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Gp130-Dependent Astrocytic Survival Is Critical for the Control of Autoimmune Central Nervous System Inflammation

Fahad Haroon,* Katrin Drögemüller,* Ulrike Hänsel,* Anna Brunn,† Dirk Reinhold,‡ Gopala Nishanth,* Werner Mueller,§ Christian Trautwein,¶ Matthias Ernst,‖ Martina Deckert,† and Dirk Schlüter*

Astrocytes are activated in experimental autoimmune encephalomyelitis (EAE) and have been suggested to either aggravate or ameliorate EAE. However, the mechanisms leading to an adaptive or protective effect of astrocytes on the course of EAE are incompletely understood. To gain insight into the astrocyte-specific function of gp130 in EAE, we immunized mice lacking cell surface expression of gp130, the signal-transducing receptor for cytokines of the IL-6 family, with myelin oligodendrocyte glycoprotein35–55 peptide. These glial fibrillary acid protein (GFAP)-Cre gp130fl/fl mice developed clinically a significantly more severe EAE than control mice and succumbed to chronic EAE. Loss of astrocytic gp130 expression resulted in apoptosis of astrocytes in inflammatory lesions of GFAP-Cre gp130fl/fl mice, whereas gp130fl/fl control mice developed astrogliosis. Astrocyte loss of GFAP-Cre gp130fl/fl mice was paralleled by significantly larger areas of demyelination and significantly increased numbers of CD4 T cells in the CNS. Additionally, loss of astrocytes in GFAP-Cre gp130fl/fl mice resulted in a reduction of CNS regulatory Foxp3+ CD4 T cells and an increase of IL-17+, IFN-γ−, and TNF-producing CD4 as well as IFN-γ− and TNF-producing CD8 T cells, illustrating that astrocytes regulate the phenotypic composition of T cells. An analysis of mice deficient in either astrocytic gp130– Src homology region 2 domain-containing phosphatase 2/Ras/ERK or gp130–STAT1/3 signaling revealed that prevention of astrocyte apoptosis, restriction of demyelination, and T cell infiltration were dependent on the astrocytic gp130– Src homology region 2 domain-containing phosphatase 2/Ras/ERK, but not on the gp130–STAT1/3 pathway, further demonstrating that gp130-dependent astrocyte activation is crucial to ameliorate EAE. The Journal of Immunology, 2011, 186: 000–000.

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; BSF, B cell-stimulating factor; CNTF, ciliary neurotrophic factor; CT, cardiotrophin; EAE, experimental autoimmune encephalomyelitis; GFAP, glial fibrillary acidic protein; HPRT, hypoxanthine phosphoribosyltransferase; iNOS, inducible NO synthase; LIF, leukemia inhibitory factor; MOG, myelin oligodendrocyte glycoprotein; OSM, oncostatin M; p.i., postimmunization; SHP2, Src homology region 2 domain-containing phosphatase 2; WT, wild-type.

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also explains why the function of gp130 is still unknown in cerebro autoimmune disorders, whereas the function of gp130 ligands and coreceptors has been partially characterized. In EAE, IL-27Ra, which forms a signaling complex in combination with gp130, plays a crucially protective role by inhibiting immunopathology mediated by IL-17–producing CD4 T cells (16). In contrast, mice lacking IL-6 developed impaired autoimmune T cell responses after immunization with MOG Ag, resulting in an increased resistance to EAE (17). Further studies with CNTF and LIF/R–/– mice have shown that gp130-dependent signal transduction is important for limiting EAE by enhancing oligodendrocyte survival (18, 19). Collectively, these data indirectly indicate a regulatory role for gp130 in autoimmune CNS disorders, but it is unknown whether responsiveness of astrocytes to gp130-stimulating cytokines impacts on the disease.

Stimulation of gp130 results in the phosphorylation of a single membrane-proximal Y residue in gp130 (Y757 in mouse gp130), which induces the recruitment of the SH2 domain-containing cytoplasmic protein tyrosine phosphatase Src homology region 2 domain-containing phosphatase 2 (SHP2), its subsequent tyrosine phosphorylation, and activation of the Ras-ERK1/2 MAPK cascade (20). In contrast, binding of STAT1 and STAT3 proteins to the membrane-distal phosphotyrosine residues within gp130 (20) results in their tyrosine phosphorylation, homo- and/or heterodimerization, nuclear translocation, and transcriptional activation of target genes. Importantly, the gp130-dependent Ras and STAT1/3 pathways negatively control each other (21). To analyze the specific function of these two signaling pathways, mice lacking either gp130-STAT or gp30-Ras signaling have been generated (21, 22).

To characterize the in vivo function of astrocytes in autoimmune CNS disorders, we induced EAE in mice that lack gp130–, gp130–SHP2/Ras/ERK–, or gp130-STAT1/3–dependent signaling specifically in astrocytes. These experiments revealed the following: 1) astrocytic gp130 expression was crucial for survival of astrocytes and development of astroglia in EAE; 2) astrocyte loss exacerbated EAE characterized by significantly larger areas of demyelination and proinflammatory T cell response; and 3) the astrocytic gp130–SHP2/Ras/ERK pathway protected astrocytes from apoptosis and ameliorated EAE.

