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Astrocytic Fas ligand expression is required to induce T-cell apoptosis and recovery from experimental autoimmune encephalomyelitis

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In T-cell-mediated autoimmune diseases of the CNS, apoptosis of Fas$^+$ T cells by FasL contributes to resolution of disease. However, the apoptosis-inducing cell population still remains to be identified. To address the role of astrocytic FasL in the regulation of T-cell apoptosis in experimental autoimmune encephalomyelitis, we immunized C57BL/6 glial fibrillary acid protein (GFAP)-Cre FasL$^{fl/fl}$ mice selectively lacking FasL in astrocytes with $MOG_{35–55}$ peptide. GFAP-Cre FasL$^{fl/fl}$ mice were unable to resolve EAE and suffered from persisting demyelination and paralysis, while FasL$^{fl/fl}$ control mice recovered. In contrast to FasL$^{fl/fl}$ mice, GFAP-Cre FasL$^{fl/fl}$ mice failed to induce apoptosis of Fas$^+$ activated CD4$^+$ T cells and to increase numbers of Foxp3$^+$ Treg cells beyond day 15 post immunization, the time point of maximal clinical disease in control mice. The persistence of activated and GM-CSF-producing CD4$^+$ T cells in GFAP-Cre FasL$^{fl/fl}$ mice also resulted in an increased IL-17, IFN-γ, TNF, and GM-CSF mRNA expression in the CNS. In vitro, FasL$^+$ but not FasL$^-$ astrocytes induced caspase-3 expression and apoptosis of activated T cells. In conclusion, FasL expression of astrocytes plays an important role in the control and elimination of autoimmune T cells from the CNS, thereby determining recovery from EAE.

Keywords: Autoimmunity · Astrocytes · EAE · FasL

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Introduction

EAE is a widely used animal model to study MS, an inflammatory demyelinating disease mediated by accumulation of T lymphocytes and macrophages in the CNS [1, 2]. EAE can be induced by either active immunization with myelin Ags including myelin oligodendrocyte glycoprotein (MOG) peptide or passive transfer of myelin-reactive CD4$^+$ T cells, which are both initiators and effectors of EAE. Among CD4$^+$ T lymphocytes, GM-CSF-producing CD4$^+$ T cells, IFN-γ-secreting Th1 cells, and IL-17-secreting Th17 cells have been identified as the most important mediators in the immunopathogenesis of EAE [3–6] and all of them can induce EAE independently, although recent studies point to an essential role of GM-CSF-producing CD4$^+$ T cells, which can induce EAE independent of IFN-γ and IL-17 [7]. Infiltrating T lymphocytes trigger an inflammatory response in the CNS culminating in demyelination and axonal damage clinically resulting in paralysis [8]. Correspondingly, recovery from EAE requires termination of inflammation and the induction of T-cell apoptosis in the CNS [9].

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Fas ligand (FasL; CD95L), a cytotoxic cytokine belonging to the TNF superfamily, acts through Fas, a death receptor of the TNFR superfamily, to induce programed cell death via caspase signaling [10]. Local expression of FasL in immunoprivileged organs including eyes, testis, and placenta is essential for deletion of infiltrating inflammatory cells [11–13]. Fas/FasL interaction is of particular importance for homeostasis of the immune system and its dysregulation has been implicated in various autoimmune diseases. Mice carrying autosomal recessive mutations in the Fas (*lpr*) and FasL (*gld*) genes develop a spontaneous autoimmune syndrome similar to human systemic lupus erythematosus [14,15]. Fas and FasL are also involved in the pathogenesis of EAE, as EAE is dramatically ameliorated in *lpr* and *gld* mice in terms of disease incidence and mean clinical score [16]. Intrathecal infusion of recombinant FasL induces apoptosis of CNS-infiltrating inflammatory cells, including T cells and macrophages, but does not exert cytotoxicity against CNS-resident cells, resulting in mitigated EAE manifestations [17].

Elimination of infiltrating T cells in the CNS by Fas/FasL-mediated apoptosis is crucial for resolution of EAE [9, 18, 19], since FasL-deficient *gld* recipients develop prolonged EAE after adoptive transfer of myelin basic protein-reactive WT Fas^+ T lymphocytes [20]. The CNS-resident cell population which induces apoptosis of $CD4^+$ T cells in EAE still remains to be identified. We hypothesize that astrocytes, which constitutively express FasL, may play a key role given that FasL-expressing astrocytes are in intimate contact with apoptotic T cells in EAE and can induce apoptosis of activated $CD4^+$ T cells in vitro [21, 22]. Consistently, our previous study also demonstrated that increased apoptosis of gp130-deficient astrocytes exacerbated EAE, partially due to an impaired elimination of $CD4^+$ T cells from the CNS [23]. However, in vivo evidence confirming that astrocytic FasL is involved in the induction of $CD4^+$ T-cell apoptosis in EAE is still lacking.

In order to determine whether $FasL^+$ astrocytes are inducers of $CD4^+$ T-cell apoptosis in EAE, we generated glial fibrillary acid protein (GFAP)-Cre $FasL^{fl/fl}$ mice that are deficient of FasL selectively in astrocytes. We show in the present study that astrocytic FasL is crucial to terminate the autoimmune T-cell response in the CNS, which allows clinical recovery from EAE.

Results

Selective deletion of FasL in astrocytes of GFAP-Cre $FasL^{fl/fl}$ mice

We generated GFAP-Cre $FasL^{fl/fl}$ mice with selective FasL deletion in the CNS (Supporting Information Fig. 1). Further PCR analysis of cultivated cells showed FasL deletion in astrocytes and to a minor extent in neurons (Fig. 1A). In contrast, microglia of GFAP-Cre $FasL^{fl/fl}$ as well as astrocytes, neurons, and microglia of $FasL^{fl/fl}$ control mice did not show deletion of FasL (Fig. 1A). To confirm astrocytic FasL deletion at the protein level, cell surface expression of FasL protein was analyzed by flow cytometry from cultivated astrocytes of GFAP-Cre $FasL^{fl/fl}$ and $FasL^{fl/fl}$ mice. As shown in Figure 1B, FasL expression was reduced on the surface of astrocytes from GFAP-Cre $FasL^{fl/fl}$ as compared to $FasL^{fl/fl}$ mice. Both GFAP-Cre $FasL^{fl/fl}$ mice and $FasL^{fl/fl}$ (control) mice were born in a normal Mendelian ratio and reached adulthood without any CNS defects.

Collectively, these findings show that astrocyte-specific deletion of FasL was achieved in our newly generated GFAP-Cre $FasL^{fl/fl}$ mice, which did not show abnormalities under physiological conditions, thereby providing a useful tool for studying the function of astrocyte-specific FasL in experimentally induced models of CNS disorders.

