significant reduction in the Mi-GFAP-IL6 mice compared with Mi-WT mice. In addition, in Mi-GFAP-IL6 mice the number of lesions was reduced significantly (74.56 ± 9.22 vs 33.00 ± 5.48 for Mi-WT vs Mi-GFAP-IL6, respectively) as was the average size of the inflammatory lesions (744.51 ± 141.21 μm² vs 203.74 ± 47.03 μm² for Mi-WT vs Mi-GFAP-IL6, respectively). In all, these findings showed that the extent of the inflammatory lesion formation and tissue destruction due to immunization with MOG peptide was reduced significantly in spinal cord from GFAP-IL6 mice compared with WT littermate controls.

As noted above, the severe ataxic phenotype observed in Mi-GFAP-IL6 mice suggested the involvement of the cerebellum. Routine H&E examination of the cerebellum of Ci-GFAP-IL6 mice (Fig. 4E) revealed that in comparison with Ci-WT animals (Fig. 4A), there was increased cellularity of the gray and white matter regions as well as the presence of perivascular leukocyte accumulations particularly in the cerebellar sulci (Fig. 4E, arrows). Examination of the myelin integrity also revealed alterations present in cerebellum from the Ci-GFAP-IL6 mice (Fig. 4F) compared with Ci-WT animals (Fig. 4B) with the myelin appearing less well-organized and focal areas of pallor visible. In Mi-WT mice, the structural integrity of the cerebellum remained largely intact (Fig. 4, C and D). Leukocyte infiltrates were observed, but these were mostly found to be associated with blood vessels (Fig. 4C, arrow), while LFB staining revealed limited disruption of the myelin proximal to the perivascular infiltrates (Fig. 4D, arrow). Compared with Mi-WT mice, cerebellum from Mi-GFAP-IL6 mice had extensive hypercellular lesions corresponding to the accumulation of large numbers of inflammatory cells which were diffusely located throughout the white matter (Fig. 4G) and associated with widespread disintegration of the myelin tracts (Fig. 4H, arrowheads).

In summary, these findings indicated that MOG immunization induced much more severe and extensive inflammation and demyelination in the cerebellum of GFAP-IL6 mice compared with WT littermates. This pathological presentation in the Mi-GFAP-IL6 mice correlated with the clinical signs of severe ataxia noted in these animals.

The distribution, numbers, and phenotype of leukocytes are altered markedly in the CNS of Mi-GFAP-IL6 mice

The results presented above indicated that the localized production of IL-6 in the cerebellum of GFAP-IL6 mice redirects the EAE inflammatory response to MOG immunization and consequent tissue destruction away from the spinal cord to the cerebellum. We next investigated how the localized production of IL-6 in the cerebellum influenced the distribution, numbers and phenotype of the infiltrating leukocyte populations.
To better characterize the leukocyte populations in situ, immunohistochemistry was performed to identify T cells and microglia/macrophages (Fig. 5). In the spinal cord, lectin-positive cells with a similar number and distribution corresponding to vascular endothelial cells and microglia were observed in both Ci-WT (Fig. 5A) or Ci-GFAP-IL6 (Fig. 5B) mice. After EAE induction, multiple lesions containing macrophage and T cells were observed in the spinal cord of Mi-WT mice (Fig. 5C); however, in clear contrast, there was a paucity of these mononuclear cell infiltrates present in the spinal cord of Mi-GFAP-IL6 mice (Fig. 5D). In the cerebellum, clear evidence of microglial activation (Fig. 5F) and modest T cell accumulation (Fig. 5H) was evident in Ni-GFAP-IL6 mice, whereas no microglial activation was observed in the spinal cords of either Ci-WT (A) or Ci-GFAP-IL6 (B) mice. Induction of EAE increased the number of perivascular infiltrates formed by CD3+ cells surrounded by lectin positive microglia/macrophages in spinal cords of Mi-WT mice (C). However, almost no infiltrates were observed in the spinal cords of Mi-GFAP-IL6 (D). Conversely, in the cerebellum there were increased numbers of lectin positive macrophage/microglia in Ni-GFAP-IL6 (F) and Ci-GFAP-IL6 mice (J), which was further increased markedly in Mi-GFAP-IL6 (N) whereas only discrete foci of lectin-macrophage/microglia were observed in Mi-WT mice (M) compared with Ni-WT (E) or Ci-WT (I) animals. No CD3-positive cells were observed in either Ni-WT (G) or Ci-WT (K) mice. In contrast, scattered CD3-positive T cells were found in the cerebellum of Ni-GFAP-IL6 (H) mice, and the number of these cells further increased after CFA treatment, in particular in the cerebellar sulci of Ci-GFAP-IL6 mice (L). At peak EAE, cerebellum from both Mi-WT and Mi-GFAP-IL6 mice showed the presence of CD3-positive T cells (O and P). At higher-magnification the CD3-positive T cells were seen to be localized in a perivascular fashion in the inflammatory foci of Mi-WT mice (O). In contrast, CD3-positive cells were found scattered throughout the parenchyma of Mi-GFAP-IL6 mice (P). Scale bar: 25 μm.