Materials and Methods

Mice

C57BL/6/human glial fibrillary acid protein (GFAP)-Cre transgenic mice (23) were bred with C57BL/6/gp130fl/fl (24) to generate GFAP-Cre Y757– gp130fl/fl mice. To obtain Synapsin-I-Cre gp130fl/fl mice, C57BL/6/Syn-Cre (25) and gp130fl/fl mice were bred. The colonies were maintained by breeding of GFAP-Cre Y757– gp130fl/fl with GFAP-Cre Y757– gp130fl/fl mice, respectively. GFAP-Cre gp130Y757F and GFAP-Cre gp130RASSTAT were generated by breeding of GFAP-Cre gp130fl/fl mice with either C57BL/6/Y757F or C57BL/6/STAT (21) mice. The colony was maintained by breeding of GFAP-Cre gp130Y757F and GFAP-Cre gp130RASSTAT with gp130fl/fl mice, respectively. The genotype of offspring was determined by PCR of tail DNA using primers for GFAP-Cre, Synapsin-Cre, gp130fl/fl, gp130Y757F, and gp130RASSTAT, respectively. C57BL/6 wild-type (WT) mice were obtained from Harlan (Borchen, Germany). Animal care and experimental procedures were performed according to European regulations and approved by state authorities (Landesverwaltungsamt Halle, Germany).

Induction and clinical evaluation of EAE

MOG35–55 (MEVGWYRRSFPSVHVLYNGK) was purchased from JPT (Berlin, Germany). EAE was induced in 8- to 12-wk-old mice by s.c. immunization with 200 μg MOG35–55 in CFA (Sigma-Aldrich, Taufkirchen, Germany) containing 800 μg killed Mycobacterium tuberculosis (Sigma-Aldrich). In addition, 200 ng pertussis toxoid (Sigma-Aldrich), dissolved in 200 μl PBS, was injected i.p. at the day of immunization and again 2 d thereafter. In indicated experiments, mice were immunized for a second time with 200 μg MOG35–55 peptide in CFA (Sigma-Aldrich) containing 800 μg killed M. tuberculosis (Sigma-Aldrich) 7 d after primary immunization. Mice were monitored daily for clinical signs of EAE and graded on a scale of increasing severity from 0 to 5, according to a previously published grading scale (26) and detailed in Supplemental Table 1. Daily clinical scores were calculated as the average of all individual disease scores within each group.

Histology

For immunohistochemistry on frozen sections, mice were perfused intracardially with 0.9% NaCl in methoxyflurane anesthesia. Brains were shock frozen, and immunohistochemistry for CD45, CD8, and GFAP was performed, as described before (27). For histology on paraffin sections, anesthetized mice were perfused with 4% paraformaldehyde in PBS; brains and spinal cords were processed and stained with hemalum and eosin, cresyl violet, luxol fast blue, and periodic acid Schiff stain, and also used for immunohistochemical demonstration of GFAP, neurofilament, Mac3, and CD3. CD3 (Serotec, Düsseldor, Germany), S100 protein (DakoCytomation, Hamburg, Germany), and GFAP were demonstrated in an ABC protocol, as described (27).

Stereochemistry

To quantify numbers of astrocytes and areas of demyelination in the spinal cord, stereology was applied using a computerized stereology system (CAST Software, Olymopus, Germany). Serial cross sections (4 μm) of the entire spinal cord, that is, cervical, thoracic, and lumbar level, were cut. Every third section was stained with hemalum, or stained with luxol fast blue and cresyl violet, respectively. The volume of the entire spinal cord and the volume of the white matter, respectively, were determined according to Cavalieri’s method (28) by estimating the area of every third section with the computerized system and multiplication with the distance to the next cross section evaluated, that is, 12 μm. Nuclei of GFAP-labeled astrocytes were counted. To avoid repetitive counting of astrocytes in adjacent cross sections, every fourth section was evaluated. Numbers of astrocytes/μm² were compared between the experimental groups. To compare areas of demyelination, the area of gray matter plus demyelinated white matter was related to the total area of the respective cross section.

Isolation of cerebral leukocytes and flow cytometry

Leukocytes were isolated from brain and spinal cord and stained for CD4 T cells, CD8 T cells, and CD45εεεε inflammatory leukocytes, as described before (29). For intracellular cytokine staining, isolated leukocytes were incubated with 50 ng/ml PMA, 500 ng/ml ionomycin, and Golgi-Plug (1 μl/ml) containing brefeldin A in MEM-α at 37°C for 4 h. Thereafter, cells were stained with CD4-FITC and CD8-allophycocyanin; fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences, Heidelberg, Germany); and stained with rat anti-mouse IL-17–PE, TNF-PE, or IFN-εεεε–PE (BD Biosciences). Controls included staining with isotype-matched control Abs. For the detection of regulatory and activated CD4 T cells, isolated leukocytes were incubated with CD25-FITC and CD4-allophycocyanin, followed by a Foxp3-PE staining kit, as recommended by the manufacturer (NatuTeC/E Biosciences, Frankfurt, Germany). Apoptotic and dead CD4 T cells were detected by staining with annexin V, 7-aminoactinomycin D (7-AAD), and CD4-allophycocyanin, fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences, Heidelberg, Germany), and stained with rat anti-mouse IL-17–PE, TNF-PE, or IFN-εεεε–PE (BD Biosciences). Controls included staining with isotype-matched control Abs. For the detection of regulatory and activated CD4 T cells, isolated leukocytes were incubated with CD25-FITC and CD4-allophycocyanin, fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences, Heidelberg, Germany), and stained with rat anti-mouse IL-17–PE, TNF-PE, or IFN-εεεε–PE (BD Biosciences). Controls included staining with isotype-matched control Abs. Flow cytometry was performed on a FACSscan (BD Biosciences), and the data were analyzed with WinMDI or CellQuest software.