Aggravated EAE of GFAP-Cre $FasL^{fl/fl}$ mice with increased inflammation and demyelination

To gain insight into the astrocyte-specific function of FasL in the pathogenesis of EAE, we actively immunized GFAP-Cre $FasL^{fl/fl}$ mice and control mice with $MOG_{35–55}$ peptide emulsified in complete Freund's adjuvant and assessed clinical disease activity daily. Both GFAP-Cre $FasL^{fl/fl}$ mice and $FasL^{fl/fl}$ control mice developed EAE starting at around day 9 post immunization (p.i.) and reaching peak disease at day 15 p.i.; over this period of time they developed similar clinical symptoms (Fig. 2A). However, beyond the maximum of disease, i.e. day 15 p.i., $FasL^{fl/fl}$ mice recovered gradually while EAE progressed in GFAP-Cre $FasL^{fl/fl}$ mice indicating a significantly more severe course of EAE in the later group of mice (Fig. 2A). Already at day 15 p.i., inflammation of GFAP-Cre $FasL^{fl/fl}$ mice was more severe and more widespread as compared with that in control animals, leading to more severe demyelination. While inflammatory foci consisting of $CD3^+$ T cells and macrophages were confined to the dorsal columns of the spinal cord in $FasL^{fl/fl}$ mice, they also infiltrated the spinocerebellar tracts in GFAP-Cre $FasL^{fl/fl}$ mice. Differences between the two mouse strains were more prominent at day 22 p.i. as compared with those at day 15 p.i. Inflammation and demyelination were mild in $FasL^{fl/fl}$ mice (Fig. 2B and D) as compared with that in GFAP-Cre $FasL^{fl/fl}$ mice, with widespread inflammatory foci consisting of $CD3^+$ T cells and $Mac3^+$ macrophages (Fig. 2C and E). In GFAP-Cre $FasL^{fl/fl}$ mice, demyelination was prominent in the posterior columns as well as in spinocerebellar tracts (Fig. 2C), which also showed evidence of a disturbed axonal transport as evidenced by axonal bulbs. Inflammation was also prominent in the dorsal horn of the spinal cord, where many infiltrates resided (Fig. 2E).

Increased numbers of infiltrating T cells in the spinal cord of GFAP-Cre $FasL^{fl/fl}$ mice

Autoimmune T cells are widely regarded as the key mediator of EAE; therefore, we analyzed T cells infiltrating the spinal cord. At day 15 p.i., flow cytometry revealed that numbers of infiltrating $CD4^+$ and $CD8^+$ T cells were slightly but not significantly

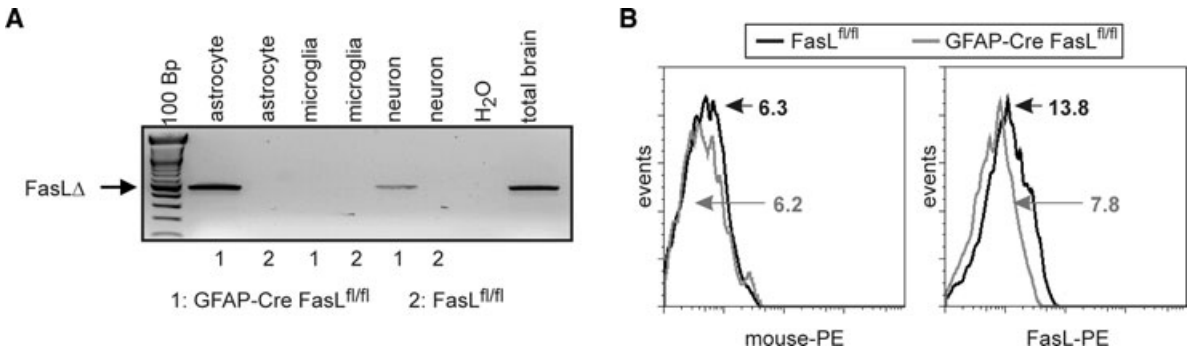

Figure 1. Organ and cell-type-specific deletion of FasL in GFAP-Cre FasL$^{fl/fl}$ mice. (A) Deletion of FasL from cultivated and FACS-sorted astrocytes, microglia, and cultivated neurons of GFAP-Cre FasL$^{fl/fl}$ (lanes labeled 1) and FasL$^{fl/fl}$ (lanes labeled 2) mice. Total brain (positive control) was from a GFAP-Cre FasL$^{fl/fl}$ mouse. (B) Cell surface expression of the FasL protein (right) was analyzed by flow cytometry from cultivated astrocytes of GFAP-Cre FasL$^{fl/fl}$ and FasL$^{fl/fl}$ mice. Staining with control mouse Ab is shown (left). Representative data from one of two experiments are shown and the MFI of FasL expression is given in each histogram.

increased in the spinal cord of GFAP-Cre FasL$^{fl/fl}$ mice as compared with those in FasL$^{fl/fl}$ mice (Fig. 3A and B), which corresponds to the similar clinical scores at this time point (Fig. 2). At day 22 p.i., significantly more CD4$^+$ and CD8$^+$ T cells were detected in the spinal cord of GFAP-Cre FasL$^{fl/fl}$ mice than in FasL$^{fl/fl}$ mice (Fig. 3A and B; $p < 0.01$ for CD4$^+$ and CD8$^+$ T cells).

As GM-CSF-producing CD4$^+$ T cells are essential for the induction of EAE [7], we determined the percentage and number of GM-CSF-producing CD4$^+$ T cells in the spinal cord of both mouse strains. Flow cytometry revealed that GM-CSF-producing CD4$^+$ T cells accounted for approximately 15% of CD4$^+$ T cells in both mouse strains; however, the absolute number of GM-CSF-producing CD4$^+$ T cells was significantly increased in GFAP-Cre FasL$^{fl/fl}$ mice as compared with that in control animals at day 22 p.i. (Fig. 3C). In addition, we compared the phenotypic composition of CD4$^+$ T cells between the two genotypes to determine whether astrocyte-specific deletion of FasL influenced the activation state of infiltrating CD4$^+$ T cells in EAE. At day 15 p.i., the percentage of Foxp3$^-$ CD25$^+$ activated CD4$^+$ T cells (Fig. 3D) and Foxp3$^+$ regulatory CD4$^+$ T cells (Fig. 3E) was similar in both strains of mice, whereas at day 22 p.i., as compared with FasL$^{fl/fl}$ mice, the percentage of Foxp3$^-$ CD25$^+$ activated CD4$^+$ T cells was increased while the percentage of Foxp3$^+$ regulatory CD4$^+$ T cells was reduced in GFAP-Cre FasL$^{fl/fl}$ mice, respectively (Fig. 3D and E). Intraspinal CD4$^+$ T cells from both mouse strains expressed Fas, as detected by flow cytometry (Fig. 3F), and, thus, they might be regulated by FasL$^+$ cells. At day 22 p.i., the percentage of 7-aminoactinomycin D (7-AAD)$^+$ CD4$^+$ T cells was significantly reduced in GFAP-Cre FasL$^{fl/fl}$ mice as compared with that in FasL$^{fl/fl}$ mice (Fig. 3G, $*p < 0.05$) suggesting that elimination of infiltrating T cells by apoptosis was impaired in GFAP-Cre FasL$^{fl/fl}$ mice in late stages of EAE. Annexin V staining was not used to detect CD4$^+$ T-cell apoptosis in vivo because previous reports showed that annexin V did not selectively detect apoptotic T cells, since it also stained activated CD4$^+$ T cells [24].

Increased proinflammatory gene transcription in the spinal cord of GFAP-Cre FasL$^{fl/fl}$ mice

To examine the impact of astrocyte-specific FasL deletion on the expression of proinflammatory genes during EAE, quantitative real-time PCR for cytokines and chemokines was performed on spinal cord tissue at day 15 p.i. and day 22 p.i. of EAE, respectively. At day 15 p.i., IFN-γ and IL-27 mRNA was significantly elevated in GFAP-Cre FasL$^{fl/fl}$ mice as compared to FasL$^{fl/fl}$ mice while mRNA levels of IL-17, TNF, IL-23, and GM-CSF did not differ between the two mouse strains (Fig. 4). In contrast, at day 22 p.i., mRNA levels of all mediators, except for IL-23, were significantly upregulated in GFAP-Cre FasL$^{fl/fl}$ mice as compared with levels in FasL$^{fl/fl}$ mice, indicating an increased proinflammatory response in the spinal cord of GFAP-Cre FasL$^{fl/fl}$ mice at this late time point (Fig. 4). Interestingly, mRNA of IL-17, a main mediator of EAE, persisted at high levels in the spinal cord of GFAP-Cre FasL$^{fl/fl}$ mice up to day 22 p.i. Taken together, these results show that astrocytic deletion of FasL resulted in an increased transcription of important proinflammatory genes in the spinal cord which induce and contribute to severity of EAE.