FIGURE 5. Altered distribution and increased accumulation of macrophage/microglia and T cells in the cerebellum of Mi-GFAP-IL6 mice. Paraffin-embedded sections of spinal cord (A–D) or cerebellum (E–P) were incubated with tomato lectin (to identify macrophage/microglia; A–F, I, J, M–P) or anti-CD3 (C, D, G, H, K, L, O, P) and stained as described in the Materials and Methods section. No microglial activation was observed in the spinal cords of either Ci-WT (A) or Ci-GFAP-IL6 (B) mice. Induction of EAE increased the number of perivascular infiltrates formed by CD3+ cells surrounded by lectin positive microglia/macrophages in spinal cords of Mi-WT mice (C). However, almost no infiltrates were observed in the spinal cords of Mi-GFAP-IL6 (D). Conversely, in the cerebellum there were increased numbers of lectin positive macrophage/microglia in Ni-GFAP-IL6 (F) and Ci-GFAP-IL6 mice (J), which was further increased markedly in Mi-GFAP-IL6 (N) whereas only discrete foci of lectin-macrophage/microglia were observed in Mi-WT mice (M) compared with Ni-WT (E) or Ci-WT (I) animals. No CD3-positive cells were observed in either Ni-WT (G) or Ci-WT (K) mice. In contrast, scattered CD3-positive T cells were found in the cerebellum of Ni-GFAP-IL6 (H) mice, and the number of these cells further increased after CFA treatment, in particular in the cerebellar sulci of Ci-GFAP-IL6 mice (L). At peak EAE, cerebellum from both Mi-WT and Mi-GFAP-IL6 mice showed the presence of CD3-positive T cells (O and P). At higher-magnification the CD3-positive T cells were seen to be localized in a perivascular fashion in the inflammatory foci of Mi-WT mice (O). In contrast, CD3-positive cells were found scattered throughout the parenchyma of Mi-GFAP-IL6 mice (P). Scale bar: 25 μm (A–D), 25 μm (E–N), or 8 μm (O and P).
mice compared with Ni-WT (Fig. 5, E and G) animals. After CFA immunization there was negligible microglial activation (Fig. 5f) or T cell infiltration (Fig. 5k) in WT mice. However, while Ci-GFAP-IL6 animals showed a similar degree of microglial activation and morphology as compared with Ni-GFAP-IL6 (Fig. 5j) mice, increased numbers of T cells were observed in Ci-GFAP-IL6 mice, in particular in the cerebellar sulci (Fig. 5l). This latter finding is consistent with the routine histological findings noted above indicating that the cerebellum of the GFAP-IL6 mice favors the accumulation and activation of inflammatory cells following peripheral immune activation. In Mi-WT mice with EAE, perivascular infiltrates consisting of T cells surrounded by activated microglia/macrophages were located in the white matter of the cerebellum (Fig. 5, M and O). In contrast to Mi-WT mice, the cerebellum of Mi-GFAP-IL6 mice showed extensive accumulation of large numbers of lectin-positive macrophage/microglia, which were distributed throughout the white matter (Fig. 5n). These macrophages had a more rounded morphology and were more densely stained compared with the more ramified morphology of the macrophage/microglia in the Mi-WT mice and were accompanied by extensive T cell infiltration into the white matter (Fig. 5p). Overall, these findings revealed that while the Mi-GFAP-IL6 mice displayed reduced disease scores and exhibited little spinal cord pathology, there was a marked increase in the distribution and severity of the EAE inflammatory response in the cerebellum of these mice.