Quantitative RT-PCR

For RT-PCR, mRNA was isolated from the spinal cord of nonimmunized and MOG35–55-immunized mice (RNeasy kit; Qiagen). mRNA was transcribed into cDNA by use of a SuperScript reverse-transcriptase kit with oligo(dT) primers (Invitrogen). Quantitative PCR for the gp130 ligands IL-6, LIF, CNTF, OSM, CT-1, BSF-3, IL-11, and IL-27 was performed with cDNA derived from C57BL/6 WT mice. The ratio between the respective cytokine and hypoxanthine phosphoribosyltransferase (HPRT) was calculated per mouse, and data are presented as the increase over nonimmunized mice. Quantitative RT-PCR for IL-17, IFN-εεεε, IL-23, inducible NO synthase (iNOS), TNF, TGF-β2, IL-27, and HPRT was performed with cDNA from GFAP-Cre gp130fl/fl and gp130fl/fl mice with the GeneAmp 5700 sequence detection system (Applied Biosystems, Foster City, CA). Quantitation was performed with the sequence detector software SDS 2.1 (Applied Bio- systems), according to the ΔΔ cycle threshold method (30) with HPRT as housekeeping gene. Data are expressed as increase of mRNA expression in...
imunized mice over nonimmunized controls of the respective mouse strain. All primers and probes were obtained from Applied Biosystems.

**Statistics**

Demyelinated areas and astrocyte numbers were evaluated on serial 4-μm cross sections of the spinal cord, which were either stained with luxol fast blue and cresyl violet or anti-GFAP and hemalum, respectively. Every third section was evaluated. For statistical evaluation of the number of GFAP+ astrocytes on GFAP-immunostained sections, at least 20 high power fields (final original magnification ×400), randomly selected from all areas of various regions of the spinal cord, were analyzed per section in three animals per group. Differences were analyzed with the nonparametric Mann-Whitney rank sum test. To test for statistical differences in the survival rate, clinical scores, and cell numbers, the two-tailed Student t test was used. The p values < 0.05 were accepted as significant. All experiments were performed at least twice.

**Results**

**Upregulation of gp130 ligands in EAE**

Because the gp130 receptor is essential for signal transduction of molecules of the IL-6 cytokine family, we analyzed whether expression of single or multiple members of this family is regulated in the spinal cord of mice suffering from EAE. At 14 d post-immunization (p.i.), expression of IL-6, LIF, OSM, and IL-27 was markedly upregulated, whereas expression of CNTF, CT-1, BSF3, and IL-11 was unaltered (Fig. 1). At this stage of EAE, mice had a mean clinical score of 2.2, and up to 25 d p.i. clinical scores declined to 0.9. In parallel to regressing disease activity, mRNA levels of IL-6, LIF, OSM, and IL-27 declined, although levels of LIF and OSM were still increased as compared with nonimmunized mice (Fig. 1).

**Aggravated EAE of GFAP-Cre gp130fl/fl**

To study the functional role of astrocytic gp130 expression, we used GFAP-Cre gp130fl/fl mice, which lack gp130 cell surface expression on astrocytes (31). We have previously shown that in this mutant, including a regular number and morphology of GFAP+ astrocytes (31).

Clinically, GFAP-Cre gp130fl/fl mice developed a significantly more severe EAE as compared with gp130fl/fl control mice upon immunization with MOG35–55 peptide. Data show the mean ± SEM. Eight to 10 (A) and six (B), respectively, mice per experimental group were analyzed. At 20 and 60 d p.i., clinical score and disease incidence were significantly increased in GFAP-Cre gp130fl/fl mice as compared with gp130fl/fl mice (*p < 0.05, **p < 0.001). Data from one of three (A) and one of two (B) independent experiments are shown.

**Upregulation of IL-6 family cytokine mRNA in EAE**

mRNA transcription of IL-6 family cytokine members in the spinal cord of mice suffering from EAE was evaluated by quantitative RT-PCR in nonimmunized and MOG35-55-immunized C57BL/6 mice at 14 and 25 d p.i. Data are expressed as increase of the respective cytokine mRNA of immunized over nonimmunized mice normalized to HPRT expression. Three mice per group were analyzed. Data represent the mean ± SEM. Data from one of two independent experiments are shown.