Reduced apoptosis of CD4$^+$ T cells in coculture with FasL-deficient astrocytes

Twenty-four hours after coculture of FasL$^{fl/fl}$ CD4$^+$ T cells with primary astrocytes isolated from the CNS of FasL$^{fl/fl}$ or GFAP-Cre FasL$^{fl/fl}$ mice, T-cell apoptosis induced by FasL-deficient astrocytes was compared to that induced by control astrocytes. In accordance with a previous report of Bechmann et al. [21], significantly lower numbers of T cells cocultured with FasL-deficient astrocytes underwent apoptosis as demonstrated by both annexin V binding and caspase staining (Fig. 5). Based on these findings, we conclude that, during EAE, astrocytic FasL-induced apoptotic elimination of T cells in the CNS of GFAP-Cre FasL$^{fl/fl}$ mice is significantly

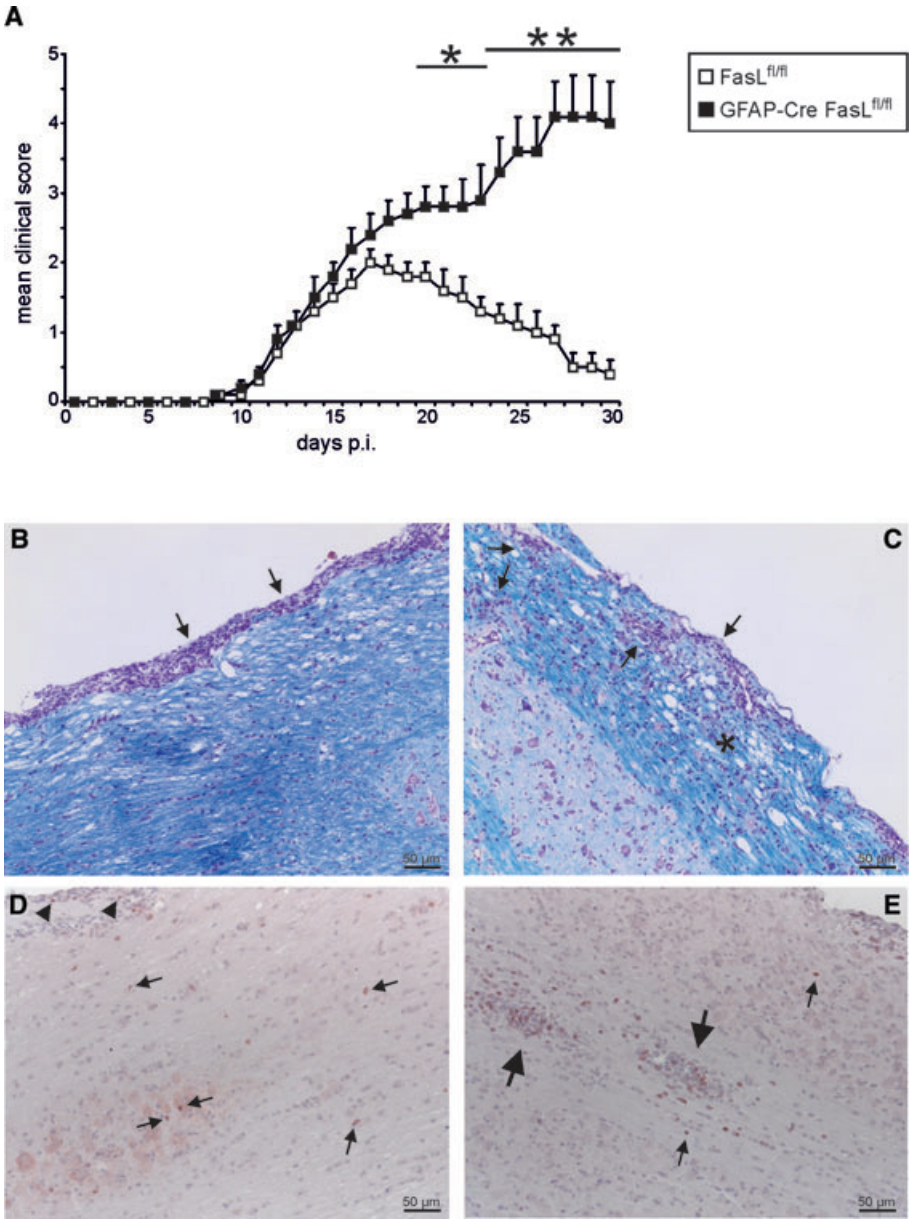

Figure 2. Aggravated EAE with increased demyelination and inflammation in GFAP-Cre FasL$^{fl/fl}$ mice. (A) Clinical symptoms of GFAP-Cre FasL$^{fl/fl}$ and FasL$^{fl/fl}$ mice were scored daily after immunization with $MOG_{35–55}$ peptide in CFA, and data are displayed as the mean ± SEM of 8–10 mice per group, from one experiment representative of four performed. $^{*}p < 0.05$, $^{**}p < 0.01$, *t*-tests. (B) Lack of ongoing demyelination in the posterior column of the spinal cord in a FasL$^{fl/fl}$ mouse, although myelin sheaths are reduced. Inflammatory infiltrates are confined to the leptomeninges (arrows), but do not infiltrate the spinal cord parenchyma. Cresyl violet—luxol fast blue staining. (C) Severe inflammation with leptomeningeal and spinal cord parenchymal infiltrates (arrows) with pronounced demyelination (*) in the posterior column of the spinal cord in a GFAP-Cre FasL$^{fl/fl}$ mouse. Note numerous myelin fragments and pronounced edema. Cresyl violet—luxol fast blue staining. (D) Some CD3^{+} T cells contributed to leptomeningeal infiltrates (arrowheads) and only single CD3^{+} T cells are still present in parenchyma of the spinal cord of a FasL$^{fl/fl}$ mouse (small arrows). Anti-CD3 immunostaining, slight counterstaining with hemalum. (E) Many CD3^{+} T cells form inflammatory cuffs in the posterior columns and around blood vessels in the posterior horn (large arrows) and are also scattered throughout the spinal cord (small arrows) in a GFAP-Cre FasL$^{fl/fl}$ mouse. Anti-CD3 immunostaining, slight counterstaining with hemalum. (B–E) Histological findings of representative mice (day 22 p.i.) from one of two independent experiments with three mice each are shown, bar 50 µm.

compromised as compared with that of control animals, resulting in a significantly enhanced disease activity.

Discussion

The present study demonstrates that elimination of infiltrating encephalitogenic T lymphocytes is a key mechanism for EAE resolution and that FasL^{+} astrocytes are crucial to induce T-cell apoptosis and elimination from the CNS in this autoimmune disease. In agreement with Bechmann et al. [21], who demonstrated that astrocytes induced apoptosis of activated T cells in a FasL-dependent way in vitro, we observed that FasL^{+} astrocytes induced apoptosis of activated Fas^{+} CD4^{+} T cells in vitro. Since astrocytes are also FasL^{+} in MS, our data suggest a similar role of astrocytes for the elimination of inflammatory leukocytes in this severe human autoimmune disease.