To aid further in the phenotypical characterization of the infiltrating cell populations, we isolated cells from Mi-GFAP-IL6 and Mi-WT (Fig. 6a) cerebellum at peak disease and performed flow cytometric analysis. In support of the immunohistochemical results, Mi-GFAP-IL6 animals displayed a 10-fold increase in all leukocyte subsets examined (Fig. 6a). Although, the relative proportion of T cell subsets (CD4+ and CD8+), dendritic cells, (CD45+/CD11c−) and macrophages (CD45+/CD11b+) remained similar between the two strains of immunized mice, there was an approximate 2-fold increase in the percentage of neutrophils (Ly6G+/CD11c−) and B220+/CD11c− cells (B-cells) isolated from Mi-GFAP-IL6 transgenic animals than from Mi-WT controls (Fig. 6b).

Inflammation-related gene expression in GFAP-IL6 and WT mice during EAE

To delineate further the nature of the immune response in the spinal cord and cerebellum of Mi-WT vs Mi-GFAP-IL6 mice, the level of mRNA transcripts for a number of key cytokine and chemokine genes was analyzed by RPA. In the spinal cord of Ci-WT mice, the levels of all the examined cytokine and chemokine transcripts were very low or undetectable (Fig. 7, A and B). A similar pattern of expression was seen in the Ci-GFAP-IL6 mice with the exception of a small but significant increase in both the IL-6 and CCL12 mRNAs. In general, this pattern of expression was maintained in the preclinical phase of disease in both Mi-WT and Mi-GFAP-IL6 mice. However, at the peak phase of disease, the expression of the TNF, TGF-β, CCL12, IL-6, IL-12, and IFN-γ mRNAs was increased markedly in Mi-WT mice and were significantly higher than were found in the Mi-GFAP-IL6 mice (Fig. 7b). These differences were particularly striking in the case of TNF, CCL5, and IFN-γ. Finally, during the chronic phase of disease, the levels of the TNF, CCL5, and IFN-γ but not the TGF-β, CCL12, and IL-6 mRNA transcripts decreased significantly in Mi-WT mice and showed a similar change in the Mi-GFAP-IL6 animals. In all, these findings showed that the levels of expression of a number of cytokine and chemokine genes was significantly reduced in the spinal cord of Mi-GFAP-IL6 mice, consistent with the reduced numbers of leukocytes and tissue destruction.

The results for the cerebellum (Fig. 7, C and D) were similar to those of the spinal cord in CFA-treated mice. Thus, low levels of all the examined cytokine and chemokine mRNA transcripts were observed in Ci-WT mice, whereas IL-6 and CCL12 mRNA transcripts were increased in the Ci-GFAP-IL6 mice. However, in contrast to the spinal cord, TNF and TGF-β were also increased in the cerebellum of the GFAP-IL6 mice. Essentially the same pattern of expression was observed in both genotypes in the preclinical phase of disease. In contrast, the expression of all the cytokine and chemokine mRNAs studied was significantly increased in Mi-WT mice at the peak phase of disease, while only TNF and CCL5 mRNAs were increased clearly in the Mi-GFAP-IL6 mice compared with the preclinical phase. Finally, during the chronic phase of disease, the levels of all transcripts were significantly reduced from their peak values.

As noted above, it is now clear that CD4+ Th17 cells are crucial effector T cells in the development of MOG-EAE. Therefore, we compared the levels of IL-17 mRNA in the cerebellum of Mi-WT and Mi-GFAP-IL6 mice. Initially, RPA was used for this purpose however, due to extremely low levels of IL-17 mRNA, we could barely detect this transcript. As an alternative, we used the more sensitive real time-PCR technique for this analysis. As shown in Fig. 7e the level of IL-17 mRNA was significantly higher in the cerebellum of Ci-GFAP-IL6 mice compared with Ci-WT littermates. During the peak disease phase, there was a significant increase of IL-17 mRNA in Mi-WT mice compared with Ci-WT, while the level of IL-17 mRNA in Mi-GFAP-IL6 mice was increased to similar levels as seen in the Ci-GFAP-IL6 controls.