**Astrocyte loss in GFAP-Cre gp130fl/fl mice with EAE**

To study the impact of astrocyte-specific gp130 expression on EAE challenge, a detailed stereological analysis was performed. Numbers of astrocytes in the spinal cord of GFAP-Cre gp130fl/fl mice were monitored daily up to 60 d after single (A) or double (B) immunization with MOG35-55 peptide. Data show the mean ± SEM. Eight to 10 (A) and six (B), respectively, mice per experimental group were analyzed. At 20 and 60 d p.i., clinical score and disease incidence were significantly increased in GFAP-Cre gp130fl/fl mice as compared with gp130fl/fl mice (*p < 0.05, **p < 0.001). Data from one of three (A) and one of two (B) independent experiments are shown.

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Clinically, GFAP-Cre gp130fl/fl mice developed a significantly more severe EAE as compared with gp130fl/fl control mice upon immunization with MOG35–55 peptide. Data show the mean ± SEM. Eight to 10 (A) and six (B), respectively, mice per experimental group were analyzed. At 20 and 60 d p.i., clinical score and disease incidence were significantly increased in GFAP-Cre gp130fl/fl mice as compared with single-immunized mice (Fig. 2B). In GFAP-Cre gp130fl/fl mice, double immunization induced a very rapid progression of clinical symptoms, and peak scores developed within 5 d after disease onset without regression thereafter (Fig. 2B). Because single immunization was sufficient to induce maximal disease scores in GFAP-Cre gp130fl/fl mice, we used the single-immunization protocol in further experiments.

To exclude that a genotoxic effect of Cre causes the more severe disease of GFAP-Cre gp130fl/fl mice, we induced EAE in normal WT (gp130wt/wt) and GFAP-Cre gp130wt/wt mice. As illustrated in Supplemental Fig. 1, the clinical course of EAE was identical in both strains of mice, excluding a genotoxic effect of GFAP-Cre in our experiments.

Because GFAP-Cre–driven deletion of gp130 also results in lack of gp130 expression in ~10% of neurons (31), we also established mice with neuron-specific, Synapsin I-Cre–mediated deletion of gp130 (Supplemental Fig. 2A–C). It has been shown before that Synapsin I-Cre efficiently deletes genes in the vast majority of neurons in all regions of the CNS, including the spinal cord (25). Importantly, Synapsin I-Cre gp130fl/fl mice showed the same course of EAE as gp130fl/fl mice (Supplemental Fig. 2D), demonstrating that the absence of gp130 on astrocytes, but not on neurons, is responsible for aggravation of EAE in GFAP-Cre gp130fl/fl mice.
mice were significantly reduced as compared with gp130fl/fl control animals (Fig. 3A). In GFAP-Cre gp130fl/fl mice, astrocyte loss was observed at all levels of the spinal cord, that is, the cervical, thoracic, and lumbar segment. In addition to this three-dimensional analysis of astrocyte numbers, standard immunohistochemistry showed a gradual loss of astrocytes within inflammatory and demyelinated lesions over time in GFAP-Cre gp130fl/fl mice (Fig. 3B). As early as 14 d.p.i., the decline in astrocyte numbers in inflammatory lesions of GFAP-Cre gp130fl/fl mice was paralleled by the frequent appearance of astrocytes with crinkled processes and a condensed nucleus, indicating their apoptosis (Fig. 3C).

Interestingly, astrocyte numbers of gp130fl/fl mice also increased in noninflamed regions of the spinal cord and were significantly higher as compared with GFAP-Cre gp130fl/fl mice at 42 d.p.i. (Fig. 3B).

As early as 14 d.p.i., the decline in astrocyte numbers in inflammatory lesions of GFAP-Cre gp130fl/fl mice was paralleled by the frequent appearance of astrocytes with crinkled processes and a condensed nucleus, indicating their apoptosis (Fig. 3C). Activated, hypertrophic GFAP+ astrocytes were only rarely observed in the lesions of GFAP-Cre gp130fl/fl mice (Fig. 3D). These findings became more prominent with disease progression, and the inflammatory lesions of GFAP-Cre gp130fl/fl mice were nearly completely devoid of astrocytes at 42 d.p.i. (data not shown). In contrast, activated astrocytes in inflammatory lesions of control
mice upregulated GFAP and were hypertrophic with long and extended cellular processes resulting in astrogliosis (Fig. 3D, 3F). Combined TUNEL and GFAP staining revealed that at all time points of EAE, astrocytes of GFAP-Cre gp130fl/fl mice underwent apoptosis. Most of the TUNEL+ cells still exhibited a faint GFAP staining, which is compatible with a rapid degradation of this cytoskeletal protein (Fig. 3E). In contrast, only a few GFAP+ astrocytes were TUNEL+ in gp130fl/fl mice (Fig. 3F). These findings indicate that gp130 expression of astrocytes is important for the protection of astrocytes against apoptosis and results in the development of astrogliosis in EAE.