Astrocytic FasL was protective for mice during EAE, since $MOG_{35–55}$-immunized mice lacking astrocyte-specific FasL suffered from a clinically more severe EAE compared with their control littermates. The onset of clinical symptoms was similar in both GFAP-Cre FasL$^{fl/fl}$ and control FasL$^{fl/fl}$ mice at day 9 p.i. indicating that homing of myelin-specific leucocytes was not regulated by astrocytic FasL expression. In addition, the clinical score of GFAP-Cre FasL$^{fl/fl}$ and FasL$^{fl/fl}$ mice increased with the same kinetics until the peak of disease in FasL$^{fl/fl}$ mice at day 15 p.i. In accordance, numbers of CD4^{+} T cells were not significantly increased in GFAP-Cre FasL$^{fl/fl}$ mice at day 15 p.i. However, during the clinical recovery phase of FasL$^{fl/fl}$ mice (day 22 p.i.), numbers of CD4^{+} T cells were significantly increased in the spinal cord

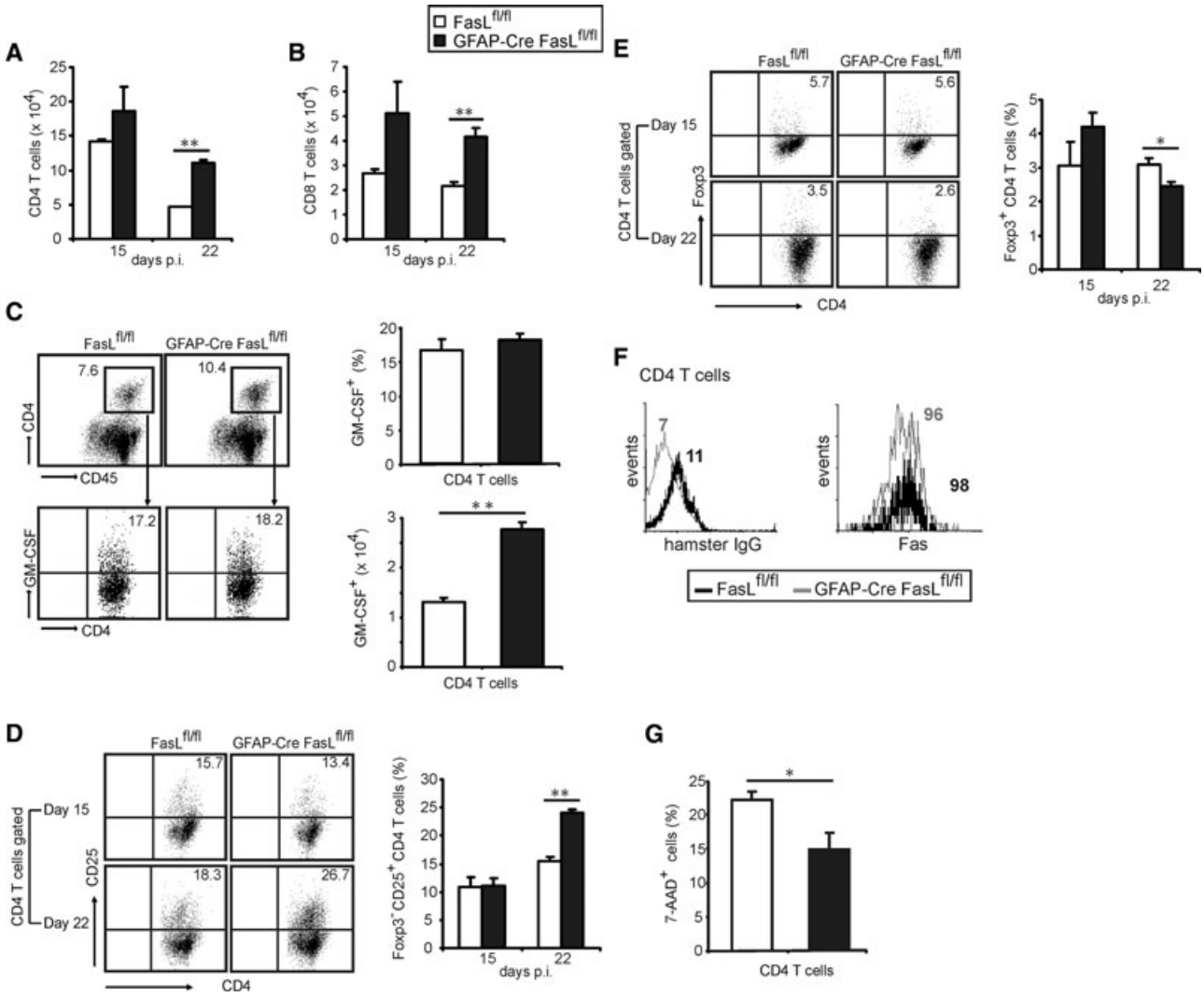

Figure 3. Increased numbers of activated and reduced numbers of Treg cells in the CNS of GFAP-Cre FasL$^{fl/fl}$ mice. (A, B) Leukocytes were isolated from the spinal cord of GFAP-Cre FasL$^{fl/fl}$ and FasL$^{fl/fl}$ mice and numbers of (A) CD4$^+$ and (B) CD8$^+$ T cells were determined by flow cytometry. (C) Leukocytes isolated from the spinal cord were stained for extracellular CD4 and CD45 and intracellular GM-CSF. Dot plots (left) show the percentage of CD4$^+$ CD45$^+$ cells (upper left), and GM-CSF$^+$ CD4$^+$ cells (lower left) at day 22 p.i., as well as the gating strategy for CD4$^+$ cells. The relative (upper right) and absolute number (lower right) of GM-CSF$^+$ CD4$^+$ cells is shown. (D, E) The percentage of (D) CD25$^+$ CD4$^+$ T cells and (E) Foxp3$^+$ regulatory CD4$^+$ T cells was analyzed by flow cytometry. (F) Cell surface expression of Fas on CD4$^+$ T cells from both GFAP-Cre FasL$^{fl/fl}$ and FasL$^{fl/fl}$ mice was analyzed by flow cytometry at day 15 p.i. and representative histograms are shown (right). Control staining was performed with hamster IgG (left). The MFI is shown in each histogram. (G) The percentage of 7-AAD$^+$ CD4$^+$ T cells was determined by flow cytometry at day 22 p.i., and the mean + SD is shown. (A–G) Data are shown as representative dot plots as well as the mean + SD of six to nine mice per experimental group from one of two independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, *t*-tests.

of GFAP-Cre FasL$^{fl/fl}$ mice as shown by both flow cytometry and histology at day 22 p.i. The reduced number of CD4$^+$ T cells positive for 7-AAD, which identifies late apoptotic and dead cells, illustrates the compromised ability of FasL-deficient astrocytes to induce apoptosis and elimination of infiltrating autoimmune T cells. The kinetics of disease and intraspinal CD4$^+$ T-cell numbers indicate that FasL-dependent elimination of CD4$^+$ T cells in EAE plays a particularly protective role in the recovery phase. Noteworthy, the more severe EAE of GFAP-Cre FasL$^{fl/fl}$ mice cannot be attributed to the GFAP-Cre transgene, since C57BL/6 GFAP-Cre mice without a loxP-flanked gene develop the same course of EAE as compared to WT mice [23].