To determine further the nature of the inflammatory response in the cerebellum of the Mi-GFAP-IL6 mice, an analysis of the expression of several host response-related genes was performed (Fig. 8, A and B). The levels of the ICAM-1, MAC1, EB22/5, and GFAP mRNA transcripts were all significantly increased in Ci-GFAP-IL6 mice compared with Ci-WT littermates (Fig. 8b). This

FIGURE 6. Markedly increased accumulation of various leukocyte subsets in the cerebellum of Mi-GFAP-IL6 mice. Tissue leukocytes were isolated and analyzed by flow cytometry as described in the Materials and Methods. Cerebellar cell counts (A) and relative ratios (B) of CD4+, CD8+, CD45+CD11b+, CD45+CD11c+, Ly6G+CD11c− and B220+ cell populations in Mi-WT and Mi-GFAP-IL6 mice. For B, quantification of each specific cell population is shown as a percentage of the total number of leukocytes gated according to side- and forward-light scatter. A near 10-fold increase of all leukocyte subsets was found in Mi-GFAP-IL6 mice compared with Mi-WT animals (A). However, only the relative proportion of Ly6G+CD11c− granulocytes and B220+ B cells was found to be increased in Mi-GFAP-IL6 compared with Mi-WT mice (B). For statistical significance, *, p < 0.05 vs WT.
pattern of expression of these host response-related mRNA transcripts was maintained in Mi-GFAP-IL6 and Ci-WT mice during the preclinical phase of disease. At peak disease in Mi-GFAP-IL6 mice, with the exception of EB22/5 mRNA, which was increased further, the ICAM-1, MAC1, and GFAP mRNAs remained elevated to similar levels while the levels of all these transcripts were significantly increased in the Mi-WT mice. Additionally, during the peak phase of disease, the levels of the iNOS and A20 mRNAs were increased significantly in both Mi-WT and Mi-GFAP-IL6 mice, however the level of iNOS mRNA lower in Mi-GFAP-IL6 mice when compared with Mi-WT animals, although this did not reach statistical significance. During the chronic phase of disease, the levels of all these mRNAs were reduced to almost control levels in the Mi-WT mice while ICAM-1, MAC1, EB22/5, and GFAP remained elevated in the Mi-GFAP-IL6 animals.

Discussion

IL-6 has emerged as a crucial cytokine in the pathogenesis of a number of experimental and human autoimmune diseases (15). IL-6 is not only multifunctional, but during the course of an autoimmune response, the production of this cytokine arises from diverse cellular sources both in the periphery and the target tissue. Although it is now known that IL-6 is crucially involved in the development of autoreactive CD4+ Th17 effector T cells in various experimental autoimmune disease models (7, 11, 12), the relative contribution of IL-6 production in the target tissue to development of autoimmunity remains uncertain. In EAE (26) and in MS (27), IL-6 or IL-6 transcripts are elevated in the CNS, which likely reflects, in part, production by intrinsic cells to the CNS such as the astrocytes and microglia (28). In this study, we assessed the role of such tissue-localized production of IL-6 in...
the induction and evolution of MOG-EAE, using transgenic mice with astrocyte-targeted and largely cerebellum-restricted production of IL-6. The results revealed a grossly exaggerated involvement of the cerebellum in Mi-GFAP-IL6 mice which developed severe ataxia concomitant with increased immune pathology and demyelination in the cerebellum. As is well known, in WT mice, MOG-EAE is characterized by an ascending paralysis reflecting the primary targeting of the autoimmune response to the spinal cord and to a lesser degree the cerebellum. However, and somewhat unexpectedly, there was only very mild inflammation in the spinal cord of Mi-GFAP-IL6 mice with EAE. The few inflammatory lesions present were small in size and accompanied by scant demyelination. Based on these findings, we conclude that the tissue-localized transgenic production of IL-6 acts as a potent stimulus to target the autoimmune response and may create a "sink" that retards leukocyte trafficking to normally preferred antigenic sites.

The mechanisms underlying the almost exclusive targeting and expansion of the EAE lesion development to the cerebellum in the Mi-GFAP-IL6 mice are likely to be complex. Astrocyte-targeted production of IL-6 in the cerebellum establishes a proinflammatory milieu that progresses with the age of the animals (19, 21). Although at the age used for the current studies, the GFAP-IL6 mice appeared physically normal and had modest pathological alterations affecting the cerebellum, the BBB integrity is compromised in the cerebellum but not in the spinal cord or in the cerebral hemispheres (18). Evidence of early inflammatory changes were also present in the cerebellum but not spinal cord of the GFAP-IL6 mice and included astrocytosis and microgliosis as well as increased expression of some proinflammatory cytokine genes. The intensity of this inflammatory process was increased after CFA immunization in these mice which produced mild clinical signs. These findings together with the grossly exaggerated accumulation of inflammatory cells following MOG-immunization indicate that the local milieu of the tissue conditioned by IL-6 can dramatically influence the development of both autoantigen-specific as well as nonspecific immune responses and associated pathology.