Increased pathology and persistence of inflammatory infiltrates in EAE of GFAP-Cre gp130fl/fl mice

A histopathological analysis of the impact of astrocytic loss on demyelination and the inflammatory response in EAE revealed that GFAP-Cre gp130fl/fl mice exhibited a remarkably more pronounced demyelination in the caudal brain stem and the spinal cord than control mice (Fig. 4A, 4C). The demyelinated area was significantly enlarged in GFAP-Cre gp130fl/fl mice as compared with control animals (Fig. 4E). Whereas inflammation and demyelination were largely confined to the dorsal columns of the spinal cord in gp130fl/fl mice, inflammatory demyelination also affected the lateral and anterior areas of the spinal cord and also extended into the gray matter of the spinal cord of GFAP-Cre gp130fl/fl mice (Fig. 4A). The more widespread inflammation and demyelination persisted in chronic EAE of GFAP-Cre gp130fl/fl mice, when gp130fl/fl mice had already clinically recovered (Fig. 4B, 4D). Flow cytometric quantification of inflammatory leukocytes confirmed that numbers of leukocytes were significantly increased in the CNS of GFAP-Cre gp130fl/fl as compared with gp130fl/fl mice (Fig. 4F).

Increased numbers of CD4 T cells were paralleled by a reduction of regulatory CD4 T cells and an increase of proinflammatory cytokine-producing T cells in GFAP-Cre gp130fl/fl mice

Because autoimmune T cells are crucial for EAE development, we studied the distribution of T cells at an early (14 d.p.i.) and late stage (42 d.p.i.) of EAE. CD3+ T cell infiltrates were present in both GFAP-Cre gp130fl/fl and control mice at 14 d.p.i. Up to 42 d.p.i., however, they had persisted only in GFAP-Cre gp130fl/fl mice, whereas they had largely been resolved in gp130fl/fl mice (Fig. 5A–D).

At 17 d.p.i., a flow cytometric analysis of T cells revealed that GFAP-Cre gp130fl/fl mice harbored 2-fold more CD4 T cells in the CNS than gp130fl/fl control animals (Fig. 5E), which already started to recover from an EAE. In contrast, numbers of CD8 T cells did not differ significantly between the two genotypes (Fig. 5E).

It has been suggested that astrocytes induce apoptosis of autoimmune CD4 T cells (32), which may explain the increased numbers of CD4 T cells in GFAP-Cre gp130fl/fl mice. However, only in two of four experiments was a reduced percentage of 7-AAD+ CD4 T cells obvious in GFAP-Cre gp130fl/fl mice. In addition, the percentage of active caspase-3+ CD4 T cells was not reduced in GFAP-Cre gp130fl/fl mice (3.3 versus 5.9% in gp130fl/fl and GFAP-Cre gp130fl/fl mice, respectively), as revealed by flow cytometry.

In addition, astrocyte apoptosis influenced the activation and the phenotypic composition of CD4 T cells in EAE. The percentage of both Foxp3+ CD25+ and Foxp3+ CD25− regulatory CD4 T cells was reduced in GFAP-Cre gp130fl/fl mice, whereas the number of activated Foxp3− CD25− effector CD4 T cells was increased as compared with control mice (Fig. 5F).

These differences in the number of regulatory and activated CD4 T cells were paralleled by an increase of IL-17−, TNF−, and IFN−γ−producing CD4 as well as TNF− and IFN−γ−producing CD8 T cells in the CNS of GFAP-Cre gp130fl/fl mice as compared with control animals (Fig. 5G, 5H).

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Increased IL-17, IFN-γ, IL-23, and iNOS mRNA production of GFAP-Cre gp130fl/fl mice

To gain further insight into the impact of astrocytes on cytokine production in EAE, cytokine mRNA transcription in the spinal cord was analyzed at an early (14 d p.i.) and a late time point (21 d p.i.) of EAE. At 14 d p.i., IL-17 mRNA was strongly upregulated in GFAP-Cre gp130fl/fl mice as compared with control animals (Fig. 6A). In contrast, IFN-γ, IL-23, TNF, iNOS, TGF-β2, and IL-27 mRNA did not differ between the two mouse strains at this time point (Fig. 6B-G). In addition to IL-17 mRNA, IFN-γ, IL-23, and iNOS mRNA were also strongly upregulated in GFAP-Cre gp130fl/fl mice at 21 d p.i. In contrast, TGF-β2 mRNA was increased in gp130fl/fl mice at this stage of EAE. No differences were observed for TNF and IL-27.

The gp130-SHP2/Ras/ERK, but not the gp130-STAT pathway ameliorates EAE, protects astrocytes from apoptosis, and reduces inflammatory infiltrates as well as demyelination