We also observed a significantly higher number of activated CD25$^+$ CD4$^+$ T cells and a significantly reduced number of Foxp3$^+$ regulatory CD4$^+$ T cells in the spinal cord of GFAP-Cre FasL$^{fl/fl}$ as compared with FasL$^{fl/fl}$ mice at day 22 p.i. Lack of astrocytic FasL expression altered the ratio of activated CD25$^+$ versus regulatory Foxp3$^+$ CD4$^+$ T cells in the spinal cord from 5:1 in FasL$^{fl/fl}$ mice to 10:1 in GFAP-Cre FasL$^{fl/fl}$ mice. These data suggest that astrocytic FasL expression predominantly contributes to elimination of activated disease-promoting CD25$^+$ CD4$^+$ T cells but not of protective regulatory Foxp3$^+$ CD4$^+$ T cells in order to recover from EAE and to achieve a restitutio ad integrum. These data are in line with previous reports illustrating that astrocytes induce a

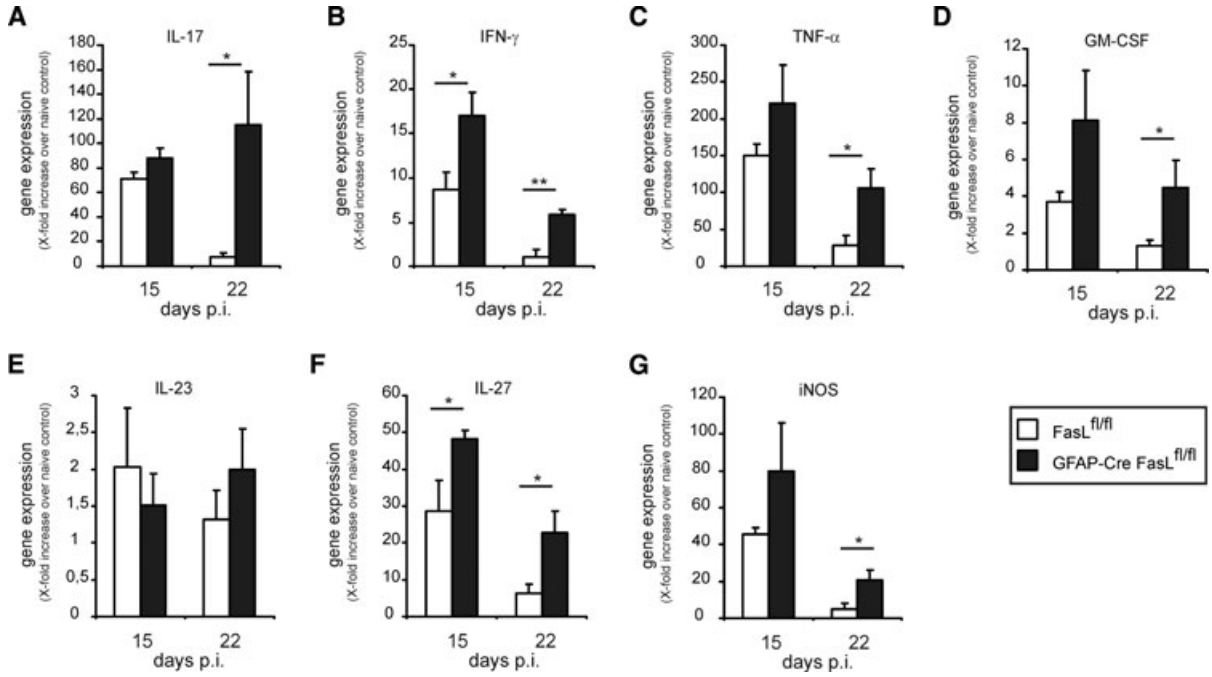

Figure 4. Increased proinflammatory gene expression in the spinal cords of GFAP-Cre FasL$^{fl/fl}$ mice. (A–G) Expression of (A) IL-17, (B) IFN-γ, (C) TNF-α, (D) GM-CSF, (E) IL-23, (F) IL-27, and (G) iNOS mRNA was determined by quantitative RT-PCR in GFAP-Cre FasL$^{fl/fl}$ and FasL$^{fl/fl}$ mice at days 15 and 22 p.i. Spinal cords of three mice per group were analyzed. Data represent the mean + SEM as relative increase over nonimmunized mice. $^{*}p < 0.05$, $^{**}p < 0.01$, *t*-tests. One of two independent experiments with three mice each is shown.

regulatory phenotype in autoimmune CD4$^+$ T cells [25], which play an important protective role in EAE [26, 27]. In agreement, the number of GM-CSF-producing CD4$^+$ T cells, i.e. the essential EAE-inducing CD4$^+$ T-cell subset [7], was significantly increased in the spinal cord of GFAP-Cre FasL$^{fl/fl}$ mice at day 22 p.i.

The increased number of infiltrating activated autoreactive CD4$^+$ T cells in GFAP-Cre FasL$^{fl/fl}$ mice was associated with an enhanced production of proinflammatory cytokines. At day 15 p.i., IFN-γ, TNF, GM-CSF, IL-27, and iNOS but not IL17 mRNA was increased in GFAP-Cre FasL$^{fl/fl}$ mice as compared with that in FasL$^{fl/fl}$ mice. IFN-γ, TNF, and GM-CSF have been reported to contribute to disease progression and demyelination in EAE [7, 28]. GM-CSF and IFN-γ are mainly produced by encephalitogenic T cells. GM-CSF sustains neuroinflammation via myeloid cells that infiltrate the spinal cord. In addition to its proinflammatory function, IFN-γ is also a potent stimulator of IL-27 production by astrocytes [29], which might explain the increased production of this immunosuppressive and protective cytokine in the spinal cord of GFAP-Cre FasL$^{fl/fl}$ mice at day 15 p.i. IL-27 can suppress IL-17 production of primed Th17 cells [29], which might explain that GFAP-Cre FasL$^{fl/fl}$ mice did not show elevated IL-17 mRNA expression in the spinal cord as compared to FasL$^{fl/fl}$ mice. IL-17 is an important cytokine contributing to demyelination and progression of EAE [30]. Comparable levels of IL-17 mRNA transcription in GFAP-Cre FasL$^{fl/fl}$ and FasL$^{fl/fl}$ mice at day 15 p.i. might, therefore, explain similar clinical scores in the two mouse strains at this stage of disease. However, at day 22 p.i., when numbers of activated CD25$^+$ and GM-CSF-producing CD4$^+$ T cells were significantly increased and numbers of Foxp3$^+$ regulatory CD4$^+$ T cells decreased in GFAP-Cre FasL$^{fl/fl}$ mice, IL-17 mRNA was very prominently increased in addition to IFN-γ, TNF, GM-CSF, and iNOS mRNA. Thus, aggravation of clinical symptoms in GFAP-Cre FasL$^{fl/fl}$ mice correlated with an increased IL-17 mRNA transcription, indicating that this cytokine was decisive for the more severe and persisting EAE in these mice. IL-23, which drives IL-17 polarization of CD4$^+$ T cells was not increased in the CNS of GFAP-Cre FasL$^{fl/fl}$ mice, which fits to the important role of IL-23 for Th17 polarization in lymphatic organs [31].

Astrocytes play both positive and negative roles in the pathogenesis and development of EAE [32]. As part of the blood-brain barrier, early chemokine release of astrocytes contributes to the recruitment of autoimmune CD4$^+$ T cells to the CNS [33]. At later stages of EAE, astrogliosis develops, which may restrict further invasion of leukocytes into the CNS parenchyma [34]. In fact, genetically induced ablation of reactive astrocytes during EAE led to widespread inflammation and more severe clinical symptoms [35]. In this regard, it is of note that gp130-dependent astrocyte survival and astrogliosis are critical to restrict CNS inflammation in EAE as well as in CNS infections, i.e. *Toxoplasma* encephalitis [23, 36]. Consistently, blocking NF-κB signaling, which is required for astrocyte activation in EAE, by tissue-specific ablation of key signaling molecules including NEMO, IKK2, and Act1 in the CNS impaired astrocytic production of inflammatory cytokines and chemokines ameliorating EAE as evidenced by decreased