It is well documented that changes in the CNS microenvironment, for example, following induction of nerve injury (29–31) or resulting from cortical cryolesion in rats (32) targets EAE inflammation to the lesion site. Similar to the GFAP-IL6 mice, focal cortical cryolesion is associated with localized breakdown of the BBB and the induction of a proinflammatory state with increased expression of a number of chemokine genes including CCL5 and CCL12 (32). However, as we have shown (supplementary Fig. 1), in contrast to Mi-GFAP-IL6 mice, cryolesion-EAE is accompanied by classical ascending spinal cord inflammatory lesions with demyelination and paralysis. Thus, a compound brain lesion caused by cryoinjury induced as described (33), while it can target EAE inflammation, does not cause the spinal cord sparing effects that were observed in the Mi-GFAP-IL6 mice and thus argues for a specific effect of IL-6 in modifying the cerebellar microenvironment. This idea that IL-6 induces a unique inflammatory environment in the cerebellum of the GFAP-IL6 mice is further reinforced by our finding that the transgenic production of the proinflammatory cytokine IL-12 in the cerebellum, while causing an earlier onset of disease, did not significantly alter the development of EAE (supplementary Fig. 1 and Ref. 34).

Mi-GFAP-IL6 mice showed a large increase in the number of mononuclear cells accumulating in the cerebellum, which led to more severe demyelination when compared with Mi-WT animals. It is likely that the increased numbers of leukocytes in the cerebellum of the Mi-GFAP-IL6 arose from increased recruitment from the circulation and/or proliferation locally. IL-6 increases the levels of cellular adhesion molecules such as ICAM-1 on endothelial cells, which is crucial for tissue leukocyte recruitment mediated by this cytokine (16). Vascular ICAM-1 and VCAM-1 levels are increased in EAE and have been shown to mediate adhesion of lymphocytes to the inflamed cerebral vessels in the spinal cord (35, 36). The loss of this interaction prevents the development of EAE and is associated with a marked reduction in the numbers of leukocytes in the CNS (37). The microenvironment of the cerebellum in the GFAP-IL6 mice is characterized by chronic activation of the vascular endothelium. In particular, the levels of the cellular adhesion molecules ICAM-1 and VCAM-1 as well as a number of integrins is increased significantly on vascular endothelium (19, 38). This pre-existing activation state of the vascular endothelium in the cerebellum of the GFAP-IL6 mice would be expected to favor the increased adhesion and subsequent transendothelial migration of leukocytes following MOG-immunization.

The BBB is a specialized and unique barrier to the CNS that regulates the flow of macromolecules and leukocytes into this tissue. In adult GFAP-IL6 mice the BBB is impaired in the cerebellum and is leaky for the life of the animal (18, 19). Therefore, it could be argued that this loss of BBB integrity would allow for

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**FIGURE 8.** Expression of selected inflammatory host response-related genes is increased in the cerebellum of GFAP-IL6 mice. Total RNA was isolated from the cerebellum of mice at the times shown and 5 μg used for analysis by RPA as specified in the Materials and Methods section. RPA for selected host response-related mRNA transcripts in cerebellum from WT and GFAP-IL6 mice (A) were quantified by densitometry and normalized to the L32 loading control (B). GFAP-IL6 animals consistently presented with an increased expression of many of the genes studied that was already evident in CFA immunized mice. After EAE immunization, Mi-WT, and to a lesser extent Mi-GFAP-IL6 mice, showed an increase in the expression of genes studied. However, overall transcript levels were found to be higher in Mi-GFAP-IL6 mice compared with Mi-WT. For statistical significance, *p < 0.05 vs WT or **p < 0.05 vs Ci mice.