Ligand engagement of the gp130 receptor results in activation of STAT1/3 and Ras/ERK pathway via specific tyrosine residues within the cytosolic portion of gp130. To evaluate the contribution...
control mice. Indeed, GFAP-Cre gp130(Y757F) mice showed similar EAE scores to gp130-deficient GFAP-Cre gp130(Y757F) mice (Fig. 7A). Histopathological analysis of GFAP-Cre gp130(Y757F) mice revealed a strong and widespread loss of astrocytes in the spinal cord (Fig. 7B) similar to that observed in GFAP-Cre gp130(Y757F) mice, whereas astrocytes of GFAP-Cre gp130(Y757F) mice were activated and hypertrophic despite a less severe disease activity (Fig. 7C). Loss of astrocytes in GFAP-Cre gp130(Y757F) mice resulted in much more severe demyelination when compared with GFAP-Cre gp130(Y757F) mice, which showed only mild demyelination (Fig. 7D, 7E). Consistent with the different extent of demyelination, inflammatory infiltrates were much more prominent and widespread in GFAP-Cre gp130(Y757F) mice than in GFAP-Cre gp130(Y757F) mice (Fig. 7F, 7G). Collectively, these observations suggest that the extent of gp130-mediated STAT activation in astrocytes, which occurs excessively in GFAP-Cre gp130(Y757F) mice and is absent from GFAP-Cre gp130(Y757F) mice, is not related to development of EAE. In contrast, the extent of EAE inversely correlates with the extent of gp130-mediated SHP2/Ras/ERK activation, which is absent in the highly disease-sensitive GFAP-Cre gp130(Y757F) and GFAP-Cre gp130(Y757F) strains, but present in gp130(Y757F) controls and GFAP-Cre gp130(Y757F) mice. Therefore, we conclude that development of a protective astrogliosis is independent of gp130-mediated STAT signaling, but requires gp130-dependent SHP2/Ras/ERK signaling.

**Discussion**

The present in vivo experiments revealed that astrocyte-specific gp130 signaling is important to protect mice from EAE, because both single and double MOG35–55-immunized mice lacking astrocyte-specific gp130 signaling developed a clinically significantly more severe EAE than control mice. The major protective function of astrocytic gp130 was to ensure astrocyte survival and to enable development of astrogliosis as important factors limiting autoimmune T cell reactions and demyelination in EAE. Such an important function of gp130 expression on astrocytes in EAE has not been observed in mice lacking individual gp130 ligands, including IL-6, LIF, and IL-27, respectively (16, 17, 34). However, all of these gp130 ligands were upregulated in EAE and, therefore, may compensate for each other in rescuing astrocytes from apoptosis under inflammatory conditions. This is in contrast to oligodendrocytes, which require CNTF and LIF to prevent their apoptosis in EAE (18, 19). The cell-type–specific importance of astrocytic gp130 is further stressed by the observation that mice lacking gp130 expression selectively in neurons developed the same course of EAE as gp130(Y757F) control mice.

Gp130 expression of astrocytes was required to prevent apoptosis of astrocytes. In good agreement with an antiapoptotic function of gp130 (35), survival of astrocytes upon in vitro stimulation with TNF is gp130 dependent (31). Because in EAE a variety of cytokines including TNF is produced (36) (Figs. 5, 6), these molecules may also contribute to loss of GFAP-Cre gp130(Y757F) astrocytes in vivo.

Astrocyte apoptosis rendered GFAP-Cre gp130(Y757F) mice unable to mount astrogliosis in EAE. Recent studies in EAE using inducible astrocyte-deficient mice demonstrated that deletion of astrocytes in EAE prevents the development of astrogliosis, resulting in a more widespread inflammation and demyelination (10). Interestingly, we demonstrated previously that in Toxoplasma encephalitis gp130-dependent astrocyte survival and astrogliosis are important to restrict and contain inflammatory lesions (31). The concept of gp130-dependent astrogliosis as a CNS intrinsic and specific mechanism to restrict inflammatory

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**FIGURE 6.** Increased IL-17, IFN-γ, IL-23, and iNOS mRNA transcription in EAE of GFAP-Cre gp130(Y757F) mice. A–F. The mRNA expression of IL-17 (A), IFN-γ (B), IL-23 (C), TNF (D), iNOS (E), TGF-β2 (F), and IL-27 (G) was analyzed by quantitative RT-PCR from GFAP-Cre gp130(Y757F) and gp130(Y757F) mice at 14 and 21 d p.i. Spinal cords of three mice per group were analyzed, and data represent the mean ± SEM as relative increase for each time point. G. iNOS expression in GFAP-Cre gp130(Y757F) mice.
lesions is supported by the more widespread inflammation and demyelination in EAE of GFAP-Cre gp130\(^{fl/Y757F}\) mice. Interestingly, patients suffering from acute multiple sclerosis are also characterized by astrocyte loss in the center of the demyelinated lesions and an astrogliosis surrounding these lesions (37).

Abrogation of gp130 signaling in astrocytes had a strong impact on the immune response in the CNS. The most striking observation was that loss of astrocytes in inflammatory lesions resulted in increased numbers of CD4 T cells in the CNS. Initially, GFAP-Cre gp130\(^{fl/Y757F}\) mice recruited CD3 T cells to the CNS similar to control mice. This observation indicates that the early recruitment of T cells to the CNS was unimpaired in GFAP-Cre gp130\(^{fl/Y757F}\) mice and that astrocytes were still able to contribute to T cell recruitment by their production of chemokines (38). However, once autoimmune inflammation was fully established, defective astrocytic gp130 signaling and rapid astrocyte apoptosis contributed to a failure to efficiently reduce CD4 T cell numbers in the CNS.