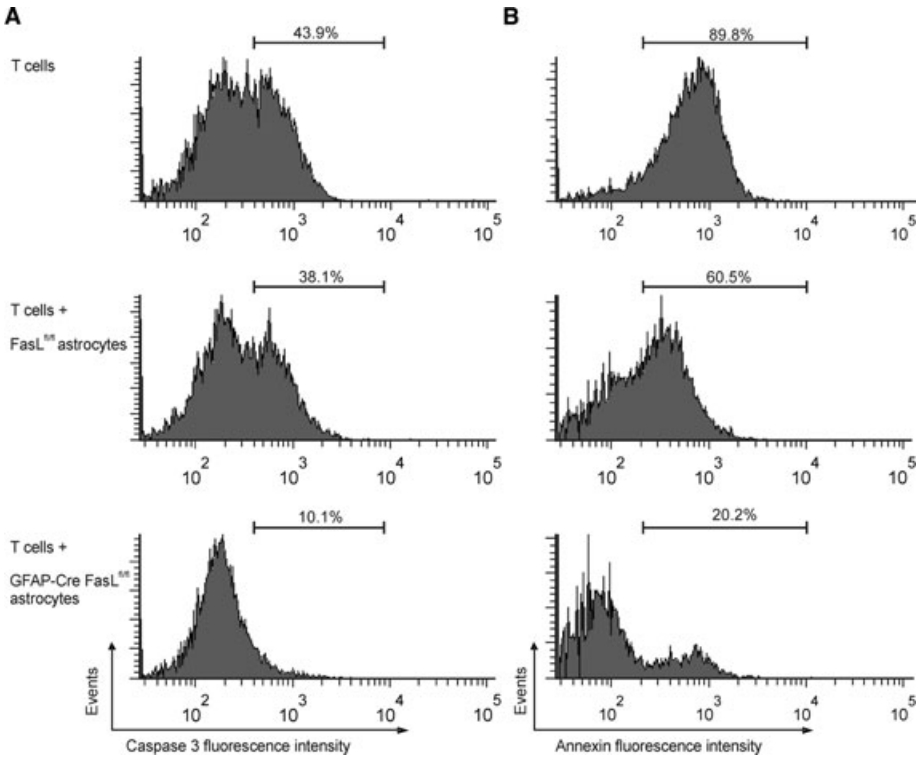

Figure 5. Astrocytes induce apoptosis of activated T cells via FasL in vitro. (A, B) After monoculture or coculture with astrocytes from FasL$^{fl/fl}$ or GFAP-Cre FasL$^{fl/fl}$ mice for 24 h, apoptosis of T cells was detected by (A) caspase 3 and (B) annexin V staining. No difference was observed in the corresponding control stainings with isotype-matched IgG (data not shown). One of two independent experiments is shown.

leukocyte infiltration and reduced demyelination [5,37,38]. Interestingly, in sharp contrast to the proinflammatory function of most astrocyte-derived chemokines, CXCL12, which is upregulated in the CNS of MS patients, particularly produced by astrocytes, suppressed ongoing EAE by redirecting the polarization of effector Th1 cells into IL10-producing Treg cells [39].

Collectively, the present study extends the in vivo function of astrocytes and illustrates that astrocytes also confer protection against EAE by the FasL-dependent apoptotic elimination of activated CD25$^+$ Foxp3$^-$ and GM-CSF-producing CD4$^+$ T cells and the concomitant inhibition of proinflammatory cytokine production. Thus, augmentation of astrocytic FasL may provide a favorable strategy for treatment of clinically active MS.

Materials and methods

Mice

GFAP-Cre$^{+/-}$ FasL$^{fl/fl}$ mice were generated by crossing C57BL/6 GFAP-Cre transgenic mice [40] with C57BL/6 FasL$^{fl/fl}$ mice [41] and the colony was maintained by breeding of GFAP-Cre$^{+/-}$ FasL$^{fl/fl}$ mice with GFAP-Cre$^{-/-}$ FasL$^{fl/fl}$ mice. Genotyping of offsprings was carried out by PCR of tail DNA with primers targeting GFAP-Cre and FasL$^{fl/fl}$. Deletion of FasL was analyzed by PCR in various organs and cell types with Del-FasL primers (5′-GTACTTCTTCTGATAAGGACC-3′ and 5′-GGAGTTGAACGAGTAGCCTC-3′). C57BL/6 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; IMMB/G/02–994/10).

Induction and assessment of EAE

$MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK) was purchased from JPT (Berlin, Germany). Active EAE was induced in 8- to 12-week-old mice by s.c. immunization with 200 μg of $MOG_{35–55}$ emulsified in complete Freund's adjuvant (Sigma, Taufkirchen, Germany) supplemented with 800 μg of killed Mycobacterium tuberculosis (Sigma). In addition, mice also received two i.p. injections of 200 ng pertussis toxin (Sigma), dissolved in 200 μL PBS, at the time of immunization as well as 48 h thereafter. Clinical signs of EAE were monitored daily and scored according to a scale of severity from 0 to 5 as described previously [23]. Daily clinical scores were calculated as the average of all individual disease scores within each group.

Isolation of intraspinal leukocytes and flow cytometry

Leukocytes were isolated from the spinal cord and stained for CD4$^+$ T cells, CD8$^+$ T cells, and CD45^{high} inflammatory leukocytes as described before [42]. For the detection of regulatory and activated CD4$^+$ T cells, isolated leukocytes were stained with CD25-FITC and CD4-allophycocyanin followed by a Foxp3-PE staining kit as recommended by the manufacturer (eBiosciences, Frankfurt, Germany). For intracellular staining of GM-CSF, isolated

leukocytes were incubated with 50 ng/mL PMA, 500 ng/mL ionomycin, Golgi-Plug (1 μL/mL) containing brefeldin A in RPMI-1640 at 37° C for 4 h. Thereafter, cells were stained with rat antimouse CD4-FITC, rat antimouse CD45-V450, fixed and permeabilized with Cytofix/Cytoperm (BD), and stained with rat antimouse GM-CSF-PE (BD). Apoptotic and dead $CD4^+$ T cells were detected by staining with 7-AAD and CD4-allophycocyanin. Fas expression on $CD4^+$ T cells was analyzed by staining with hamster antimouse Fas-PE and CD4-FITC. Controls were stained with isotype-matched control antibodies. All antibodies and reagents were obtained from BD Biosciences (Heidelberg, Germany) unless otherwise mentioned. Flow cytometry was performed on a FACScan (BD Biosciences), and the data were analyzed with WinMDI or Cell Quest software.

In vitro culture, DNA isolation, and flow cytometry of astrocytes, microglia, and neurons

Primary astrocytes were isolated from 1- to 2-day-old newborn mice and cultured as published before [43]. To obtain pure astrocytes, cells were harvested from astrocyte cultures and stained with rat antimouse CD11b-PE. Pure astrocytes ($CD11b^-$) were then separated from $CD11b^+$ microglia with a FACSVantage cell sorter (BD). Neuronal cultures were obtained according to Lenz et al. [44] with slight modifications. Briefly, pregnant female mice were sacrificed by cervical dislocation at gestational day 18.5, and dissociated cells of each embryonic brain were cultivated in flasks coated with poly-L-lysine in Neurobasal medium supplemented with B27 (Invitrogen) and 500 μM L-glutamine (Gibco). The purity of cultures for neurons was ≥98%, as determined by immunofluorescence staining for neuron-specific class III β-tubulin. DNA was isolated from sorted astrocytes and microglia, respectively, as well as from cultured neurons using a DNA isolation kit (Qiagen, Germany). For the detection of FasL expressed on the surface of astrocytes, mixed astrocyte/microglia cultures were stained with mouse antimouse FasL-PE and CD11b-FITC. Controls were stained with isotype-matched control antibodies.