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9 The online version of this article contains supplemental material.
more indiscriminate entry of immunocompetent T cells and other leukocytes into the brain of the Mi-GFAP-IL6 mice. Indeed, our results showed a clear increase in the number of T cells present in the cerebellum of Ci-GFAP-IL6 mice but not Ci-WT mice. However, the role of the BBB in this context is not so clear. It has been shown that activated T cells are capable of freely entering the CNS after migrating across the healthy intact BBB (39, 40). In EAE, disease severity was reported to correlate closely with the degree of BBB permeability suggesting that loss of BBB integrity is integral to the development of EAE (41). However, the relationship between BBB integrity and inflammatory cell accumulation in the CNS in EAE is not straightforward. Areas of the CNS of naive mice that have constitutively greater BBB permeability do not necessarily overlap with lesion distribution in EAE suggesting that BBB integrity in of itself does not determine lesion establishment (42). Moreover, accumulation of inflammatory cells in the brain parenchyma in EAE can occur before detectable loss of BBB integrity (42).

Another plausible mechanism that could contribute to the targeted recruitment of inflammatory cells to the cerebellum in the Mi-GFAP-IL6 mice may involve chemokines signaling. In this regard, we identified CCL12 (MCP-5) mRNA as being markedly increased in the cerebellum of the GFAP-IL6 mice compared with WT. In addition, the expression of some other chemokine genes including CXCL10, CCL2, and CCL5 is elevated in the cerebellum of the GFAP-IL6 mice (43) however, in comparison with CCL12, the level of expression of these other chemokine transcripts is considerably lower (I.L. Campbell, unpublished observation). Interestingly, CCL12 gene expression was also increased in the cerebellum and spinal cord of WT mice before onset of EAE and increased further during peak disease suggesting there may be a role for this chemokine in leukocyte trafficking to the CNS in WT mice with EAE. Studies in vitro have shown that CCL12 is a potent chemoattractant for monocyte/macrophages (44, 45). This function of CCL12 appears to be mediated primarily by the receptor CCR2 (45). Studies using CCR2 KO mice have failed to produce a unifying conclusion as to the function of CCR2 signaling in MOG-EAE. Initial reports concluded that CCR2 KO mice were resistant to EAE and exhibited a marked disease reduction (46, 47), however this was challenged in a later study (48). Given the well-known promiscuity of this chemokine receptor family (49, 50), it is conceivable the function of CCL12 in the pathogenesis of EAE could be mediated via alternative CCRs but this, as well as the functions of CCL12 in EAE, remain to be determined.

Adoptive transfer studies in IL-6 KO mice suggest that local production of IL-6 in the CNS is required for the optimal development of EAE through the local priming of vascular function e.g., by up-regulation of adhesion molecules (13). It is now clear that IL-6 together with TGF-β can drive the formation of effector Th17 cells (7, 10, 12) and these cytokines are essential for the induction of EAE. Interestingly, in addition to IL-6, we observed that the level of TGF-β gene expression was also increased in the cerebellum of the Mi-GFAP-IL6 mice. This raised the question as to whether the local production of these cytokines might also modulate effector T cell function during an autoimmune response. The spontaneous development of arthritogenic Th17 cells and augmentation of disease by zymosan in SKG mice was shown to be dependent on the synergistic interaction between T cell-derived and non-T cell-derived IL-6 (4). However, our findings here showed that, while elevated, the CNS levels of IL-17 mRNA were little altered between Ci-GFAP-IL6 and Mi-GFAP-IL6 mice while increased IFN-γ mRNA levels at peak disease were similar for Mi-WT and Mi-GFAP-IL6 mice. Furthermore, although increased significantly in number in the Mi-GFAP-IL6 mice, the relative proportions of CD4+ T cells and CD11b+ microglia/macrophages were similar between Mi-WT and Mi-GFAP-IL6 mice. The findings suggest that the major impact of site-specific tissue production of IL-6 is in the targeting of the inflammatory response and not the subsequent modulation of effector T cell function in the lesion.

In this study, we have shown that localized transgenic production of IL-6 in the cerebellum targets and enhances the inflammatory response directed against a CNS-specific autoantigen. Moreover, this local IL-6 milieu created a leukocyte “sink” that diverted trafficking from and inflammation in the normally dominant antigenic site of the spinal cord. The mechanisms underlying these responses to IL-6 in the cerebellum likely include the induction of specific cytokines and chemokines as well as increased vascular activation and loss of integrity of the BBB. These findings establish for the first time that intrinsic production of IL-6 in a target tissue can dramatically target and augment the development of an autoimmune response.

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Disclosures
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References