Previous experiments have demonstrated that the elimination of autoimmune T cells by apoptosis is important for EAE resolution (39, 40). Although it is at present unclear which cell types induce CD4 T cell apoptosis in EAE, astrocytes may be key inducers because of the following: 1) astrocytes are in intimate contact with apoptotic T cells and express Fas ligand (9); 2) autoreactive CD4 T cells are Fas\(^+\) (41); and 3) Fas ligand\(^+\) astrocytes induce apoptosis of MOG-specific T cells in vitro (42). However, flow cytometry showed only reduced numbers of 7-AAD\(^-\), that is, late apoptotic or dead, CD4 T cells and failed to detect reduced active

**FIGURE 7.** Aggravated EAE with widespread astrocyte loss, severe demyelination, and increased T cell infiltration in GFAP-Cre gp130\(^{fl/Y757F}\) mice. A, The clinical score of GFAP-Cre gp130\(^{fl/Y757F}\) and gp130\(^{fl/Y757F}\) as well as GFAP-Cre gp130\(^{fl/Y757F}\) and GFAP-Cre gp130\(^{fl/Y757F}\) mice was monitored daily up to 40 d p.i. with MOG\(_{35–55}\) peptide. Six to eight mice per experimental group were analyzed. Data show the mean ± SEM. The clinical score was significantly increased in GFAP-Cre gp130\(^{fl/Y757F}\) and GFAP-Cre gp130\(^{fl/Y757F}\) mice as compared with gp130\(^{fl/Y757F}\) controls (\(*p < 0.01\)). The clinical score of GFAP-Cre gp130\(^{fl/Y757F}\) mice did not differ significantly from control mice. B, Partial loss of GFAP-expressing astrocytic processes perivascularly (arrows) and in a subleptomeningeal infiltrate in a GFAP-Cre gp130\(^{fl/Y757F}\) mouse. Note the crinkled cellular processes and the condensed shrunken nuclei of astrocytes (arrowheads). C, Astrocytes of a GFAP-Cre gp130\(^{fl/Y757F}\) mouse were normally activated (arrows) in the spinal cord. B and C, Anti-GFAP immunostaining, slight counterstaining with hemalum, original magnification ×200. D, Loss of myelin and vacuolation in a subleptomeningeal infiltrate in a GFAP-Cre gp130\(^{fl/Y757F}\) mouse. Note the intense perivascular and intraparenchymal inflammatory reaction (arrows). E, Regular myelination in the spinal cord with only slight leptomeningeal infiltrate in GFAP-Cre gp130\(^{fl/Y757F}\) mouse. D and E, Cresyl violet with luxol fast blue staining, original magnification ×100. F, CD3 T cells in a subleptomeningeal perivascular infiltrate and scattered throughout the brain parenchyma in a GFAP-Cre gp130\(^{fl/Y757F}\) mouse. G, Single CD3 T cells in the leptomeninges and the spinal cord parenchyma of a GFAP-Cre gp130\(^{fl/Y757F}\) mouse. F and G, Anti-CD3 immunostaining, slight counterstaining with hemalum, original magnification ×200. B–G, Three mice were analyzed per experimental group at 17 d p.i. A–G, Representative data from one of two independent experiments are shown.
caspase-3 expression in CNS CD4 T cells of GFAP-Cre gp130<sup>0/0</sup> mice in two of four experiments. These data indicate that gp130 expression of astrocytes is not crucial for the induction of CD4 T cell apoptosis, although they do not principally rule out the possibility that astrocytes may induce apoptosis of autoimmune CD4 T cells.

The increased number of CD4 T cells in the CNS might also be caused by an increased homing of these cells to the spinal cord of GFAP-Cre gp130<sup>0/0</sup> mice. Our observation of a strong reduction in the number of perivascular astrocytes in inflamed areas of the spinal cord of GFAP-Cre gp130<sup>0/0</sup> mice and the observation of Yoskuhl et al. (10) that depletion of proliferating astrocytes in EAE results in an increased influx of inflammatory leukocytes into the spinal cord indicate that perivascular astrocytes restrict the recruitment of CD4 T cells to the CNS. Thus, both activated perivascular and parenchymal astrocytes surrounding inflammatory lesions appear to restrict homing and spread of CD4 T cells to and within the CNS. Mutually not exclusive, the elevated and sustained production of proinflammatory mediators in the spinal cord of GFAP-Cre gp130<sup>0/0</sup> mice (Fig. 5) may contribute to the increased recruitment of autoimmune CD4 T cells and may induce a self-amplifying loop sustaining ongoing inflammation and demyelination. Interestingly, reactive astrocytes also restrict leukocyte infiltration, demyelination, tissue destruction, and motor deficits after traumatic spinal cord injury (43), which further argues for an immunosuppressive function of astroglialosis. However, astroglialosis may not always be protective, because reactive astrocytes inhibit axonal regeneration after spinal cord injury (44) and may prevent oligodendrocyte invasion into demyelinated areas in EAE (7).