Histology

For histology on paraffin sections, mice anesthetized with methoxyflurane were perfused with 0.1 M PBS followed by 4% paraformaldehyde in PBS, spinal cords were processed and stained with hemalum and eosin, cresyl violet, and luxol fast blue. In addition, paraffin sections were used for immunohistochemical demonstration of GFAP, neurofilament, Mac3, and CD3 (Serotec, Düsseldorf, Germany) by an ABC protocol as described [45].

Quantitative RT-PCR

Total mRNA was isolated from the spinal cords of nonimmunized and $MOG_{35–55}$- immunized mice (RNeasy kit, Qiagen, Germany) at day 15 and day 22 p.i., respectively. SuperScript reverse transcriptase kit with oligo (dT) primers (Invitrogen, Germany) was used to generate cDNA from total mRNA. Quantitative RT-PCR for IL-17, IFN-γ, IL-23, IL-27, TNF, GM-CSF, iNOS, and hypoxanthine phosphoribosyltransferase was performed on the light cycler 480 real-time PCR system (Roche). The ratio between the respective gene and corresponding hypoxanthine phosphoribosyltransferase was calculated per mouse according to the ΔΔ cycle threshold method [46], and data were expressed as the increase of mRNA expression in immunized mice over non immunized controls of the respective mouse strain. All primers and probes were obtained from Applied Biosystems.

Coculture of $CD4^+$ T cells with astrocytes

$CD4^+$ T cells were isolated from spleens and LNs of C57BL/6 mice by MACS (Miltenyi Biotec, Germany) according to the manufacturer' instructions. Purified $CD4^+$ T cells were activated for 48 h by culturing in anti-CD3 (BD, 5 μg/mL) and anti-CD28 (eBiosciences, 2 μg/mL) coated 96-well plates at $1–2 \times 10^5$ cells/well in 200 μL of RPMI-1640 (Gibco) supplemented with 10% FCS (Gibco), 1% L-glutamine (Gibco), 100 U/mL penicillin (Sigma), and 0.1 mg/mL streptomycin (Sigma). For coculture, 1×10^5 activated T cells were inoculated onto the astrocytic monolayers in six-well plates. After 24 h incubation, T cells were collected and apoptosis was detected by staining cells with Annexin-allophycocyanin, Caspase 3-PE, and CD4-Pacific Blue.

Statistics

To test for statistical differences in the clinical scores and cell numbers, the two-tailed Student's *t*-test was used. p values < 0.05 were accepted as significant. All experiments were performed at least twice.

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References

1 **McFarland, H. F. and Martin, R.**, Multiple sclerosis: a complicated picture of autoimmunity. *Nat. Immunol.* 2007. **8**: 913–919.

2 **Baxter, A. G.**, The origin and application of experimental autoimmune encephalomyelitis. *Nat. Rev. Immunol.* 2007. **7**: 904–912.

3 **Lees, J. R., Golumbek, P. T., Sim, J., Dorsey, D. and Russell, J. H.**, Regional CNS responses to IFN-gamma determine lesion localization patterns during EAE pathogenesis. *J. Exp. Med.* 2008. **205**: 2633–2642.

4 **Stromnes, I. M., Cerretti, L. M., Liggitt, D., Harris, R. A. and Goverman, J. M.**, Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. *Nat. Med.* 2008. **14**: 337–342.

5 **Kang, Z., Altuntas, C. Z., Gulen, M. F., Liu, C., Giltiay, N., Qin, H., Liu, L. et al.**, Astrocyte-restricted ablation of interleukin-17-induced Act1-mediated signaling ameliorates autoimmune encephalomyelitis. *Immunity* 2010. **32**: 414–425.

6 **Domingues, H. S., Mues, M., Lassmann, H., Wekerle, H. and Krishnamoorthy, G.**, Functional and pathogenic differences of Th1 and Th17 cells in experimental autoimmune encephalomyelitis. *PLoS ONE* 2010. **5**: e15531.

7 **Codarri, L., Gyulveszi, G., Tosevski, V., Hesske, L., Fontana, A., Magnenat, L., Suter, T. et al.**, RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat. Immunol.* 2011. **12**: 560–567.

8 **Gold, R., Linington, C. and Lassmann, H.**, Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. *Brain* 2006. **129**: 1953–1971.

9 **Schmied, M., Breitschopf, H., Gold, R., Zischler, H., Rothe, G., Wekerle, H. and Lassmann, H.**, Apoptosis of T lymphocytes in experimental autoimmune encephalomyelitis. Evidence for programmed cell death as a mechanism to control inflammation in the brain. *Am. J. Pathol.* 1993. **143**: 446–452.

10 **Strasser, A., Jost, P. J. and Nagata, S.**, The many roles of FAS receptor signaling in the immune system. *Immunity* 2009. **30**: 180–192.

11 **Griffith, T. S., Brunner, T., Fletcher, S. M., Green, D. R. and Ferguson, T. A.**, Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* 1995. **270**: 1189–1192.

12 **Bellgrau, D., Gold, D., Selawry, H., Moore, J., Franzusoff, A. and Duke, R. C.**, A role for CD95 ligand in preventing graft rejection. *Nature* 1995. **377**: 630–632.

13 **Hunt, J. S., Vassmer, D., Ferguson, T. A. and Miller, L.**, Fas ligand is positioned in mouse uterus and placenta to prevent trafficking of activated leukocytes between the mother and the conceptus. *J. Immunol.* 1997. **158**: 4122–4128.

14 **Watanabe-Fukunaga, R., Brannan, C. I., Copeland, N. G., Jenkins, N. A. and Nagata, S.**, Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* 1992. **356**: 314–317.

15 **Takahashi, T., Tanaka, M., Brannan, C. I., Jenkins, N. A., Copeland, N. G., Suda, T. and Nagata, S.**, Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell* 1994. **76**: 969–976.

16 **Waldner, H., Sobel, R. A., Howard, E. and Kuchroo, V. K.**, Fas- and FasL-deficient mice are resistant to induction of autoimmune encephalomyelitis. *J. Immunol.* 1997. **159**: 3100–3103.

17 **Zhu, B., Luo, L., Chen, Y., Paty, D. W. and Cynader, M. S.**, Intrathecal Fas ligand infusion strengthens immunoprivilege of central nervous system and suppresses experimental autoimmune encephalomyelitis. *J. Immunol.* 2002. **169**: 1561–1569.

18 **Pender, M. P., Nguyen, K. B., McCombe, P. A. and Kerr, J. F.**, Apoptosis in the nervous system in experimental allergic encephalomyelitis. *J. Neurol. Sci.* 1991. **104**: 81–87.

19 **Tabi, Z., McCombe, P. A. and Pender, M. P.**, Apoptotic elimination of V beta 8.2+ cells from the central nervous system during recovery from experimental autoimmune encephalomyelitis induced by the passive transfer of V beta 8.2 +encephalitogenic T cells. *Eur. J. Immunol.* 1994. **24**: 2609–2617.

20 **Sabelko-Downes, K. A., Cross, A. H. and Russell, J. H.**, Dual role for Fas ligand in the initiation of and recovery from experimental allergic encephalomyelitis. *J. Exp. Med.* 1999. **189**: 1195–1205.

21 **Bechmann, I., Steiner, B., Gimsa, U., Mor, G., Wolf, S., Beyer, M., Nitsch, R. et al.**, Astrocyte-induced T-cell elimination is CD95 ligand dependent. *J. Neuroimmunol.* 2002. **132**: 60–65.

22 **Kohji, T. and Matsumoto, Y.**, Coexpression of Fas/FasL and Bax on brain and infiltrating T cells in the central nervous system is closely associated with apoptotic cell death during autoimmune encephalomyelitis. *J. Neuroimmunol.* 2000. **106**: 165–171.