In good agreement with Trajkovic et al. (12), who showed that astrocytes can induce regulatory T cell mitigating EAE, we observed that astrocyte loss in GFAP-Cre gp130<sup>0/0</sup> mice did not only result in an increase of activated CD25<sup>+</sup>CD4 T cells, but also in a decrease of Foxp3<sup>+</sup> regulatory CD4 T cells. Because regulatory CD4 T cells play an important protective role in EAE (45), their decline in GFAP-Cre gp130<sup>0/0</sup> mice most likely directly contributes to the more severe course of EAE. These phenotypic changes were associated with a significant increase of IL-17<sup>+</sup>, IFN-γ<sup>+</sup>, and TNF-producing CD4 T cells in GFAP-Cre gp130<sup>0/0</sup> mice. This increase clearly exceeded the absolute increase of CD4 T cell number in GFAP-Cre gp130<sup>0/0</sup> mice as compared with control animals, which indicates that gp130 signaling in astrocytes is important to shift the balance between protective and EAE-inducing CD4 T cells toward protective T cells. Interestingly, previous in vitro data also provided evidence that astrocytes induce a regulatory phenotype in autoimmune CD4 T cells (12). In addition, CD8 T cells of GFAP-Cre gp130<sup>0/0</sup> mice produced more IFN-γ and TNF. Thus, although gp130-dependent astrocyte loss did not affect the absolute number of CD8 T cells, their proinflammatory cytokine production was increased.

Quantitative RT-PCR revealed that transcription of IL-17, IFN-γ, IL-23, and TNF mRNA was also increased in the CNS of GFAP-Cre gp130<sup>0/0</sup> mice suffering from EAE. IL-17 (46) and IL-23 (47) are key cytokines contributing to EAE. Although IFN-γ is not strictly required for the induction of EAE (48), the ongoing elevated IFN-γ production in GFAP-Cre gp130<sup>0/0</sup> mice may induce the sustained production of iNOS, which can also significantly contribute to development and severity of EAE (49). Thus, the increased Th1/Th17 response combined with an increased transcription of iNOS and IL-23 mRNA by CNS parenchymal cells is likely to account for the more widespread and ongoing demyelination in the absence of astroglialosis in GFAP-Cre gp130<sup>0/0</sup> mice. Noteworthily, transcription of TNF and IL-27 was not elevated and TGF-β2 was even reduced in GFAP-Cre gp130<sup>0/0</sup> mice. This may be explained by the loss of astrocytes in these animals, because astrocytes are a source of these cytokines in EAE (11, 50, 51). Obviously, IL-27 and TGF-β2 transcription by other cells than astrocytes was too low to compensate for loss of astrogliosederived IL-27 and TGF-β2 and to suppress immunopathology induced by autoimmune T cells, as has been reported before (16, 52).

In this study, we extended our previous genetic approach to dissect the contribution of the two major intracellular signaling cascades engaged by gp130 by creating compound mutant mice that are heterozygous for the corresponding gp130 signaling mutant alleles. These alleles become functionally dominant in astrocytes upon GFAP-Cre–mediated tissue-specific ablation of the paired, conditional gp130<sup>0/</sup> WT allele. Diminished activation of the gp130-SHP2/Ras/ERK pathway in GFAP-Cre gp130<sup>0/0</sup><sup>R/Y757F</sup> mice reproduced all pathological features observed in gp130 signaling-deficient GFAP-Cre gp130<sup>0/</sup> mice, including astrocyte loss, lack of astrogliosis, a significantly more severe clinical course, increased T cell infiltration, and severe demyelination. Noteworthily, mice lacking gp130-SHP2/Ras/ERK signaling in all cells may develop spontaneous autoimmune arthritis due to excessive activation of the STAT1/3 pathway in nonhematopoietic cells (53). Although in our model GFAP-Cre gp130<sup>0/0</sup><sup>R/Y757F</sup> mice with excessive activation of STAT in astrocytes, that is, a nonhematopoietic cell type, showed an exaggerated course of EAE and astrocyte apoptosis, the augmented autoimmune reaction was largely independent of astrogliosederived gp130 signaling (53). In sharp contrast, GFAP-Cre gp130<sup>0/0</sup><sup>R/E757T</sup> mice with intact gp130-SHP2/Ras/ERK signaling developed remarkably milder clinical symptoms as compared with GFAP-Cre gp130<sup>0/0</sup><sup>R/Y757F</sup> and GFAP-Cre gp130<sup>0/0</sup> mice due to an astrocyte-dependent reduction of autoimmune T cells in the CNS. Noteworthily, our genetic approach in GFAP-Cre gp130<sup>0/0</sup><sup>R/E757T</sup> mice is designed to leave STAT1/3 activation by gp130-independent pathways, including the epidermal growth factor and IL-10 signaling, intact. Because STAT3 can be activated by various signaling pathways (reviewed in Ref. 54), STAT3-dependent astrogliosis after spinal cord injury (55) may be caused by the cumulative defective signaling of various pathways and explains why GFAP-Cre gp130<sup>0/0</sup><sup>R/E757T</sup> mice still develop astrogliosis in our model. Interestingly, apoptosis of astrocytes in EAE was suppressed by gp130-mediated activation of the SHP2/Ras/ERK pathway and not that of STAT1/3 despite the observation that excessive STAT3 activation provides an antiapoptotic signal in many other cell types (56). In contrast, both astrogliosis and LIF-mediated cardiomyocyte hypertrophy are dependent on the gp130-SHP2/Ras/ERK signaling pathway (57). Collectively, this points to cell-type– and disease-specific functions of these two signaling pathways and establishes astrocytes as an important regulator of cerebral autoimmune reactions.

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**Disclosures**

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