23 **Haroon, F., Drögemüller, K., Händel, U., Brunn, A., Reinhold, D., Nishanth, G., Mueller, W. et al.**, Gp130-dependent astrocytic survival is critical for the control of autoimmune central nervous system inflammation. *J. Immunol.* 2011. **186**: 6521–6531.

24 **Elliott, J. I., Surprenant, A., Marelli-Berg, F. M., Cooper, J. C., Cassady-Cain, R. L., Wooding, C., Linton, K. et al.**, Membrane phosphatidylserine distribution as a non-apoptotic signalling mechanism in lymphocytes. *Nat. Cell Biol.* 2005. **7**: 808–816.

25 **Trajkovic, V., Vuckovic, O., Stosic-Grujicic, S., Miljkovic, D., Popadic, D., Markovic, M., Bumbasirevic, V. et al.**, Astrocyte-induced Treg cells mitigate CNS autoimmunity. *Glia* 2004. **47**: 168–179.

26 **Gultner, S., Kuhlmann, T., Hesse, A., Weber, J. P., Riemer, C., Baier, M. and Hutloff, A.**, Reduced Treg frequency in LFA-1-deficient mice allows enhanced T effector differentiation and pathology in EAE. *Eur. J. Immunol.* 2010. **40**: 3403–3412.

27 **Tsakiri, N., Papadopoulos, D., Denis, M. C., Mitsikostas, D. D. and Kollias, G.**, TNFR2 on non-haematopoietic cells is required for Foxp3 +Treg-cell function and disease suppression in EAE. *Eur. J. Immunol.* 2012. **42**: 403–412.

28 **Petermann, F. and Korn, T.**, Cytokines and effector T-cell subsets causing autoimmune CNS disease. *FEBS Lett.* 2011. **585**: 3747–3757.

29 **Fitzgerald, D. C., Zhang, G. X., El-Behi, M., Fonseca-Kelly, Z., Li, H., Yu, S., Saris, C. J. et al.**, Suppression of autoimmune inflammation of the central nervous system by interleukin 10 secreted by interleukin 27-stimulated T cells. *Nat. Immunol.* 2007. **8**: 1372–1379.

30 **Komiyama, Y., Nakae, S., Matsuki, T., Nambu, A., Ishigame, H., Kakuta, S., Sudo, K. et al.**, IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J. Immunol.* 2006. **177**: 566–573.

31 **Kobayashi, T., Okamoto, S., Hisamatsu, T., Kamada, N., Chinen, H., Saito, R., Kitazume, M. T. et al.**, IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease. *Gut* 2008. **57**: 1682–1689.

32 **Giraud, S. N., Caron, C. M., Pham-Dinh, D., Kitabgi, P. and Nicot, A. B.**, Estradiol inhibits ongoing autoimmune neuroinflammation and NFkappaB-dependent CCL2 expression in reactive astrocytes. *Proc. Natl. Acad. Sci. USA* 2010. **107**: 8416–8421.

33 **Zhou, Y., Sonobe, Y., Akahori, T., Jin, S., Kawanokuchi, J., Noda, M., Iwakura, Y. et al.**, IL-9 promotes Th17 cell migration into the central nervous system via CC chemokine ligand-20 produced by astrocytes. *J. Immunol.* 2011. **186**: 4415–4421.

34 **Toft-Hansen, H., Fuchtbauer, L. and Owens, T.**, Inhibition of reactive astrocytosis in established experimental autoimmune encephalomyelitis favors infiltration by myeloid cells over T cells and enhances severity of disease. *Glia* 2011. **59**: 166–176.

35 **Voskuhl, R. R., Peterson, R. S., Song, B., Ao, Y., Morales, L. B., Tiwari-Woodruff, S. and Sofroniew, M. V.**, Reactive astrocytes form scar-like perivascular barriers to leukocytes during adaptive immune inflammation of the CNS. *J. Neurosci.* 2009. **29**: 11511–11522.

36 **Drögemüller, K.**, **Helmuth, U.**, **Brunn, A.**, **Sakowicz-Burkiewicz, M.**, **Gutmann, D. H.**, **Mueller, W.**, **Deckert, M. et al.**, Astrocyte gp130 expression is critical for the control of Toxoplasma encephalitis. *J. Immunol.* 2008. **181**: 2683–2693.

37 **van, L. G.**, **De, L. R.**, **Schmidt, H.**, **Huth, M.**, **Mildner, A.**, **Schmidt-Supprian, M.**, **Lassmann, H. et al.**, Inhibition of transcription factor NF-kappaB in the central nervous system ameliorates autoimmune encephalomyelitis in mice. *Nat. Immunol.* 2006. **7**: 954–961.

38 **Brambilla, R.**, **Persaud, T.**, **Hu, X.**, **Karmally, S.**, **Shestopalov, V. I.**, **Dvoriantchikova, G.**, **Ivanov, D. et al.**, Transgenic inhibition of astroglial NF-kappa B improves functional outcome in experimental autoimmune encephalomyelitis by suppressing chronic central nervous system inflammation. *J. Immunol.* 2009. **182**: 2628–2640.

39 **Meiron, M.**, **Zohar, Y.**, **Anunu, R.**, **Wildbaum, G. and Karin, N.**, CXCL12 (SDF-1alpha) suppresses ongoing experimental autoimmune encephalomyelitis by selecting antigen-specific Treg cells. *J. Exp. Med.* 2008. **205**: 2643–2655.

40 **Bajenaru, M. L.**, **Zhu, Y.**, **Hedrick, N. M.**, **Donahoe, J.**, **Parada, L. F. and Gutmann, D. H.**, Astrocyte-specific inactivation of the neurofibromatosis 1 gene (NF1) is insufficient for astrocytoma formation. *Mol. Cell Biol.* 2002. **22**: 5100–5113.

41 **Karray, S.**, **Kress, C.**, **Cuvellier, S.**, **Hue-Beauvais, C.**, **Damotte, D.**, **Babinet, C. and Levi-Strauss, M.**, Complete loss of Fas ligand gene causes massive lymphoproliferation and early death, indicating a residual activity of gld allele. *J. Immunol.* 2004. **172**: 2118–2125.

42 **Schlüter, D.**, **Oprisiu, S. B.**, **Chahoud, S.**, **Weiner, D.**, **Wiestler, O. D.**, **Hof, H. and Deckert-Schlüter, M.**, Systemic immunization induces protective $CD4^+$ and $CD8^+$ T cell-mediated immune responses in murine *Listeria monocytogenes* meningoencephalitis. *Eur. J. Immunol.* 1995. **25**: 2384–2391.

43 **Frei, K.**, **Bodmer, S.**, **Schwerdel, C. and Fontana, A.**, Astrocyte-derived interleukin 3 as a growth factor for microglia cells and peritoneal macrophages. *J. Immunol.* 1986. **137**: 3521–3527.

44 **Lenz, G. and Avruch, J.**, Glutamatergic regulation of the p70S6 kinase in primary mouse neurons. *J. Biol. Chem.* 2005. **280**: 38121–38124.

45 **Schlüter, D.**, **Löhler, J.**, **Deckert, M.**, **Hof, H. and Schwendemann, G.**, Toxoplasma encephalitis of immunocompetent and nude mice: immunohistochemical characterisation of Toxoplasma antigen, infiltrates and major histocompatibility complex gene products. *J. Neuroimmunol.* 1991. **31**: 185–198.

46 **Livak, K. J. and Schmittgen, T. D.**, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001. **25**: 402–408.

Abbreviations: **7-AAD**: 7-aminoactinomycin D · **FasL**: Fas ligand · **GFAP**: glial fibrillary acid protein · **MOG**: myelin oligodendrocyte glycoprotein · **p.i.**: post immunization

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